Discovering, Characterizing, and Applying Acyl Homoserine Lactone-Quenching Enzymes to Mitigate Microbe-Associated Problems Under Saline Conditions

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Quorum quenching (QQ) is proposed as a new strategy for mitigating microbe-associated problems (e.g., fouling, biocorrosion). However, most QQ agents reported to date have not been evaluated for their quenching efficacies under conditions representative of seawater desalination plants, cooling towers or marine aquaculture. In this study, bacterial strains were isolated from Saudi Arabian coastal environments and screened for acyl homoserine lactone (AHL)-quenching activities. Five AHL quenching bacterial isolates from the genera *Pseudoalteromonas*, *Pontibacillus*, and *Altererythrobacter* exhibited high AHL-quenching activity at a salinity level of 58 g/L and a pH of 7.8 at 50 °C. This result demonstrates the potential use of these QQ bacteria in mitigating microbe-associated problems under saline and alkaline conditions at high (>37 °C) temperatures. Further characterizations of the QQ efficacies revealed two bacterial isolates, namely, *Pseudoalteromonas* sp. L11 and *Altererythrobacter* sp. S1-5, which could possess enzymatic QQ activity. The genome sequences of L11 and S1-5 with a homologous screening against reported AHL quenching genes suggest the existence of four possible QQ coding genes in each strain. Specifically, two novel AHL enzymes, AiiA\textsubscript{S1-5} and Est\textsubscript{S1-5} from *Altererythrobacter* sp. S1-5, both contain signal peptides and exhibit QQ activity over a broad range of pH, salinity, and temperature values. In particular, AiiA\textsubscript{S1-5} demonstrated activity against a wide spectrum of AHL molecules. When tested against three bacterial species, namely, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Vibrio alginolyticus*, AiiA\textsubscript{S1-5} was able to inhibit the motility of all three species under saline conditions. The enzyme did not impose any detrimental effects on cell growth, suggesting a lower potential
INTRODUCTION

Seawater is increasingly being used as an alternative water resource for various purposes to alleviate water demands in water-stressed countries (Elimelech and Phillip, 2011). However, marine microorganisms can attach onto surfaces, propagate and establish biofilm matrices, and frequently, this attachment can result in detrimental consequences. For example, biofilm formation can foul membranes in a seawater desalination plant, in turn reducing the flux (Al-Ahmad et al., 2000). Biofilm formation can also accelerate the biocorrosion of metal pipelines (Enning et al., 2012). Marine pathogens form biofilms on fish and shrimp, which can lead to the mortality and morbidity of these livestock and cause economic losses in marine aquaculture (Mizan et al., 2015). In all instances, biofilm formation increases the capital and operational costs associated with seawater usage.

Conventional antifouling strategies include the use of toxic biocides and coating materials such as tributyltin, copper, chlorine and ozone (Amara et al., 2018). However, there are health, safety and environmental concerns associated with the incessant use of these chemicals. Recently, a quorum quenching (QQ) strategy was proposed as an eco-friendly way to inhibit biofouling by blocking the cell-to-cell communication ability of bacteria (which is also known as quorum sensing, or QS). QS is a cell density-dependent regulatory mechanism used by bacteria to coordinate group behavior in response to QS signals secreted by the cell population. The concentration of QS signals increases as the cell population grows, which, upon reaching a certain threshold value, will trigger the expression of certain genes, including one related to pathogenicity, biofilm formation, spore germination and other functions (De Foeridt, 2018). QQ bacteria and QQ enzymes have been demonstrated to be effective in the membrane fouling mitigation of lab-scale membrane bioreactors (MBRs) used for wastewater treatment (Lee et al., 2018; Oh and Lee, 2018), and in a pilot-scale MBR (Lee et al., 2016).

Several studies have also presented the QQ strategy for mitigating biofouling in marine environments or in seawater desalination plants (Dobretsov et al., 2011; Katebian et al., 2016, 2018). The QQS discovered and applied to date have primarily been restricted to QS inhibitors (e.g., vanillin, cinnamaldehyde, and kojic acid). Nevertheless, several QQ bacteria were tested for biocontrol in marine environments. Thinh et al. (2008) enriched a complex bacterial consortium that exhibited AHL degradation activity, and they introduced this enrichment culture to colonize larval fish guts and demonstrated an improved survival rate from AHL-induced virulence traits by opportunistic bacteria. Torres et al. (2016) further screened for QQ enzymatic activity among 450 bacterial strains that were isolated from a mollusk hatchery, and they identified Alteromonas stellipolaris PQQ-42 as a potential AHL-degrading bacterium that increased the survival rate of corals against Vibrio. QQ bacteria and enzymes were subsequently shown to be widely distributed in marine sources (Romero et al., 2011, 2012). Marine isolates belonging to the Erythrobacter, Labrenzia, and Bacterioplanes genera were capable of degrading AHL molecules (Rehman and Leiknes, 2018). However, none of these studies took advantage of the special traits of marine QQ enzyme-secreting bacteria to mitigate marine biofouling. Moreover, these studies did not evaluate if the QQ enzymes were able to mitigate biofouling under the harsh environmental conditions representative of industrial seawater applications. These environmental conditions include high salinity (of up to 58 g/L), high temperatures (of up to 45°C) and an alkaline pH ranging from 7.2 to 8.0 (Scarascia et al., 2016).

To address this knowledge gap, during this study, bacteria isolated from a Saudi coastal habitat and the Red Sea were first screened for their AHL-quenching ability. Five AHL-quenching bacterial isolates from the genera Pseudoalteromonas, Pontibacillus, and Altererythrobacter were determined to exhibit high AHL quenching at a salinity value of 58 g/L and a pH of 7.8 at 50°C. The genomes of two bacterial isolates were sequenced, and the potential QQ genes from the genomes were screened based on their homologies with reported QQ genes. The possible AHL-quenching genes were verified by expressing the potential QQ genes in recombinant E. coli to obtain enzymatically active recombinant proteins for testing. Subsequently, two enzymes (N-acyl homoserine lactonase, AiiA and esterase, Est) from Altererythrobacter sp. S1-5 were biochemically characterized at different pH, salinity and temperature. The AiiA from Altererythrobacter sp. S1-5 was further demonstrated to inhibit marine biofilm formation and the virulence of the opportunistic bacteria Aeromonas hydrophila, Pseudomonas aeruginosa, and Vibrio alginolyticus under saline conditions. The findings from this study demonstrate the potential feasibility of using QQ bacteria and enzymes to mitigate biofouling under saline conditions. Specifically, it is the first study to demonstrate the presence of AHL-quenching activity in Altererythrobacter.

MATERIALS AND METHODS

Sample Collection, Strain Isolation, and Identification

High-salinity artificial seawater (HSAS: 51.5 g/L NaCl, 0.74 g/L KCl, 0.99 g/L CaCl₂, 2.85 g/L MgCl₂, and 1.92 g/L MgSO₄;
salinity: 58 g/L), high-salinity marine broth medium (HSMB: HSAS containing 1 g/L yeast extract, 5 g/L peptone), and high-salinity marine agar (HSMA: HSMB with 15 g/L agar) were used for strain isolation and screening. Marine aquaculture sludge from the Jeddah Fisheries Research Center (JFRC) as well as beach sand and seawater from the Red Sea were collected for the isolation of AHL-quenching bacteria. A 50 mL aliquot of seawater was transferred into a sterile flask. For the sludge and sand, 15 g of each sample was individually poured into separate sterile flasks that contained 50 mL of sterile HSAS and 2 g of glass beads (Sigma, St. Louis, MO, United States; diameter: 5 mm). All the inoculated flasks were cultivated at 40°C and 180 rpm for 24 h. Following that incubation, the cultures were serially diluted with HSAS and plated onto HSMA plates. The agar plates were further incubated at 40°C for 24 h. Colonies showing different morphologies were picked and purified by plate streaking.

The bacterial isolates were identified through partial length 16S rRNA gene sequencing. A single colony of marine isolates was scraped with sterile toothpicks, suspended in 20 µL of sterile water and heated at 95°C for 5 min to achieve cell lysis. One microliter of supernatant was used as the DNA template for polymerase chain reactions (PCRs). A partial 16S rRNA gene was amplified using the universal primers 11F (5′-GTTGYGATYCTGG CTCAG-3′) and 1492R (5′-GYYTACCTTGTAGACGT-3′). The PCR was performed at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 52°C and 2 min at 72°C, and a final elongation for 5 min at 72°C. The PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States) and submitted to the KAUST Bioscience core lab for Sanger-based sequencing using the primers 11F, 1492R, 338F (5′-ACTCCTACGGGAGGCAGCAG-3′), and 592R (5′-GWATTACGGCGGCKGCTG-3′). The sequences were paired and searched against the NCBI GenBank database using the BLASTn search algorithm.

### Screening for AHL Quenching Strains From Marine Isolates Under High Salinity

Single colonies of tested strains were inoculated into individual wells of sterile 96-well microtiter plates, and each well contained 200 µL of autoclaved HSMB medium. The 96-well plates were incubated at 50°C and 120 rpm for 24 h. After the incubation, the plates were centrifuged at 2,300 × g for 10 min. The supernatant in each well was discarded and the cell pellet was washed once with HSAS. The centrifugation process was repeated and the resulting cell pellets were individually resuspended in 200 µL of HSAS containing an AHL mixture (0.29 mM C4-HSL, 0.25 mM C6-HSL, 0.22 mM C8-HSL and 0.17 mM 3-oxo-C12-HSL). After incubating at 50°C for 24 h, the culture was centrifuged again at 2300 × g for 10 min. Ten microliters of supernatant was collected for residual AHL quantification using an Agrobacterium tumefaciens bioassay (Tang et al., 2013). In brief, A. tumefaciens NT1 (traR, tra::lacZ749) (Piper et al., 1993) was grown in AT minimal medium (10.7 g/L KH2PO4, 2 g/L (NH4)2SO4, 78 mg/L MgSO4, 13 mg/L CaCl2, H2O, 5 mg/L FeSO4, 7H2O, 1.4 mg/L MnSO4·H2O, pH 6.7, and 0.5% filtered glucose, pH = 6.7) at 28°C and 150 rpm overnight. The culture was diluted 1:500 into fresh AT minimal medium containing 250 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma, St. Louis, MO, United States) to make the A. tumefaciens bioassay solution. Ten microliters of each sample used to analyze the residual AHL concentrations were pipetted into individual wells of a sterile 96-well-plate, with each well containing 190 µL of A. tumefaciens bioassay solution. After an incubation at 28°C for 12 h, the absorbance of each well at 492 nm and 630 nm was recorded using a SpectraMax 340 PC 384 microplate reader (Molecular Devices LLC, San Jose, CA, United States). The absorbances at 492 nm and 630 nm are a combination of absorption and light scattering by indigo (which is an X-gal degradation product) and biosensor cells (Tang et al., 2013). The residual AHL activity in each sample was expressed as normalized β-galactosidase activity as described in Tang et al. (2013). The quantification was performed in triplicate. The same concentration of AHL mixture in abiotic HSAS solution was incubated under the same conditions described above and used as a negative control when determining the residual AHL activity. The relative AHL quenching efficiency (QE) for each strain was determined using Eq. (1) as follows:

$$\text{QE} = \frac{\text{AHLn} - \text{AHLs}}{\text{AHLn}} \times 100\%$$

where AHLn denotes the residual AHL activity of the negative control after the reaction and AHS denotes the residual AHL activity of bacterial samples after the reaction with potential QQ enzymes. It was previously shown that AHS can be rather unstable in alkaline and high temperature environments (Yates et al., 2002). Therefore, to eliminate the instability of AHS caused by abiotic factors after long-term reactions and to denote the AHL activity changes arising from the potential QQ enzymes more accurately, we use AHLn instead of the initial AHL activity spiked into the prior reaction mixture. Strains with a relative AHL QE ≥ 90% were selected for further QQ enzyme screening.

### Localization of AHL Quenching Enzyme in AHL Quenching Isolates

Artificial seawater (AS: 29.5 g/L NaCl, 0.74 g/L KCl, 0.99 g/L CaCl2, 2.85 g/L MgCl2, and 1.92 g/L MgSO4; salinity: 36 g/L), marine broth medium (MB: AS with 1 g/L yeast extract, 5 g/L peptone), and marine agar (MA: MB with 15 g/L agar) were used for the AHL quenching enzyme experiment. Selected strains were grown in MB medium at 37°C overnight. To obtain the potential AHL quenching enzymes present in the intracellular fraction of each bacterial isolate, 5 mL of pure culture was centrifuged at 20,000 × g for 10 min, and the cell pellet was suspended in phosphate-buffered saline (PBS) (Fisher Scientific, Hampton, NH, United States). The cells were lysed using a Q500 sonicator (Qsonica, Newtown, CT, United States) at a 45% amplitude for 5 min, with repetitive 15 s pulsating sonication at 45 s intervals. The fraction was centrifuged at 10,000 × g for 10 min and filtered through a 0.2 µm cellulose acetate membrane to obtain the lysed cellular extract as filtrate. To obtain the potential AHL-quenching enzymes secreted extracellularly by the bacterial isolates, a cell pellet made from 5 mL of overnight
culture was resuspended in 5 mL of AS. The cell suspension was incubated at 37°C for 12 h and centrifuged at 20,000 × g for 10 min to obtain the supernatant fraction. The cellular extract and supernatant fraction of each strain were fractionated separately using centrifugal filters (MWCO: 10 kDa, Merck, Darmstadt, Germany) prior to the determination of the potential AHL-quenching activity. To demonstrate whether the quenching activity arose from enzymatic activity, the cellular extract and supernatant fraction were also heat-inactivated at 100°C for 30 min and tested for AHL quenching activity. Each sample was mixed with an AHL mixture (0.15 mM C4-HSL, 0.13 mM C6-HSL, 0.11 mM C8-HSL, and 0.08 mM 3-oxo-C12-HSL) and incubated at 37°C for 18 h for the residual AHL determination. PBS buffer and AS were used to replace the cell extract and supernatant to create the negative control.

**Genome Sequencing of AHL-Quenching Strains**

Two strains (L11 and S1-5) that exhibit potential enzymatic AHL-quenching activity were sequenced for protein-coding genes (CDS) that encode the AHL quenching enzyme. The genomic DNA from L11 and S1-5 was extracted with a QIAGEN Genomic Tips kit (Qiagen, Hilden, Germany). A 20 kb DNA library was prepared for each strain by genomic DNA fragmentation with a Pippin HT size-selection system (Sage Science, Beverly, MA, United States). Single molecular real-time (SMART) sequencing was performed with a PacBio RS II platform (Pacific Biosciences, Menlo Park, CA, United States). The raw reads were assembled into contigs using a Canu assembler (Koren et al., 2017). The assembled contigs were examined for integrity using dotplots with a Genome PAir-Rapid Dotter tool (Krumsieck et al., 2007). The assembled contigs were annotated with a RASTtk server (Brettin et al., 2015). The potential AHL quenching ORFs in L11 and S1-5 were screened with NCBI tBLASTn using published QQ sequences was performed using ESPript (Gouet et al., 1999). Highly homologous ORFs (identify ≥ 25%, coverage ≥ 40%) that share a conserved domain with the reported QQ protein were selected for gene expression evaluation and to test for quenching activity.

**Expression of AHL Quenching Genes in Escherichia coli**

Eight potential QQ gene sequences from strains L11 and S1-5 were amplified with Q5-hot start polymerase (New England BioLabs, Beverly, MA, United States) using the primers listed in **Supplementary Table 1**. The PCR amplicons of the QQ genes were either ligated into a pTrcHis A vector (Thermo Fisher, Waltham, MA, United States) by T4 DNA ligase (Thermo Fisher Waltham, MA, United States) or into pET-20b (+) vectors (Merck, Darmstadt, Germany) by Gibson Assembly Master Mix (New England BioLabs, Beverly, MA, United States). Recombinant pTrcHis A and pET-20b(+) vectors with correct insertions were transformed into E. coli TOP10 (Thermo Fisher, Waltham, MA, United States) and E. coli BL21 (DE3) (Merck, Darmstadt, Germany), respectively, for protein expression. The blank vectors pTricHisA and pET-20b(+) were also transformed into E. coli TOP10 and E. coli BL21 (DE3) and denoted as negative recombinant controls (NC-1 and NC-2). The AiiA gene from Bacillus mycoides ATCC 6462 was inserted into pET-20b(+) and expressed in E. coli BL21 (DE3) as a positive control sample for QQ activity evaluation.

Overnight cultures of recombinant strains were diluted 1:100 into fresh LB medium supplemented with 100 µg/mL ampicillin (Sigma, St. Louis, MO, United States). The culture was incubated at 30°C and 160 rpm until the cells reached exponential growth (OD₆₀₀ = 0.8). Subsequently, induction was initiated by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Thermo Fisher, Waltham, MA, United States) to the culture prior to further incubation at 30°C 120 rpm for 16 h. After the induction, the cell pellet was collected and suspended in PBS buffer. The cell suspension was sonicated, and the supernatant was passed through a 0.2 µm filter to remove any cell debris. The crude enzyme was subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis. Each target protein band was excised from the SDS-PAGE gel and fragmented into short peptides by in-gel digestion treatment with trypsin (Promega, Madison, WI, United States). The digested peptides were subjected to NanoLC MS/MS analysis using a nanospray UltiMate 3000 ultra-high-performance liquid chromatography (UHPLC) binary HPLC system coupled to a Q-Exactive HF mass spectrometer (Thermo Fisher, Waltham, MA, United States). The target proteins were identified by searching against the Swiss-Prot protein sequence database using the Mascot v 2.6 search engine from Matrix Science.

To evaluate the quenching performance of recombinant QQ enzymes, a reaction system containing 10 µM 3-oxo-C12-HSL, 50 µL of crude enzyme, and PBS buffer was incubated at 37°C for 3 h. Ten microliters of reaction mixture was tested for residual AHL activity using the A. tumefaciens bioassay. The crude extract of the control strain (NC-1 and NC-2) was used as a negative control. The substrate specificity of the crude enzyme was tested with C4-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, and 3-oxo-C12-HSL (Sigma, St. Louis, MO, United States). The crude enzyme was mixed with different AHL molecules in PBS buffer, and after the reaction at 37°C, the residual AHL was determined by bioassay.

**Purification of Recombinant QQ Enzyme**

The recombinant strain that was verified to possess AHL-quenching activity was induced in 100 mL of LB medium. The crude enzyme was prepared as described above and the recombinant enzyme was purified by affinity chromatography using a 5 mL HisTrap HP column (GE Healthcare, Piscataway, NJ, United States). The column was equilibrated with binding buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 20 mM imidazole). The crude enzyme was diluted with binding buffer and loaded onto the column. The target protein was eluted with 200 mM imidazole; the eluted fractions were pooled and dialyzed in a dialysis tubing (Fisher Scientific, Hampton, NH, United States) with a molecular weight cutoff (MWCO) of 3.5 kDa. The PBS buffer was used as the dialysis medium, and
the entire procedure was performed three times to remove the extra imidazole and salt. The solution was then concentrated using a centrifugal filter with a MWCO of 3.5 kDa (Merck, Darmstadt, Germany).

**Biochemical Characterization of Recombinant QQ Enzymes**

The optimal pH of the QQ enzyme was tested by incubating a purified enzyme with 5 μM oxo-C12-HSL in 0.1 M citrate-phosphate buffer (pH 3.0–8.0) and 0.1 M glycine-NaOH buffer (pH 9.0–10.0) at 37°C for 1 h. The effect of the salinity on the enzyme activity was evaluated by incubating the enzyme with 5 μM oxo-C12-HSL under the optimal pH for each enzyme and under different temperatures ranging from 20 to 100°C for 10 min. The same amount of PBS buffer was used instead of the purified enzyme as a negative control for each test. After the reaction, the residual AHL was quantified using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. All the reactions were performed in triplicate. For the biochemical characterization of the NAD(P)-dependent enzymes SDRS1−5 and SDRL11, 1 mM NADPH or NADH (Sigma, St. Louis, MO, United States) was added to each reaction to facilitate the enzymatic activity.

**AHL Quantification by LC-MS/MS**

The AHL molecule oxo-C12-HSL was determined with an Agilent 1260 Infinity quaternary liquid chromatograph (Agilent, Santa Clara, CA, United States) equipped with an Agilent Pursuit C18 column (3 μm particle size, 2.0 × 150 mm) and SCIEX Q-TRAP 5500 mass spectrometer (AB SCIEX, Foster City, CA, United States). The separation was performed at room temperature at a mobile phase flow rate of 250 μL/min using the following gradient elution profile: t = 0 min, 95% solution A (LC-MS grade water with 0.1% formic acid), 5% solution B (LC-MS grade methanol); t = 2 min, 95% solution A, 5% solution B; t = 4 min, 50% solution A, 50% solution B; t = 6 min, 5% solution A, 95% solution B; t = 10 min, 5% solution A, 95% solution B, t = 12 min, 95% solution A, 5% solution B; t = 18 min, 95% solution A, 5% solution B. The detection was performed in positive ion mode using the parameters listed in Supplementary Table 2.

**Enzymatic Effect on the Motility and Biofilm Formation of Opportunistic Marine Pathogens**

The effect of AiiAS1−5 on the motility and biofilm formation of Aeromonas hydrophila, Pseudomonas aeruginosa, and Vibrio alginolyticus (Supplementary Table 3), was investigated. Five microliters of overnight culture for each marine strain was spotted in the center of the plate containing marine broth-soft agar (0.3% and 0.5% agar) prior to the addition of 5 μL of purified AiiAS1−5 (0.2 μg). The plate was cultured at 40°C overnight. The slightly higher mesophilic temperature of 40°C was chosen to mimic the temperature experienced by bacteria during industrial processes (e.g., in cooling towers, seawater desalination plants and tropical marine aquaculture). The experiment was repeated six times. The migration of the culture was evaluated by calculating the diameters of the haloes around the spotted area. In addition, the effect of the AiiAS1−5 on the biofilm formation was also evaluated. Single colonies of A. hydrophila, P. aeruginosa, and V. alginolyticus were cultured in AS with 2% peptone and incubated at 40°C overnight. Thereafter, 2 × 105 colony forming units (CFU)/mL of cells were mixed with purified AiiAS1−5 (0, 5, 10, and 20 μg/mL). The cultures were transferred into 96-well microtiter plates and further incubated at 40°C for 24 h. Thereafter, the supernatants were carefully removed, and the biofilms were stained with 0.2% crystal violet at room temperature for 30 min. Extra dye was removed with AS and the stained cells were solubilized with 200 μL of 30% acetic acid (O’Toole, 2011). The absorbance of the solution was measured at 595 nm. PBS buffer was used instead of purified AiiAS1−5 as a negative control.

**Transcription of Virulence Genes in Marine Strains in the Presence of Purified AiiAS1−5**

A. hydrophila, P. aeruginosa, and V. alginolyticus were grown in marine media at 37°C and 180 rpm overnight. The overnight culture was diluted 1:100 in MB medium as mentioned before (salinity: 36 g/L), and 50 μg/mL of purified AiiAS1−5 was added to the pathogenic strain culture at the inoculation time. The bacterial cultures were grown at 37°C and 180 rpm. The growth curve of the marine strain with AiiAS1−5 or PBS buffer was monitored based on the optical density at 600 nm. The bacterial cultures were sampled at the mid-exponential, late exponential and stationary phases of growth. The samples were used for RNA extraction (RNasey Mini Kit, Qiagen). cDNA was synthesized with a SuperScript III First-Strand Synthesis Supermix (Thermo Fisher, Waltham, MA, United States). The transcriptional level of each gene (Supplementary Table 4) was calculated using the relative standard curve method. To obtain the standard curve, the PCR amplification product of each gene was first cloned into a PCR Blunt II-TOPO vector (Thermo Fisher, Waltham, MA, United States). Plasmid DNA containing the target gene was serially diluted based on the copy number and used as the standard. Real-time PCR was performed on a 7900HT Fast Real-Time PCR system (Thermo Fisher, Waltham, MA, United States). The reaction system contained 5 μL of Fast SYBR Green master mix (Thermo Fisher, Waltham, MA, United States), 0.2 μL each of the forward and reverse primers (10 μM), and 2 μL of cDNA template (2 ng/μL). The quantification of the target gene was normalized to the reference gene (rpoB) in each sample.

**Statistical Analysis**

The statistical analysis was performed with Minitab 17. A paired t-test was used to determine the statistical significance of the difference. The difference was defined as statistically significant when P < 0.05.
RESULTS

Bacterial Screening From Marine Sources and the AHL Quenching Test

A total of 51 bacterial isolates that can grow at a high salinity (58 g/L) and a high temperature of 50°C were obtained for QQ screening (Supplementary Table 5). The AHL mixture (0.29 mM C4-HSL, 0.25 mM C6-HSL, 0.22 mM C8-HSL and 0.17 mM 3-oxo-C12-HSL) was used to screen the QQ bacteria under high salinity (58 g/L) and a high temperature (50°C). These QQ isolates showed the ability to inhibit AHL activity before and after heat inactivation (QSIs). The supernatant of the isolates maintained the same portion, suggesting the presence of quorum signal inhibitors (Supplementary Figure 1). Figure 1B corresponded with the size fraction experiment for the three phyla: Firmicutes (Staphylococcus, Bacillus, Halobacillus, Virgibacillus, Pontibacillus, Aquibacillus, and Halobacillus), Bacteroidetes (Titalama, Tenacibaculum, Vibrio), and Proteobacteria (Delftia, Bacteriostrategon, Altererythrobacter, Devosia, Halomonas, and Erythrobacteraceae). Among the QQ isolates, five showed ≥90% relative AHL QE toward AHL mixtures (Supplementary Table 1 and Supplementary Figure 1). These five QS-quenching bacteria belong to the Pseudomonadaceae (Proteobacteria), Bacillaceae (Firmicutes), and Erythrobacteraceae (Proteobacteria), with three of them belonging to the genus Altererythrobacter in the Erythrobacteraceae (Table 1).

Size Fractionation of the Bacterial Cell Extract and Supernatant Contents to Identify the AHL-Quenching Enzymes

Both the cell extract and supernatant fraction obtained from the five bacterial isolates showed the ability to inhibit AHL (Figure 1), with the cell extract having a higher AHL inhibition performance than the supernatant (Figures 1A,C). After heat inactivation, the cell extracts of L11 and S1-5 showed decreased AHL inhibition activity, while no decrease in AHL inhibition activity was detected for the other isolates (Figure 1A). This result corresponds with the size fraction experiment for the L11 and S1-5 cell extract (Figure 1B), in which a higher relative AHL QE was observed in the >10 kDa portion. This size fraction was generally found to contain potential QQ enzymes (Czajkowski and Jafra, 2009). For the other isolates, the relative AHL quenching activity was higher in the <10 kDa portion, suggesting the presence of quorum signal inhibitors (QSIs). The supernatant of the isolates maintained the same AHL quenching activity before and after heat inactivation (Figure 1C), and the <10 kDa fractions in the supernatant contributed more to the quenching effect than the larger size fractions (Figure 1D). Both the cell extract and the supernatant of the five strains showed similar QQ performance at 50°C (Supplementary Figures 2A,C) except for the presence of QQ activity found in the >3 kDa fraction of the S1-5 supernatant (Supplementary Figures 2B,D).

Genomic Sequencing of the Two Bacterial Isolates S1-5 and L11 That Potentially Possess Enzymatic QQ Activity

One closed contig was assembled from S1-5 raw reads, and it had a sequence length of 3.35 Mbp and a GC content of 66.3%. Two contigs were assembled from the L11 raw reads; both contigs were predicted to be closed, with no gap detected (Supplementary Figure 3). Contig 1 has a sequence length of 2.89 Mbp (GC content of 46%) and Contig 2 has a full length of 0.64 Mbp (GC content of 45.2%). The sequencing files were deposited in the European Nucleotide Archive (ENA) under study accession number PRJEB30480. Eight potential QQ enzyme genes from S1-5 and L11 (Table 2) were classified as N-acyl homoserine lactonase (AiiA), oxidoreductase (SDR), esterase (Est), hydrolase (Hyd), a penicillin acylase family protein (PvdQ), and a hypothetical protein (HP). These ORFs all shared a conserved motif with the reference AHL-quenching enzymes (Supplementary Figure 4).

Verification of Identified QQ Enzymes for AHL Quenching Activity and Specificity

The identified ORFs were expressed in recombinant Escherichia coli. The crude enzyme and cell lysate from recombinant E. coli, along with the E. coli host that contained blank expression vectors (NC-1 and NC-2), were individually mixed with 5 μM 3-oxo-C12-HSL and tested for residual AHL concentration. Four ORFs, namely, AiiA51−5, SDR51−5, Est5−5 and SDR11−11, were verified to be AHL quenchers. The enzymes showed 26.5%, 40.1%, 13.8%, and 38.0% relative AHL QE compared with the negative controls (Figure 2). The positive control made up of AiiA from B. mycoides ATCC 6462 showed a 37.9% relative AHL QE compared with the negative control. The level of activity exhibited by the positive control was comparable to that observed for both SDRs from L11 and S1-5. The AHL specificity test further showed that AiiA51−5 showed catalytic activity toward all the tested AHLs, and Est5−5 can quench all the AHLs except C4-HSL and C6-HSL. Both SDR51−5 and SDR11−11 can quench all

| Strain name | Best matched 16S rRNA gene identification | Accession number for 16S rRNA gene sequences | E-value | Similarity percentage |
|-------------|------------------------------------------|---------------------------------------------|---------|-----------------------|
| L11         | *Pseudaltheromonas* sp. (JQ237129.1)     | MK575497                                    | 0.0     | 98%                   |
| L12         | *Pontibacillus* sp. (MG252492.1)         | MK575888                                    | 0.0     | 99%                   |
| S1-1        | *Altererythrobacter marinus* (MF716636.1)| MK578236                                    | 0.0     | 99%                   |
| S1-5        | *Altererythrobacter* sp. (KC169804.1)   | MK574878                                    | 0.0     | 98%                   |
| S1-6        | *Altererythrobacter marinus* (NR_116432.1) | MK578235                                  | 0.0     | 99%                   |

TABLE 1 | Marine bacteria that showed 90–100% relative AHL quenching efficiency under saline condition and at high temperature.
FIGURE 1 | Presence of AHL quenching enzymes in each fraction of AHL quenching bacteria. (A) Evaluation of AHL quenching activity in cell extracts and heat-inactivated cell extracts, (B) separation of cell extract based on molecular size (10 kDa) and evaluation of the AHL quenching effect in each fraction, (C) detection of AHL quenching activity in bacterial supernatant and heat-inactivated supernatant, and (D) separation of bacterial supernatant based on the molecular size and evaluation of the AHL quenching effect in each fraction. Each fraction was mixed with AHL mixture (0.15 mM C4-HSL, 0.13 mM C6-HSL, 0.11 mM C8-HSL, and 0.08 mM 3-oxo-C12-HSL) and incubated at 37°C for 18 h for residual AHL determination using a biosensor. The relative AHL quenching efficiency of each sample was calculated based on Eq. (1), as stated in the section “Materials and Methods.” PBS buffer and AS were used to replace the cell extract and supernatant to create the negative control. Three biological replicates were performed, and the results were expressed as the means ± standard error.

the 3-oxo-AHLs. Moreover, SDR$_{S1-5}$ showed a quenching effect on C8-HSL (Supplementary Table 6).

Biochemical Characterization of Recombinant QQ Enzymes
AiiA$_{S1-5}$ and Est$_{S1-5}$, SDR$_{S1-5}$ and SDR$_{L11}$ were further induced and purified for biochemical characterization at varying pH values, salt concentrations and temperatures. However, during the course of this 1-month experiment, the purified SDR$_{S1-5}$ and SDR$_{L11}$ did not exhibit good stability while they were in storage compared to AiiA$_{S1-5}$ and Est$_{S1-5}$. The purified SDR$_{S1-5}$ and SDR$_{L11}$ consistently did not show any AHL-quenching activity after storage despite the addition of NADH or NADPH (data not shown). Hence, the biochemical characterization of only AiiA$_{S1-5}$ and Est$_{S1-5}$ was performed using 3-oxo-C12-HSL as the substrate. AiiA$_{S1-5}$ exhibited >25% relative activity against 3-oxo-C12-HSL between pH 7 and 9, with the highest activity observed at pH 8.0 (Figure 3A). AiiA$_{S1-5}$ showed the maximum relative activity in PBS buffer, but it was still able to retain more than 63% of its activity at 0.1–2 M NaCl concentrations (Figure 3B). AiiA$_{S1-5}$ showed the highest activity at 0.5 M KCl, and it maintained >70% activity at 0–2 M KCl (Supplementary Figure 5A). The relative activity of AiiA$_{S1-5}$ increased from 10 to 50°C (the optimal temperature range) before decreasing rapidly to 7.8%, when the temperature was further increased to 70°C. The enzyme was fully inactivated at 80°C (Figure 3C). Est$_{S1-5}$ exhibited the highest relative activity at pH 9.0, and it retained 26.9% of its activity at pH 10.0 (Figure 3D). Est$_{S1-5}$ showed optimal activity at 0.1 M NaCl, and it retained more than 83% of its activity at NaCl concentrations between 0 and 0.5 M (Figure 3E). The relative activity of Est$_{S1-5}$ decreased with the increasing KCl concentration (Supplementary Figure 5B). The enzyme showed the highest activity at both 30 and 40°C, and it was still able to maintain >30% of its relative activity at high temperatures of 50 and 60°C (Figure 3F).

Effect of Purified AiiA$_{S1-5}$ on Marine Bacteria
Purified AiiA$_{S1-5}$ was selected for further testing due to its stability, broad substrate specificity and high enzymatic activity in comparison to Est$_{S1-5}$. The swarming and swimming of A. hydrophila, P. aeruginosa, and V. alginolyticus all decreased in the presence of AiiA$_{S1-5}$ compared with the negative control (Figure 4A and Supplementary Figure 6). Purified AiiA$_{S1-5}$ reduced swarming more significantly than swimming. There were observed reductions of 48.9%, 35.4%, and 70.2% in swarming in A. hydrophila, P. aeruginosa, and V. alginolyticus, respectively, compared to their respective controls, which were not exposed to the enzyme. Purified AiiA$_{S1-5}$ reduced swimming...
TABLE 2

| Strain | ORF name | Signal peptide | Glu (%) | Hypothetical function | Superfamily |
|--------|----------|----------------|---------|-----------------------|------------|
| L1     | AiiA     | +              | 31.8    | N-acyl homoserine lactonase (AiiA) | Metallo-hydrolase-like_MBL-fold |
| S1     | AiiA     | +              | 31.8    | N-acyl homoserine lactonase (AiiA) | Metallo-hydrolase-like_MBL-fold |
| L1     | Hyd      | +              | 13.3    | Hydrolase family | α/β-hydrolase family |
| S1     | Hyd      | +              | 13.3    | Hydrolase family | α/β-hydrolase family |
| L1     | PvdQ     | +              | 9.9     | PvdQ protein (PvdQ) | Ntn_hydrolase superfamily |
| S1     | HP       | +              | 13.3    | Hypothetical protein (HP) | Hypothetical protein (HP) |

Potential QQ ORFs were screened by tBLASTn using known QQ enzymes published as references.

Potential QQ ORFs from strains L1 and S1−5 were screened using the reported AHL quenching enzymes.

DISCUSSION

Microbe-associated problems in the marine environment (e.g., seawater desalination plants, seawater cooling towers, and aquaculture) are conventionally mitigated by means of biocides or antibiotics that cause health, safety and environmental concerns. QS inhibitors are increasingly being explored as alternative agents to mitigate these problems (Dobretsov et al., 2011). However, research has shown that bacteria can develop resistance to QS inhibitors (Maeda et al., 2012; García-Contreras et al., 2013), rendering the treatment ineffective over the long term. In comparison, QQ enzymes that degrade extracellular QS signals are viewed as imposing less of a burden on cellular metabolism (Fetzner, 2015), and hence, they minimize the development of resistance toward these greener inhibitory agents. In most instances, QQ enzymes or bacteria with enzymatic activity from marine sources (Huang et al., 2012; Mayer et al.,...
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FIGURE 2 | AHL quenching activity comparison of eight potential protein-coding genes expressed in recombinant Escherichia coli cells. The relative AHL quenching efficiency of each sample was calculated based on Eq. (1), as stated in the “Materials and Methods” section. The E. coli TOP10 carrying vector pTricHisA and E. coli BL21 (DE3) carrying pET-20b(+) were used as negative controls. AiiA\textsubscript{S1\textminus5} (acyl homoserine lactonase), SDR\textsubscript{S1\textminus5} (oxidoreductase), Est\textsubscript{S1\textminus5} (esterase), and Hyd\textsubscript{S1\textminus5} (hydrolase) are possible AHL quenching ORFs from Altererythrobacter sp. S1–5; Hyd\textsubscript{L11} (hydrolase), PvdQ\textsubscript{L11} (penicillin acylase family protein), SDR\textsubscript{L11} (oxidoreductase), and H\textsubscript{PL11} (hypothetical protein) are possible AHL quenching ORFs from Pseudoalteromonas sp. L11. PC denotes a positive control, which is obtained by inserting an AiiA gene from Bacillus mycoides ATCC 6462 into pET-20b(+) and expressing it in E. coli BL21 (DE3). Two independent biological replicates with three technical replicates in each biological replicate were performed for this experiment.

FIGURE 3 | Biochemical properties of purified AiiA\textsubscript{S1\textminus5} and Est\textsubscript{S1\textminus5}. The relative enzyme activity of AiiA\textsubscript{S1\textminus5} in/at different (A) pH buffers (0.1 M citrate-phosphate buffer, pH 3.0–8.0; 0.1 M glycine-NaOH buffer, pH 9.0–10.0), (B) NaCl concentrations (0, 0.1, 0.5, 1, and 2 M), and (C) temperatures of 10–80°C. The relative enzyme activity of Est\textsubscript{S1\textminus5} in/at different (D) pH buffers, (E) NaCl concentrations, and (F) temperatures. The relative enzyme activity of the purified enzymes was normalized using the activity of the purified enzymes at the optimal pH, salinity, and temperature.

2015; Tang et al., 2015; Liu et al., 2017; Rehman and Leiknes, 2018) were demonstrated for their efficiencies to quench QS in only minimal or nutrient medium, which deviate significantly from conditions in marine industrial systems such as seawater desalination plants (salinity: 46,400 ppm, temperature: 22–33°C, and pH 8.1–8.3) (Khawaji et al., 2007) and cooling
FIGURE 4 | Effect of purified $\text{AiiA}_{31-5}$ on the motility and biofilm formation of marine strains. (A) Swarming and swimming of $\text{A. hydrophila}$, $\text{P. aeruginosa}$, and $\text{V. alginolyticus}$ on soft MB agar (MB medium with 0.3% and 0.5% agar) in the presence of PBS or 0.2 $\mu$g of purified $\text{AiiA}_{31-5}$ at 40°C for 12 h. The red and green boxes represent the swarming diameters of the marine strains in the presence of PBS and $\text{AiiA}_{31-5}$. The blue and magenta boxes represent the swimming diameters of the marine strains in the presence of PBS and $\text{AiiA}_{31-5}$. The inhibition efficiency of $\text{AiiA}_{31-5}$ on the swarming and swimming of the strains under saline conditions is shown in the right y-axis, and the inhibition efficiency was calculated using the following equation: $(P_m - E_m)/P_m \times 100\%$, where $P_m$ and $E_m$ represent the motility halos of each strain in centimeters in the presence of PBS buffer and the purified enzyme $\text{AiiA}_{31-5}$. (B) The effect of 0, 5, 10, and 20 $\mu$g/mL of purified $\text{AiiA}_{31-5}$ on the biofilm formation of $\text{A. hydrophila}$, $\text{P. aeruginosa}$, and $\text{V. alginolyticus}$. The biofilm formation of the marine bacteria was developed on artificial seawater (AS) with 2% peptone extracted from casein in 96-well polystyrene plates at 40°C for 24 h. $^*P < 0.05; ^{**}P < 0.01; ^{***}P < 0.001$.

FIGURE 5 | RT-qPCR analysis on the relative expression of selected virulence-associated genes in $\text{A. hydrophila}$, $\text{P. aeruginosa}$, and $\text{V. alginolyticus}$ on MB medium in the presence of PBS (negative control) or $\text{AiiA}_{31-5}$ at 37°C. (A) Growth curves of $\text{A. hydrophila}$, $\text{P. aeruginosa}$, and $\text{V. alginolyticus}$ in the presence of PBS or $\text{AiiA}_{31-5}$. The relative transcription levels of genes from (B) $\text{A. hydrophila}$, (C) $\text{P. aeruginosa}$, and (D) $\text{V. alginolyticus}$ in the presence of PBS and purified $\text{AiiA}_{31-5}$. The transcriptional levels of pathogenicity-related genes at different growth stages were normalized to the reference gene $\text{rpoB}$. Information on these genes can be found in Supplementary Table 4. The results are presented as the means ± standard deviation ($n = 3$). $^*P < 0.05; ^{**}P < 0.01; ^{***}P < 0.001$.

towers (e.g., salinity: >35,000 ppm, and temperature 32–48°C) (Al-Bloushi et al., 2018).

In this study, five bacterial isolates belonging to $\text{Pseudoalteromonas}$, $\text{Pontibacillus}$, and $\text{Altererythrobacter}$ demonstrated high QQ activity at a salinity of 58 g/L, 50°C, and a pH of 7.8. To the best of our knowledge, this is the first report on the existence of QQ activity in $\text{Pontibacillus}$ and $\text{Altererythrobacter}$. The enzymatic QQ activity was primarily discovered in the intracellular fraction of $\text{Pseudoalteromonas}$ sp. L11 and to a certain extent, it was also found in $\text{Altererythrobacter}$ sp. S1-5 (Figure 1). So far, many bacteria with either intracellular or extracellular QQ enzymatic activity (Kim et al., 2014; Fetzner, 2015) have been reported, but the extracellular activity is more feasible. For example, Cheong et al. (2013) entrapped the bacteria in a vessel that allows only the extracellular enzymes to pass through and react with AHL in a lab-scale membrane bioreactor used for wastewater treatment. This exclusion eliminated the need for an additional step of lysing the bacterial hosts to retrieve
the intracellular enzymes. However, no similar demonstration has been performed in saline environments.

To look for possible QQ enzymes for marine application purposes, the genes identified in L11 and S1-5 that shared homologies with the existing known QQ genes were first expressed in recombinant E. coli, purified and characterized further. Using this approach, we found four protein coding genes that shared high homology with the existing QQ genes in Alterederythrobacter sp. S1-5, but only three demonstrated QQ enzymatic activity when expressed in recombinant E. coli. Similarly, we found four homologous QQ ORFs in L11. Both PvdQ_{L11} and HP_{L11} shared 24% and 48% of their identity with the reported pmfA and QQ-16d enzymes from the same genera (Weiland-Bräuer et al., 2015; Liu et al., 2017). However, a further examination of each of the individual purified enzymes from L11 showed that only SDRL_{L11} was positive for AHL quenching activity. This may be because the enzyme from the halophilic microorganism was folded incorrectly or maintained poor stability during recombinant expression in its mesophilic host under low-salt conditions (Madern et al., 2000). Among the three ORFs in Alterederythrobacter S1-5 that showed QQ activity, an N-terminal peptide was predicted for both AiiA_{S1-5} and Est_{S1-5}, suggesting the possibility that the extracellular enzymes would be feasible for use in practical applications. Most QQ enzymes reported thus far do not have a signal peptide, except for AiiA in the marine organisms Muricauda olearia (Tang et al., 2015) and Erythrobacter flavus (Rehman and Leiknes, 2018) and dhlR in Rhizobium sp. (Krysciak et al., 2011).

After purification, only the AiiA_{S1-5} and Est_{S1-5} belonging to the AHL lactonase showed a capacity to degrade AHL. To date, the reported AHL lactonases primarily belong to the metallo-β-lactamase superfamily, the phosphotriesterase family and the α/β hydrolase family. The phosphotriesterase family from the archaeon Sulfolobus solfataricus and the crenarchaeon Vulcansisata moutnovskia (Hiblot et al., 2015) are the most thermophilic QQ enzyme group discovered so far, and they showed QQ activity, an N-terminal peptide was predicted for its mesophilic host under low-salt conditions (Madern et al., 2000; Takahashi et al., 2018). The 3D structural modeling of AiiA described in an earlier study (Easwaran et al., 2015).

AiiA_{S1-5} outperformed the other enzymes discovered in this study, and it was further chosen to demonstrate its QQ efficacy against A. hydrophila, P. aeruginosa, and V. alginolyticus. Both P. aeruginosa and A. hydrophila were previously reported to be commonly associated with fouled membranes in a seawater desalination plant (Hong et al., 2016; Yap et al., 2017; Nagaraj et al., 2018), while V. alginolyticus (Bermont-Bouis et al., 2007) and P. aeruginosa (Hamzah et al., 2014) were reportedly possibly linked to pipeline biocorrosion by seawater. Furthermore, these three bacterial species constitute the dominant pathogenic bacterial group reported in marine aquaculture (Sindermann, 1984). It was observed that AiiA_{S1-5} did not impose detrimental effects on cell growth, which reiterates its lower possibility of developing resistance against QQ enzymes over long-term usage. Instead, purified AiiA_{S1-5} showed an inhibitory effect against bacterial motility, biofilm formation and virulence transcription in three marine bacteria under saline conditions (salinity: 36 g/L). The biofilm inhibitory effect of AiiA_{S1-5} in P. aeruginosa is more significant than that of the other two bacteria. This distinction was likely caused by the substrate affinity of AiiA_{S1-5} toward the different AHLs secreted by the tested strains (Supplementary Table 3). A 10 μg/mL concentration of AiiA_{S1-5} inhibited the biofilm formation of P. aeruginosa to ca. 80% under saline conditions. Similarly, purified AHL-lactonase (100 μg/mL) from Enterobacter aerogenes VT66 inhibited >70% of the P. aeruginosa PAO1 biofilm (Rajesh and Rai, 2015), Labrenzia sp. VG12 cells with AHL lactonase activity also reduced the P. aeruginosa biofilm by 25% (Rehman and Leiknes, 2018), although the exact enzymatic concentration used here was not made known. These earlier studies, along with that reported here, demonstrated the inhibition effect of AHL lactonases on QS-regulated biofilm formation in P. aeruginosa.

In most instances, the transcribed levels of QS-coordinated receptor genes and virulence genes were significantly decreased by AiiA_{S1-5} at the late exponential phases of A. hydrophila and P. aeruginosa (Figure 5). However, the inhibitory effect was no longer observed at the stationary phase. This observation is consistent with that reported in an earlier study (Park et al., 2005; Sivakumar et al., 2019), and it was probably due
to the accumulation of enzymatic inhibitors in the culture over time, or to a decrease in the metabolic activities of the cultures at the stationary phase. QQ enzymes should therefore be deployed as an environmentally benign approach for controlling microbe-associated problems at the early stages of growth and biofilm formation.

CONCLUSION

In this study, bacterial species that proliferate under saline conditions were isolated and screened to select the ones that are positive for QQ under high salinity and high temperatures. Further genomic and biochemical characterization revealed a particularly promising AiiA from the Altererythrobacter sp. S1-5 that demonstrated a broad AHL substrate specificity range and enzymatic activity at an optimal pH of 8 and 50°C. AiiAS1-5 was also able to maintain good relative QQ activity with increasing salt concentrations up to 2 M. Its QQ efficacy against P. aeruginosa, A. hydrophila, and V. alginolyticus was further demonstrated, in that the motility traits and virulence gene cascades were detrimentally impacted, especially at the mid to late exponential phases of bacterial growth.

These findings collectively suggest that this QQ enzyme would be feasible for use in mitigating membrane biofouling under saline conditions and/or QS-associated pathogenic infections in marine aquaculture.

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AUTHOR CONTRIBUTIONS

T-NW designed and performed the experiments, performed the data analysis, and wrote the manuscript with P-YH, who also supervised the research and provided reagents and materials. Q-TG and AP assisted with the genomic DNA sequence assembly. AK helped to write the manuscript.

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SUPPLEMENTARY MATERIAL

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