Journal of Bioscience and Bioengineering
VOL. 127 No. 4, 411–417, 2019

www.elsevier.com/locate/jbiosc

Distribution and characterization of *N*-acylhomoserine lactone (AHL)-degrading activity and AHL lactonase gene (*qsdS*) in *Sphingopyxis*

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Received 9 July 2018; accepted 7 October 2018
Available online 31 October 2018

***N*-Acylhomoserine lactone (AHL)-degrading enzyme is identified from the various environments and applied for quorum-sensing inhibition. In this study, we isolated two AHL-degrading strains, *Sphingopyxis* sp. EG6 and FD7, from the industrial cooling water samples. When the eight *Sphingopyxis* type strains were checked for the AHL-degrading activity, two strains, *Sphingopyxis alaskensis* DSM 13593 and *Sphingopyxis bauzanensis* DSM 22271, showed high AHL-degrading activity. The complete genome sequences of EG6 and FD7 revealed the presence of gene homolog of *qsdS*, which encodes AHL-lactonase in *Sphingomonas ursincola*. The *qsdS* gene is seated between putative gene homologs involved in 3-isopropylmalate dehydratase large (*leuC2*) and small (*leuD*) subunits in the genome of EG6, FD7, DSM 13593, and DSM 22271, but completely disappeared between *leuC2* and *leuD* in the genome sequences of *Sphingopyxis* type strains without AHL-degrading activity. Purified His-tagged QsdS showed high AHL-degrading activity and catalyzed AHL ring opening by hydrolyzing lactones. In addition, heterologous expression of *qsdS* in *Pseudomonas aeruginosa* resulted in reduction of biofilm formation. These results suggested that the AHL-degrading activity in *Sphingopyxis* is useful as an effective agent for biofilm inhibition.**

[**Keywords:** Quorum sensing; *N*-Acyl-homoserine lactone; *Sphingopyxis*; Quorum quenching; Lactonase]

During cell-to-cell communication in bacteria, groups of bacteria communicate with one another to coordinate their behavior and function, similar to a multicellular organism (1). Quorum sensing is one of the bacterial cell-to-cell communication processes that is stimulated in response to an increase in population density (2). Bacteria release the chemical signaling molecules called autoinducers and respond to the accumulation of autoinducers. In several Proteobacteria, LuxI family protein catalyzes the biosynthesis of *N*-acyl-L-homoserine lactone (AHL) as an autoinducer. LuxI catalyzes the formation of AHL from *S*-adenosyl-L-methionine (SAM) and acyl—acyl carrier protein or CoA-aryl/acyl moieties (2,3). AHL in the environment diffuses into a cell and binds to the LuxR family protein. LuxR-AHL complexes can bind to a specific promoter called the *lux* box to activate the transcription of target genes (2). AHL-mediated quorum sensing regulates the expression of genes involved in various processes (2,4). Furthermore, there have been many studies demonstrating that biofilm formation is regulated by AHL-mediated quorum sensing in several gram-negative bacteria (5). In biofilms, bacterial cells are attached to surfaces by an exopolymeric matrix and interact with each other (5). For instance, in *Pseudomonas aeruginosa*, quorum sensing modulates the transcription of genes required for biosynthesis of the exopolysaccharide surrounding the cells in a biofilm (6).

Quorum quenching is a method for preventing quorum sensing by disrupting signaling (7). So far, various AHL-degrading genes and enzymes have been cloned and characterized from bacteria, fungi, and mammalian cells (7). AHL-degrading enzymes can be categorized into two major functional groups. AHL lactonases catalyze a hydrolysis reaction of the lactone ring in the homoserine moiety of AHLs. The bacterial AHL lactonases have been classified into three major protein families, metallo-β-lactamase superfamily, α/β-hydrolase-fold family, and phosphotriesterase family (7). Paraoxonases observed in the mammalian species have AHL-lactonase activities and to interfere with bacterial quorum sensing (8). AHL acylase hydrolyzes the amide bond of AHL to yield a homoserine lactone and the corresponding fatty acid chain. The AHL acylases have been also classified into three major protein families, penicillin G acylase family, aculeacin A acylase family, and amidase family (9). Cytochrome P450 from *Bacillus megaterium* catalyzes oxidation of AHL and interfere with bacterial quorum sensing (10). AHL-degrading enzymes have been investigated as an approach for controlling biofilm formation, which causes membrane biofouling in water treatment plant (9).

In a previous study, we have demonstrated that genes encoding AHL-degrading enzymes were transferred among the same species by transpositional or non-transpositional events. In strains of *Microbacterium*, which have higher AHL-degrading activity, it has been assumed that the AHL-lactonase gene *aiiM* was transferred from other *Microbacterium* strains by non-transpositional transmission (11). In strains of *Acinetobacter*, it has been suggested that the *amiE* gene, which encodes an AHL acylase belonging to the

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.10.005

amidase family, was transferred to the other *Acinetobacter* strains by a putative transposon (9). We have also previously identified a novel AHL-lactonase gene, *qsdS*, from *Sphingomonas ursincola* isolated from the industrial cooling water systems (12). In this study, we report the identification and presence of a *qsdS* homologous gene in the genus *Sphingopyxis* isolated from the industrial cooling water systems, the distribution of AHL-degrading activity and the *qsdS* gene in strains of *Sphingopyxis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, compounds, and growth conditions *Escherichia coli* DH5α and *P. aeruginosa* PAO1 (13) were grown at 37 °C in Luria–Bertani (LB) medium. Eight *Sphingopyxis* type strains, *Sphingopyxis alaskensis* DSM 13593 (14), *Sphingopyxis bauzanensis* DSM 22271 (15), *Sphingopyxis chilensis* DSM 14889 (16), *Sphingopyxis macrogoltabida* NBRC 15033 (17), *Sphingopyxis soli* DSM 25337 (18), *Sphingopyxis terrae* NBRC 15098 (17), *Sphingopyxis taejonensis* DSM 15583 (19), and *Sphingopyxis witflariensis* DSM 14551 (20) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany or NITE Biological Resource Center (NBRC), Japan. *Sphingopyxis* strains were grown at 30 °C in 1/5 diluted tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD, USA) (12). Two AHL reporters, *Chromobacterium violaceum* CV026 (21) and VIR07 (22), which respond to short-chain and long-chain AHLs, respectively, were grown at 30 °C in LB medium. Solid bacterial media were prepared by adding agar to a final concentration of 1.5%. Antibiotics were added as required at final concentrations of 100 and 50 μg/mL for ampicillin and gentamycin. AHLs used in this study, *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-decanoyl-L-homoserine lactone (C10-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), and *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), were synthesized using a previously described method (23). AHLs were dissolved in dimethyl sulfoxide (DMSO) to prepare 10 mM stock solutions. The primer sequences used in this study are shown in Table S1.

Isolation and identification of bacteria Water samples were collected from the industrial cooling water systems of factory E and factory F. The samples were serially diluted with distilled water, spread on 1/5 TSB agar plates, and incubated at 30 °C for 72 h. Colonies from each sample were randomly selected and transferred onto fresh 1/5 TSB agar medium. For the identification of bacterial species, the 16S rRNA gene was amplified by PCR with Blend Taq Plus DNA polymerase (Toyobo, Osaka, Japan) and the previously described universal primers, 27f and 1525r (24). After electrophoretic separation, amplified 16S rDNA was purified and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500 Series Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The phylogenetic tree was constructed using the neighbor-joining method with the ClustalW program of MEGA software (25).

Assay for AHL-degrading activity Bacterial strains were inoculated into the appropriate medium and incubated for 18 h at 30 °C with shaking. The overnight cultures were resuspended to a final OD_{600} of 0.1 in fresh medium. AHL was added to the medium to a final concentration of 20 μM. Cultures were incubated at 30 °C with shaking, and aliquots were removed at intervals and centrifuged to obtain supernatant. The AHL remaining in the supernatant was detected on LB agar plates containing CV026 or VIR07. Briefly, an overnight culture of CV026 or VIR07 was added to 25 mL of melted LB agar medium and solidified in a petri dish. Supernatants were applied to paper discs (8-mm diameter; Advantec, Tokyo, Japan), and the discs were placed on the LB agar plates mixed with CV026 or VIR07. The plates were incubated overnight at 30 °C, and the appearance of pigment was assessed. The amount of residual AHL was calculated using an equation describing the relationship between the size of the purple zone and the amount of AHL (26).

Genome sequencing Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan). Sequencing was performed on the PacBio RS instrument (Pacific Biosciences, Menlo Park, CA, USA) using libraries prepared with the SMRTbell Template Prep Kit 1.0 (Pacific Biosciences) by Eurofins Genomics (Tokyo, Japan). The sequencing reads were assembled using the PacBio SMRT Portal version 2.3.0 (27). Prediction of putative coding sequences and gene annotations were performed using the Microbial Genome Annotation Pipeline (http://www.migap.org/). Briefly, protein-coding sequences were predicted using a combination of MetaGeneAnnotator (28), RNAmmer (29), tRNAScan (30), and BLAST (31).

Identification of the internal sequences between *leuC2* and *leuD* genes in *Sphingopyxis* strains Genomic DNA of *Sphingopyxis* type strains were extracted using DNeasy Blood & Tissue Kit. Degenerate PCR primers, DG-F1 and DG-R2, were designed based on the most conserved nucleic acid sequences of the *leuC2* and *leuD* genes from the complete genome sequences of *Sphingopyxis*. Degenerate PCR was performed with KOD FX Neo DNA polymerase (Toyobo) using the following cycling parameters: 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 2 min for 30 cycles. After electrophoretic separation, amplified DNA fragments were purified using NucleoSpin Gel and PCR Clean-up (Takara Bio, Shiga, Japan) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500 Series Genetic Analyzer.

Cloning of the *qsdS* gene from *Sphingopyxis* strains The *qsdS* genes from EG6, FD7, DSM 13593, and DSM 22271 were amplified by PCR with KOD FX Neo DNA polymerase and specific primers. PCR was performed with the following cycling parameters: 98 °C for 10 s and 68 °C for 1 min for 30 cycles. The PCR fragments were digested with *Hin*dIII and *Eco*RI for *qsdS* from EG6, or *Eco*RI and *Pst*I for *qsdS* from FD7, DSM13593, and DSM 22271. The digested PCR fragments were inserted into the same restriction sites of the broad-host-range vector pBBR1MCS5 (32). The resulting plasmids containing the *qsdS* genes from EG6, FD7, DSM 13593, and DSM 22271 were designated pBBR-E6Q, pBBR-F7Q, pBBR-A1Q, and pBBR-B2Q, respectively. The constructed plasmids were transformed into *E. coli* DH5α, and transformants were used for the AHL degradation assay. The plasmids pBBR1MCS5, pBBR-E6Q, and pBBR-F7Q were also transformed into *P. aeruginosa* PAO1 via electroporation (33).

Expression and purification of His-tagged AHL lactonases For expression of His-tagged QsdS from EG6 and FD7, the *qsdS* genes were amplified with KOD FX Neo DNA polymerase and specific primers. The pMAL-c2x vector (New England Biolabs, Tokyo, Japan) was used for the expression of His-tagged QsdS under the *tac* promoter. PCR was performed with the following cycling parameters: 94 °C for 30 s, 50 °C for 30 s, and 74 °C for 1 min for 30 cycles. The PCR products were digested by *Nde*I and *Hin*dIII (for EG6) or *Bam*HI (for FD7). The digested PCR fragments were inserted into the same restriction sites on pMAL-c2X for the construction of pQSD-EG6H and pQSD-FD7H. For the expression and purification of His-tagged QsdS, the full-grown culture of *E. coli* DH5α harboring QsdS-expressing plasmid was inoculated into 100 mL of LB medium containing ampicillin and 1 mM isopropyl-β-D-thiogalactoside (IPTG). After incubation for 18 h at 30 °C, the cells were harvested by centrifugation and resuspended with lysis buffer (50 mM Tris–HCl buffer and 150 mM NaCl, pH 8.0). Lysozyme from the egg white (Wako, Osaka, Japan) was added to the suspension at a final concentration of 500 μg/mL. After incubation for 10 min on ice, TritonX-100 was added to the suspension to a final concentration of 1%, sonicated, and centrifuged at 12,000 ×*g* for 5 min to remove the cell debris. Protein purification was performed with the ÄKTA start systems (GE Healthcare, Tokyo, Japan). The protein sample was loaded on a HisTrap affinity chromatography column (GE Healthcare) equilibrated with a loading buffer (20 mM sodium phosphate buffer, 500 mM NaCl, pH 7.4) containing 20 mM imidazole and eluted with the loading buffer containing 500 mM imidazole after washing off unbound bacterial proteins. The expression and purification of the His-tagged QsdS were confirmed by SDS-PAGE analysis.

HPLC analysis HPLC analysis was determined by a previously described method, with slight modifications (34,35). Briefly, to analyze AHL degradation products, 1 μL of the purified protein solution was mixed with 149 μL of the phosphate buffer (1/15 M, pH 7.0, Wako Pure Chemical, Tokyo, Japan) containing 2 mM C8-HSL. After incubation at 20–80 °C for 5 min, the reactions were stopped with 300 μL of acetonitrile, vortexed, and centrifuged to pellet the precipitated protein. Samples (20 μL) were chromatographed on an HPLC system (Jasco, Tokyo, Japan) with a UV/VIS detector set at 205 nm by using a Mightysil RP-18GP column (250 mm × 4.6 mm, 5 μm particle diameter; Kanto Kagaku, Tokyo, Japan). Samples were eluted isocratically with water:acetonitrile:acetic acid (50:50:0.1 [vol/vol/vol]) at 2 mL/min. The amount of C8-HSL was estimated by comparing the reduction in peak areas for a given retention time with a C8-HSL solution of known concentration. The data were reproduced at least three times.

Biofilm formation assay Overnight cultures of PAO1 derivatives were diluted to an OD600 of 0.1 in R2A medium (Becton, Dickinson and Company, Tokyo, Japan). Subsequently, 100 μL of this dilution was transferred to a 96-well polystyrene plate (AsOne, Osaka, Japan). After incubation at 37 °C for 24 h, 25 μL of a 0.1% crystal violet solution was added to each well. The plates were incubated at room temperature for 15 min and rinsed twice with distilled water. The crystal violet was dissolved in 100 μL of 99.5% ethanol, and biofilm formation was analyzed at 595 nm using an Infinite M200 microplate reader (Tecan Japan, Kanagawa, Japan). Seven samples were averaged and standard deviations were calculated. Means were separated using Tukey's honest significant difference (HSD) test ($P < 0.01$).

Elastase assay Overnight cultures of PAO1 derivatives were inoculated into 4 mL of PTSB medium (36) (a 1% inoculum) and incubated for 15 h at 37 °C. Then, 250 μL of each culture supernatant was transferred into a 1.5 mL microtube, and 10 mg of elastin-Congo red and 500 μL of buffer (0.1 M Tris–HCl, 1 mM $CaCl_2$, pH 8.0) were added. The microtubes were incubated for 4 h at 37 °C with shaking. The precipitate was removed by centrifugation, and the absorbance was measured at 495 nm. The relative elastase activity (A_{495}/OD_{600}) was calculated and the control value was set to 100%. Triplicate samples were averaged and standard deviations were calculated. Means were separated using Tukey's HSD test ($P < 0.01$).

AHL production assay Overnight cultures of PAO1 derivatives were inoculated into 50 mL of LB medium (a 1% inoculum) and incubated for 15 h at 37 °C. Cells were removed by centrifugation at 10,000 ×*g* for 5 min. The culture supernatant was extracted with an equal volume of ethyl acetate. The ethyl acetate layer was evaporated to dryness and dissolved in 500 μL of DMSO. AHL production was detected using the AHL reporter strains as described above.

Nucleotide sequence accession number The complete genome of *Sphingopyxis* sp. EG6 was deposited in the DDBJ/ENA/GenBank databases under accession numbers AP017603 (chromosome) and AP017604 (plasmid pSREG01). The complete genome of *Sphingopyxis* sp. FD7 was deposited in the DDBJ/ENA/GenBank databases under accession numbers AP017898 (chromosome) and AP017899 (plasmid pSFD01). The partial sequences between *leuC2* and *leuD* from *Sphingopyxis* type strains were deposited under accession numbers, LC325440 (*S. bauzanensis* DSM 22271), LC325441 (*S. witflariensis* DSM 14551), LC325442 (*S. chilensis* DSM 14889), LC325443 (*S. taejonensis* DSM 15583), and LC325444 (*S. soli* DSM 25337).

RESULTS AND DISCUSSION

Identification of novel AHL-degrading strains from cooling water systems We have isolated *S. ursincola* A1 as an AHL-degrading strain from cooling water samples (12). In this study, approximately 100 colonies were isolated from cooling water samples from other two factories and checked for AHL-degrading activity after incubation with 20 μM C10-HSL. Two strains, EG6 from factory E, and FD7 from factory F, completely degraded C10-HSL within 5 h incubation. To identify the bacterial species, the 16S rRNA genes were amplified and sequenced. Phylogenetic analysis of the genes revealed that EG6 and FD7 showed high similarity to *S. bauzanensis* DSM 22271^T (98.4% identity) and *S. soli* DSM 25337^T (98.7% identity), respectively (Fig. 1). Although these two isolates were assigned to the family *Sphingomonadaceae* as well as *S. ursincola* A1, 16S rRNA sequences of EG6 and FD7 were phylogenetically different from that of *S. ursincola* A1 (Fig. 1). To determine the time course of AHL degradation, EG6 and FD7 were inoculated into 1/5 TSB medium containing 20 μM C6-HSL or C10-HSL. After incubation with shaking at 30 °C, the remaining AHLs in the culture supernatants were detected using AHL reporter strains. EG6 and FD7 completely degraded 20 μM C6-HSL and C10-HSL within 3 and 1 h, respectively (Fig. 2). Both strains showed slightly higher degradation activity against C6-HSL than C10-HSL. The AHL-degrading activity of EG6 was almost the same to that of *S. ursincola* A1, which completely degraded 20 μM C6-HSL and C10-HSL after 3 h at the same condition (12).

AHL-degrading activity in the type strains of genus *Sphingopyxis* To examine the presence of AHL-degrading activity in *Sphingopyxis*, the eight *Sphingopyxis* type strains were obtained and used for the AHL-degrading assay. *Sphingopyxis* strains were cultivated in 1/5 TSB containing 20 μM C6-HSL or C10-HSL. After incubation, the remaining AHLs were visualized using an AHL-reporter strain CV026 and VIR07. The results are shown in Fig. 3A and B. Two strains, *S. alaskensis* DSM 13593 and *S. bauzanensis* DSM 22271, showed high degradation activity against C6-HSL. In contrast, other type strains did not show clear activity against C6-HSL. *S. alaskensis* DSM 13593 and *S. bauzanensis* DSM 22271 also showed high degradation activity against C10-HSL. Although *S. terrae* NBRC 15098 and *S. soli* DSM 25337 showed moderate degradation activity against C10-HSL, other type isolates did not show C10-HSL-degrading activity.

The complete genome revealed the presence of AHL-degrading genes in *Sphingopyxis* isolates To identify the genes involved in AHL degradation, we obtained the complete genomes of EG6 and FD7 using the PacBio RSII platform. For the EG6 results, 149,426 reads were produced, which sequenced approximately 1.56 Gbp. After assembly, the genome of EG6 included a single chromosome and one plasmid. The complete circular chromosome and the endogenous plasmid pSREG01 were 3,848,331 and 28,666 bp in size, respectively. For FD7, 148,716 reads were produced, which sequenced approximately 1.57 Gbp. After assembly, the genome of FD7 also included a single chromosome and one plasmid. The complete circular chromosome and the endogenous plasmid pSFD01 were 3,695,614 and 242,612 bp in size, respectively. The general features of the genome are listed in Table S2.

The homologs of all genes known to encode AHL-degrading enzymes were searched for in the complete genomes of EG6 and FD7. As results of the homology search, SPYCW_0330 from EG6 and SPYCA_2833 from FD7 showed higher similarities to known AiiA-

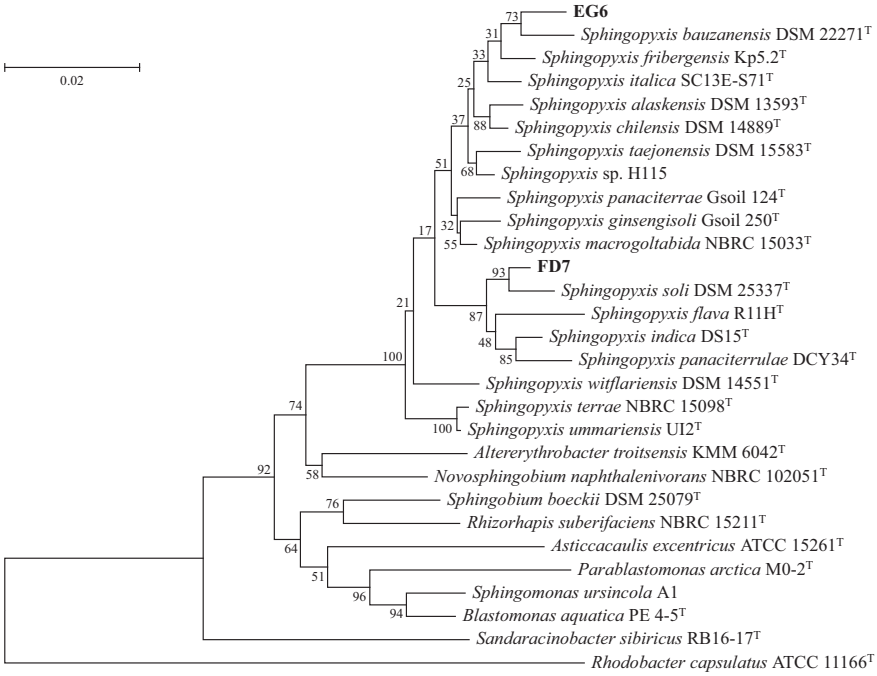

FIG. 1. Phylogenetic tree of 16S rRNA gene sequences from *Sphingopyxis* strains. The bacteria isolated in this study are shown in bold. The phylogenetic tree was constructed by the neighbor-joining method with the ClustalW program of MEGA. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The scale bar represents 0.02 substitutions per nucleotide position.

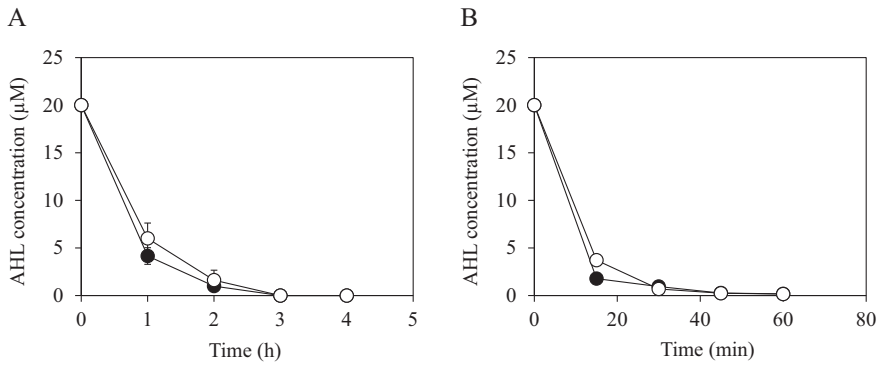

FIG. 2. AHL-degrading activity of *Sphingopyxis* sp. EG6 (A) and FD7 (B). Overnight cultures were inoculated into 1/5 TSB medium containing 20 μM C6-HSL (filled circles) or C10-HSL (open circles) and incubated at 30 °C with shaking. The AHLs remaining in culture supernatants were visualized by spotting the supernatants onto agar mixed with *C. violaceum* CV026 or VIR07. Residual amounts of AHLs were calculated using an equation based on the size of the purple zone. The data were reproduced at least three times and error bars indicate standard deviations.

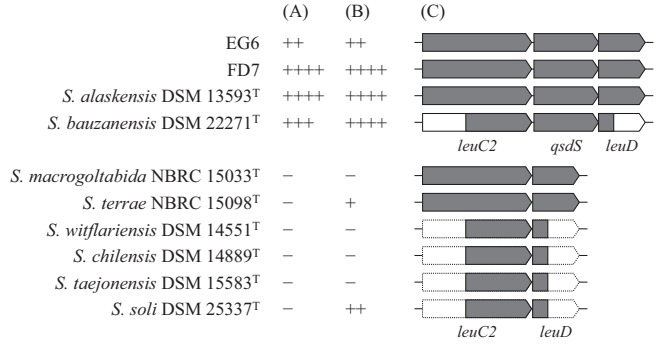

FIG. 3. AHL-degrading activity and the chromosomal locus of the *qsdS* gene in *Sphingopyxis* strains. The degradation activity against 20 μM C6-HSL (A) and C10-HSL (B) are represented as follows: AHL completely degraded within 4 h (+), 3 h (++), 2 h (+++), and 1 h (++++); AHL remaining after 4 h incubation (–). (C) The position and orientation of the *leuC2*, *qsdS*, and *leuD* genes are represented by pentagons. The filled parts indicate regions in which the nucleotide sequences have been obtained. The open parts surrounded by dotted lines indicate regions that were predicted to be coding sequences.

like AHL lactonases. In a previous study, we have identified and characterized a novel AHL lactonase, designated QsdS, from *S. ursincola* A1 (12). The amino acid sequence of SPYCW_0330 and SPYCA_2833 showed 58.8% and 58.3% identities to QsdS from *S. ursincola* A1, respectively. A conserved zinc-binding motif (HXHXDH) is essential for AHL degradation by AiiA-like AHL lactonases (37). Multiple sequence alignment revealed that this motif is found in the amino acid sequence of QsdS from four *Sphingopyxis* strains and *S. ursincola* A1 (Fig. 4). It has been demonstrated that QsdS from *S. ursincola* A1 was secreted and function outside of the cell (12). QsdS homologs from EG6 and FD7 were also predicted to be extracellular with an N-terminal signal peptide of 21 amino acid residues based on SignalP analysis (38) (Fig. 4). In fact, AHL was drastically decreased in the cell-free supernatant of EG6 and FD7 (data not shown). These results assumed that QsdS from EG6 and FD7 functions as an extracellular AHL-degrading enzyme similar to that of *S. ursincola* A1.

Genes encoding an AHL-degrading homolog were present in specific *Sphingopyxis* strains with higher AHL-degrading activity The complete genome of *S. alaskensis* DSM 13593 (synonym strain RB2256) has been reported (39). We found that the *qsdS* homologous gene (Sala_2130) was present in the genome of DSM 13593. The *qsdS* gene was seated between putative gene homologs involved in the large and small subunits of 3-isopropylmalate dehydratase (*leuC2* and *leuD*, respectively) in the genomes of EG6, FD7, and DSM 13593 (Fig. 3C). In contrast, the *qsdS* gene homolog was completely absent between *leuC2* and *leuD* in the complete genomes of *S. macrogoltabida* NBRC 15033 (synonym strain 203) (40) and *S. terrae* NBRC 15098 (41), both of which lack C6-HSL-degrading activity (Fig. 3C). Based on these results, the partial sequence between *leuC2* and *leuD* was amplified from the genome of additional five *Sphingopyxis* type strains. The *qsdS* gene homolog was also found between *leuC2* and *leuD* in *S. bauzanensis* DSM 22271, which show higher C6-HSL-degrading activity (Fig. 3C). In contrast, the *qsdS* gene homolog was completely absent between *leuC2* and *leuD* in *S. witflariensis* DSM 14551, *S. chilensis* DSM 14889, *S. taejonensis* DSM 15583, and *S. soli* DSM 25337, all of which lack C6-HSL-degrading activity (Fig. 3C). These results assumed that the higher degradation activity against C6-HSL was due to the presence of the *qsdS* gene homolog between *leuC2* and *leuD* in genus *Sphingopyxis*.

The *qsdS*-harboring *Sphingopyxis* strains, EG6, DSM 22271, and DSM 13593, were closely related on the 16S rRNA-based phylogenetic tree (Fig. 1). In contrast, *S. soli* DSM 25337, which is closely related to FD7, did not have *qsdS* gene homolog. As the result of a BLAST search, we found the *qsdS* gene homolog on the draft genome of *Sphingopyxis* sp. H115, which was isolated from a chlorinated drinking water distribution system simulator (42). However, the 16S rRNA sequence of H115 did not show any similarities with those of *qsdS*-harboring *Sphingopyxis* strains identified in this study (Fig. 1). We have previously reported that three *Acinetobacter* strains, which were phylogenetically different each other, shared an almost completely identical AHL-acylase gene (*amiE*), and many transposase-like genes were found around the *amiE* sequences (9). In contrast, the transposase-like sequences were not present around the *qsdS*-cording region in *Sphingopyxis* strains, and it was found that the stop codon of *leuC2* overlaps the start codon of *leuD2* in the genomes of *Sphingopyxis* strains, which did not contain *qsdS* gene. We also investigated the presence of a *qsdS* gene homolog in the complete genomes of other *Sphingomonadaceae* species. The *qsdS* gene homolog was located between the *leuC2* and *leuD* genes in the chromosome of *Sphingomonas sanxanigenens* DSM 19645 (accession no. CP006644), *Sphingobium* sp. SYK-6 (AP012222), and *Blastomonas* sp. RAC04 (CP016460), but not in those of *Sphingobium indicum* B90A (CP013070), *Sphingorhabdus* sp. M41 (CP014545), and *Novosphingobium aromaticivorans* DSM 12444 (CP000248). From these results, we assumed that the *qsdS* gene was obtained or missed by a non-transpositional event in the process of evolution among the family *Sphingomonadaceae*.

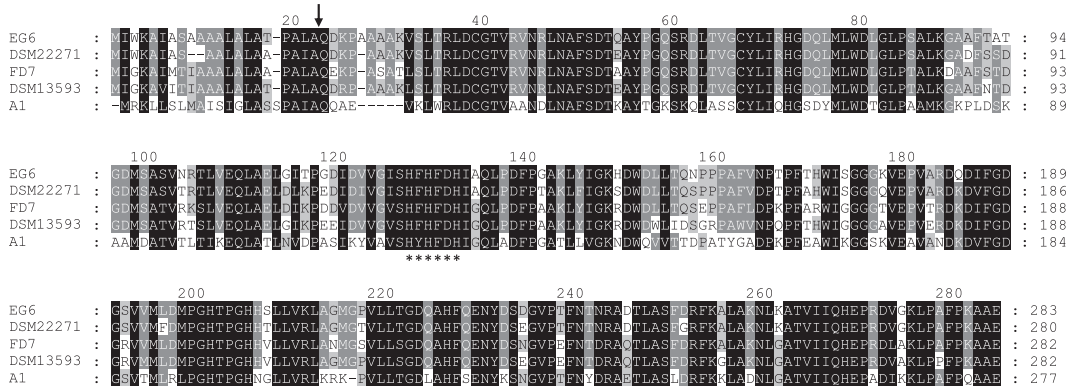

FIG. 4. Comparison of the amino acid sequences of QsdS from *Sphingopyxis* sp. EG6 and FD7, *S. alaskensis* DSM 13593, *S. bauzanensis* DSM 22271, and *S. ursincola* A1. Sequences were aligned using ClustalW and shaded using GeneDoc software. The consensus amino acid sequence (HXHXDH) is marked with asterisks. QsdS harbors an N-terminal signal peptide, and the predicted cleavage site was labeled by arrow.

Characterization and application of AHL-degrading activity of QsdS The *qsdS* genes were cloned from the four *Sphingopyxis* strains and then evaluated for their AHL-degrading activities. *E. coli* harboring QsdS-expressing plasmid from these strains showed higher degradation activity against AHL with a short acyl chain than that with a long acyl chain (Table 1). Although *E. coli* that expressed QsdS from EG6, FD7, or DSM 22271 showed similar AHL-degrading properties, *E. coli* that expressed QsdS from DSM 13593 showed slightly higher AHL-degrading activity compared with other QsdS.

QsdS from EG6 and FD7 was expressed in *E. coli* as His-tagged fusion proteins, and then purified by immobilized-metal affinity chromatography. The results from the SDS-PAGE analysis revealed that the overexpressed products were approximately 30 kDa in size as expected (data not shown). To determine whether QsdS functions as an AHL-lactonase, the structure of C8-HSL treated with QsdS was analyzed by HPLC. Fractionation of QsdS-treated C8-HSL revealed two HPLC peaks, which corresponded to those of the remaining C8-HSL and the lactone ring-opened C8-HSL (Fig. S1). These results indicated that QsdS works as an AHL-lactonase that catalyzes AHL ring opening by hydrolyzing lactones. To estimate the optimal temperature of QsdS from EG6 and FD7, C8-HSL was degraded by QsdS and analyzed by HPLC at various temperatures. QsdS from EG6 displayed over 80% of its maximum activity at 50–70 °C, but the relative activity was greatly reduced at over 80 °C (Fig. 5A). QsdS from FD7 displayed over 80% of its maximum activity at 60–70 °C, but its activity was completely diminished after pre-incubation at 70 °C (Fig. 5B). We have previously compared the optimal temperature and thermostability of various AHL lactonases (34). Although AiiT lactonase from *Thermaerobacter marianensis*, which is an extremely thermophilic bacterium, showed a higher optimal temperature and better thermostability, other AHL lactonases showed AHL-degrading activities at temperatures ranging from 40 to 60 °C. QsdS from EG6 and FD7 displayed over 80% of its maximum activity at 60–70 °C at a slightly higher optimal temperature than that of other bacteria.

The opportunistic pathogen *P. aeruginosa* produces two AHLs, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL), and regulates biofilm formation and expression of elastase by AHL-mediated quorum sensing (6). For the application of *qsdS* for quorum-sensing

TABLE 1. AHL-degrading activity of *E. coli* harboring QsdS-expressing plasmid.

	Control	Sources of QsdS			
		EG6	FD7	DSM 22271	DSM 13593
C6-HSL	–[a]	++	++	++	+++
3-oxo-C6-HSL	–	++	++	++	+++
C10-HSL	–	+	+	+	++
3-oxo-C10-HSL	–	+	+	+	++

[a] AHLs are completely degraded within 8 h (+), 6 h (++), and 4 h (+++); AHLs are remained after 8 h incubation (–).

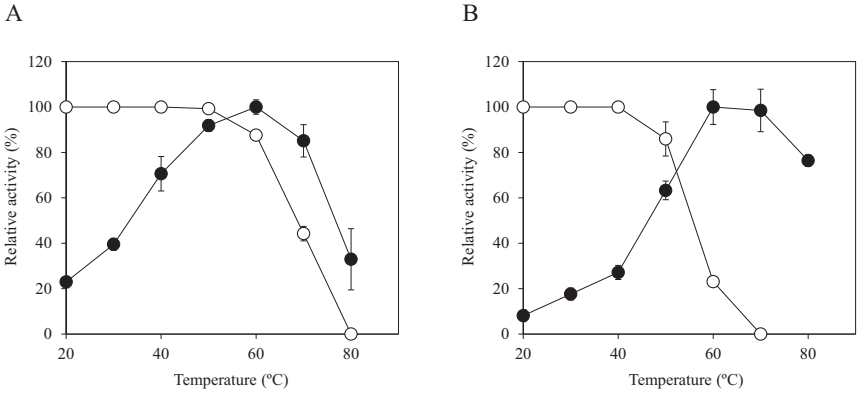

FIG. 5. Assay for optimal temperature (filled circles) and thermostability (open circles) of QsdS from EG6 (A) and FD7 (B). To assay for the optimal temperature, purified QsdS was mixed with C8-HSL and incubated at temperatures ranging from 20 to 90 °C. To assay for thermostability, QsdS was pre-incubated at temperatures ranging from 20 to 90 °C for 10 min. The pre-incubated proteins were mixed with C8-HSL and incubated at the optimal temperature. After incubation for 5 min, the residual substrate was quantified by HPLC. The maximum activity of each AHL lactonase was defined as 100%. The data were reproduced at least three times, and error bars indicate standard deviations.

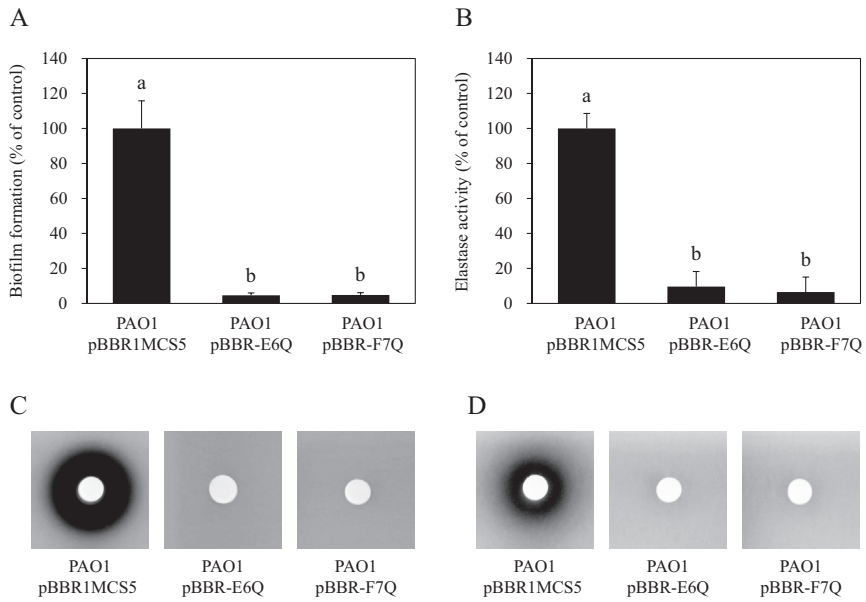

FIG. 6. Biofilm formation (A) and elastase activity (B) of PAO1 harboring pBBR1MCS5, pBBR-E6Q, or pBBR-F7Q were assayed as above. The maximum value was defined as 100%. The data were reproduced at least three times and error bars indicate standard deviations. Different letters above the bars indicate significant differences among treatments according to Tukey's HSD test ($P < 0.01$). (C) C4-HSL production by PAO1 derivatives was detected using *C. violaceum* CV026. (D) 3-oxo-C12-HSL production by PAO1 derivatives was detected using *C. violaceum* VIR07.

inhibition, we evaluated the potential use of the *qsdS* gene for interfering with quorum sensing in *P. aeruginosa* PAO1. Although PAO1 harboring pBBR1MCS5 showed a certain degree of biofilm formation and elastase activity, they were markedly reduced in PAO1 harboring pBBR-E6Q or pBBR-F7Q (Fig. 6A,B). C4-HSL and 3-oxo-C12-HSL were detected in the culture supernatant of PAO1 harboring pBBR1MCS5, but not in that of PAO1 harboring pBBR-E6Q and pBBR-F7Q (Fig. 6C,D). These results indicated that the expression of QsdS in PAO1 contributed to the self-degradation of AHLs and the interruption of biofilm formation in PAO1.

In conclusion, our study demonstrated that the *qsdS* gene, which encodes an AHL lactonase, was widely conserved in AHL-degrading *Sphingopyxis* and other *Sphingomonadaceae* strains. In general, *Sphingomonadaceae* strains are well-known oligotrophic bacteria that are isolated from oligotrophic water environments such as drinking water treatment plants, tap water, and water demineralization filters (43). In such environments, there are concerns that the development of biofilms may cause significant problems such as pipe corrosion, consumption of residual disinfectants, and the generation of tastes and odors (44). It is possible that interrupting the quorum sensing is an effective method to control bacterial biofilm formation in the oligotrophic environments. The AHL-degrading *Sphingomonadaceae* strains might be useful as an effective microbial agent, which has both inhibitory activity of biofilm formation and viability in oligotrophic water environments.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.10.005.

ACKNOWLEDGMENTS

T.M. reports grants from Kurita Water Industries Ltd. (Tochigi, Japan), during the conduct of the study; and T.I. is employee of Kurita Water Industries Ltd. The authors declare no other conflicts of interest.

References

1. **Bassler, B. L.:** Small talk. Cell-to-cell communication in bacteria, Cell, **109**, 421–424 (2002).
2. **Parsek, M. R. and Greenberg, E. P.:** Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms, Proc. Natl. Acad. Sci. USA, **97**, 8789–8793 (2000).
3. **Dong, S. H., Frane, N. D., Christensen, Q. H., Greenberg, E. P., Nagarajan, R., and Nair, S. K.:** Molecular basis for the substrate specificity of quorum signal synthases, Proc. Natl. Acad. Sci. USA, **114**, 9092–9097 (2017).
4. **Waters, C. M. and Bassler, B. L.:** Quorum sensing: cell-to-cell communication in bacteria, Annu. Rev. Cell. Dev. Biol., **21**, 319–346 (2005).
5. **Davey, M. E. and O'Toole, G. A.:** Microbial biofilms: from ecology to molecular genetics, Microbiol. Mol. Biol. Rev., **64**, 847–867 (2000).
6. **De Kievit, T.:** Quorum sensing in *Pseudomonas aeruginosa* biofilms, Environ. Microbiol., **11**, 279–288 (2009).
7. **Uroz, S., Dessaux, Y., and Oger, P.:** Quorum sensing and quorum quenching: the yin and yang of bacterial communication, Chembiochem, **10**, 205–216 (2009).
8. **Ozer, E. A., Pezzulo, A., Shih, D. M., Chun, C., Furlong, C., Lusis, A. J., Greenberg, E. P., and Zabner, J.:** Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing, FEMS Microbiol. Lett., **253**, 29–37 (2005).
9. **Ochiai, S., Yasumoto, S., Morohoshi, T., and Ikeda, T.:** AmiE, a novel *N*-acyl-homoserine lactone acylase belonging to the amidase family, from the activated-sludge isolate *Acinetobacter* sp. strain Ooi24, Appl. Environ. Microbiol., **80**, 6919–6925 (2014).
10. **Chowdhary, P. K., Keshavan, N., Nguyen, H. Q., Peterson, J. A., Gonzalez, J. E., and Haines, D. C.:** *Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines, Biochemistry, **46**, 14429–14437 (2007).
11. **Wang, W.-Z., Morohoshi, T., Someya, N., and Ikeda, T.:** Diversity and distribution of *N*-acylhomoserine lactone (AHL)-degrading activity and AHL-lactonase (AiiM) in genus *Microbacterium*, Microbes Environ., **27**, 330–333 (2012).
12. **Morohoshi, T., Sato, N., Iizumi, T., Tanaka, A., and Ikeda, T.:** Identification and characterization of a novel *N*-acyl-homoserine lactonase gene in *Sphingomonas ursincola* isolated from industrial cooling water systems, J. Biosci. Bioeng., **123**, 569–575 (2017).
13. **Holloway, B. W., Krishnapillai, V., and Morgan, A. F.:** Chromosomal genetics of *Pseudomonas*, Microbiol. Rev., **43**, 73–102 (1979).
14. **Vancanneyt, M., Schut, F., Snauwaert, C., Goris, J., Swings, J., and Gottschal, J. C.:** *Sphingomonas alaskensis* sp. nov., a dominant bacterium from a marine oligotrophic environment, Int. J. Syst. Evol. Microbiol., **51**, 73–79 (2001).
15. **Zhang, D. C., Liu, H. C., Xin, Y. H., Zhou, Y. G., Schinner, F., and Margesin, R.:** *Sphingopyxis bauzanensis* sp. nov., a psychrophilic bacterium isolated from soil, Int. J. Syst. Evol. Microbiol., **60**, 2618–2622 (2010).
16. **Godoy, F., Vancanneyt, M., Martinez, M., Steinbuchel, A., Swings, J., and Rehm, B. H.:** *Sphingopyxis chilensis* sp. nov., a chlorophenol-degrading bacterium that accumulates polyhydroxyalkanoate, and transfer of *Sphingomonas alaskensis* to *Sphingopyxis alaskensis* comb. nov., Int. J. Syst. Evol. Microbiol., **53**, 473–477 (2003).

17. **Takeuchi, M., Kawai, F., Shimada, Y., and Yokota, A.:** Taxonomic study of polyethylene glycol-utilizing bacteria: emended description of the genus *Sphingomonas* and new descriptions of *Sphingomonas macrogoltabidus* sp. nov., *Sphingomonas sanguis* sp. nov. and *Sphingomonas terrae* sp. nov., Syst. Appl. Microbiol., **16**, 227–238 (1993).
18. **Choi, J. H., Kim, M. S., Jung, M. J., Roh, S. W., Shin, K. S., and Bae, J. W.:** *Sphingopyxis soli* sp. nov., isolated from landfill soil, Int. J. Syst. Evol. Microbiol., **60**, 1682–1686 (2010).
19. **Lee, J. S., Shin, Y. K., Yoon, J. H., Takeuchi, M., Pyun, Y. R., and Park, Y. H.:** *Sphingomonas aquatilis* sp. nov., *Sphingomonas koreensis* sp. nov., and *Sphingomonas taejonensis* sp. nov., yellow-pigmented bacteria isolated from natural mineral water, Int. J. Syst. Evol. Microbiol., **51**, 1491–1498 (2001).
20. **Kampfer, P., Witzenberger, R., Denner, E. B., Busse, H. J., and Neef, A.:** *Sphingopyxis witflariensis* sp. nov., isolated from activated sludge, Int. J. Syst. Evol. Microbiol., **52**, 2029–2034 (2002).
21. **McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S., and Williams, P.:** Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones, Microbiology, **143**, 3703–3711 (1997).
22. **Morohoshi, T., Kato, M., Fukamachi, K., Kato, N., and Ikeda, T.:** *N*-acylhomoserine lactone regulates violacein production in *Chromobacterium violaceum* type strain ATCC 12472, FEMS Microbiol. Lett., **279**, 124–130 (2008).
23. **Chhabra, S. R., Harty, C., Hooi, D. S., Daykin, M., Williams, P., Telford, G., Pritchard, D. I., and Bycroft, B. W.:** Synthetic analogues of the bacterial signal (quorum sensing) molecule *N*-(3-oxododecanoyl)-L-homoserine lactone as immune modulators, J. Med. Chem., **46**, 97–104 (2003).
24. **Devereux, R. and Willis, S. G.:** Amplification of ribosomal RNA sequences, pp. 277–287, in: Akkermans, A. D. L., van Elsas, J. D., and De Bruijn, F. J. (Eds.), Molecular microbial ecology manual. Springer, Dordrecht (1995).
25. **Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S.:** MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol., **30**, 2725–2729 (2013).
26. **Morohoshi, T., Nakazawa, S., Ebata, A., Kato, N., and Ikeda, T.:** Identification and characterization of *N*-acylhomoserine lactone-acylase from the fish intestinal *Shewanella* sp. strain MIB015, Biosci. Biotechnol. Biochem., **72**, 1887–1893 (2008).
27. **Chin, C. S., Alexander, D. H., Marks, P., Klammer, A. A., Drake, J., Heiner, C., Clum, A., Copeland, A., Huddleston, J., Eichler, E. E., Turner, S. W., and Korlach, J.:** Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data, Nat. Methods, **10**, 563–569 (2013).
28. **Noguchi, H., Taniguchi, T., and Itoh, T.:** MetaGeneAnnotator: detecting species-specific patterns of ribosomal binding site for precise gene prediction in anonymous prokaryotic and phage genomes, DNA Res., **15**, 387–396 (2008).
29. **Lagesen, K., Hallin, P., Rodland, E. A., Staerfeldt, H. H., Rognes, T., and Ussery, D. W.:** RNAmmer: consistent and rapid annotation of ribosomal RNA genes, Nucleic Acids Res., **35**, 3100–3108 (2007).
30. **Lowe, T. M. and Eddy, S. R.:** tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence, Nucleic Acids Res., **25**, 955–964 (1997).
31. **Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J.:** Basic local alignment search tool, J. Mol. Biol., **215**, 403–410 (1990).
32. **Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd, and Peterson, K. M.:** Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes, Gene, **166**, 175–176 (1995).
33. **Smith, A. W. and Iglewski, B. H.:** Transformation of *Pseudomonas aeruginosa* by electroporation, Nucleic Acids Res., **17**, 10509 (1989).
34. **Morohoshi, T., Tominaga, Y., Someya, N., and Ikeda, T.:** Characterization of a novel thermostable *N*-acylhomoserine lactonase from the thermophilic bacterium *Thermaerobacter marianensis*, J. Biosci. Bioeng., **120**, 1–5 (2015).
35. **Wang, W. Z., Morohoshi, T., Ikenoya, M., Someya, N., and Ikeda, T.:** AiiM, a novel class of *N*-acylhomoserine lactonase from the leaf-associated bacterium *Microbacterium testaceum*, Appl. Environ. Microbiol., **76**, 2524–2530 (2010).
36. **Ohman, D. E., Cryz, S. J., and Iglewski, B. H.:** Isolation and characterization of *Pseudomonas aeruginosa* PAO mutant that produces altered elastase, J. Bacteriol., **142**, 836–842 (1980).
37. **Dong, Y. H., Xu, J. L., Li, X. Z., and Zhang, L. H.:** AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*, Proc. Natl. Acad. Sci. USA, **97**, 3526–3531 (2000).
38. **Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H.:** SignalP 4.0: discriminating signal peptides from transmembrane regions, Nat. Methods, **8**, 785–786 (2011).
39. **Lauro, F. M., McDougald, D., Thomas, T., Williams, T. J., Egan, S., Rice, S., DeMaere, M. Z., Ting, L., Ertan, H., Johnson, J., and other 11 authors:** The genomic basis of trophic strategy in marine bacteria, Proc. Natl. Acad. Sci. USA, **106**, 15527–15533 (2009).
40. **Ohtsubo, Y., Nagata, Y., Numata, M., Tsuchikane, K., Hosoyama, A., Yamazoe, A., Tsuda, M., Fujita, N., and Kawai, F.:** Complete genome sequence of *Sphingopyxis macrogoltabida* type strain NBRC 15033, originally isolated as a polyethylene glycol degrader, Genome Announc., **3**, e01401-15 (2015).
41. **Ohtsubo, Y., Nonoyama, S., Nagata, Y., Numata, M., Tsuchikane, K., Hosoyama, A., Yamazoe, A., Tsuda, M., Fujita, N., and Kawai, F.:** Complete genome sequence of *Sphingopyxis terrae* strain 203-1 (NBRC 111660), a polyethylene glycol degrader, Genome Announc., **4**, e00530-16 (2016).
42. **Gomez-Alvarez, V., Pfaller, S., and Revetta, R. P.:** Draft genome sequence of two *Sphingopyxis* sp. strains, dominant members of the bacterial community associated with a drinking water distribution system simulator, Genome Announc., **4**, e00183-16 (2016).
43. **Vaz-Moreira, I., Nunes, O. C., and Manaia, C. M.:** Diversity and antibiotic resistance patterns of Sphingomonadaceae isolates from drinking water, Appl. Environ. Microbiol., **77**, 5697–5706 (2011).
44. **Donlan, R. M.:** Biofilms: microbial life on surfaces, Emerg. Infect. Dis., **8**, 881–890 (2002).