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AmiE, a Novel *N*-Acylhomoserine Lactone Acylase Belonging to the Amidase Family, from the Activated-Sludge Isolate *Acinetobacter* sp. Strain Ooi24

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Many Gram-negative bacteria use *N*-acyl-L-homoserine lactones (AHLs) as quorum-sensing signal molecules. We have reported that *Acinetobacter* strains isolated from activated sludge have AHL-degrading activity. In this study, we cloned the *amiE* gene as an AHL-degradative gene from the genomic library of *Acinetobacter* sp. strain Ooi24. High-performance liquid chromatography analysis revealed that AmiE functions as an AHL acylase, which hydrolyzes the amide bond of AHL. AmiE showed a high level of degrading activity against AHLs with long acyl chains but no activity against AHLs with acyl chains shorter than eight carbons. AmiE showed homology with a member of the amidases (EC 3.5.1.4) but not with any known AHL acylase enzymes. An amino acid sequence of AmiE from Ooi24 showed greater than 99% identities with uncharacterized proteins from *Acinetobacter ursingii* CIP 107286 and *Acinetobacter* sp. strain CIP 102129, but it was not found in the draft or complete genome sequences of other *Acinetobacter* strains. The presence of transposase-like genes around the *amiE* genes of these three *Acinetobacter* strains suggests that *amiE* is transferred by a putative transposon. Furthermore, the expression of AmiE in *Pseudomonas aeruginosa* PAO1 reduced AHL accumulation and elastase activity, which were regulated by AHL-mediated quorum sensing.

Quorum sensing is a cell-to-cell communication system that is used by many species of bacteria and that depends on their population densities (1). Their communications are regulated by an autoinducer (AI), a self-produced signal molecule specific for a certain bacterium (1). Once the AI reaches a threshold concentration, gene transcription is activated, resulting in the expression of phenotypes such as motility, adhesion, biofilm formation, toxicity, and pathogenicity (2). *N*-Acyl-L-homoserine lactone (AHL) is one of the most common AIs produced by Gram-negative bacteria (3). It consists of an alkyl chain of various lengths appended to a lactone ring via an amide bond. The variation in length, saturation, and side chain substitution in the alkyl chain gives specificity to the signal molecule, which enables intraspecies communication (3). During the last decade, many AHL-degradative genes have been cloned from various microorganisms (4). AHL lactonase catalyzes AHL ring opening by hydrolyzing lactones, and AHL acylase hydrolyzes the amide bond of AHL (4).

The activated sludge process is a wastewater treatment widely used for sewage and human waste (5). Recent studies of the activated sludge process have focused on its microbiological aspects to improve the biodegradation capability and process management techniques (6, 7). The internal environment of these activated sludge flocs is similar to that of biofilms; high cell densities are present, allowing bacterial activation via bacterial cell-to-cell communication (6). It has been reported that the AHL-mediated quorum-sensing system affects the wastewater treatment process that uses activated sludge (8). It has been observed that quorum sensing is associated with the formation of a biofouling layer on the membrane surface in a membrane bioreactor (MBR) wastewater treatment system (7). The use of AHL-degrading enzymes has been investigated as an approach to controlling membrane biofouling in wastewater treatment systems (9).

AHL-producing and AHL-degrading bacteria coexist in various ecosystems, including the fish intestine and plant surfaces, and have various strategies for gaining a competitive advantage (10, 11). In a previous study, we reported that AHL-producing and AHL-degrading strains coexist in activated sludge obtained from sewage treatment plants in Japan, and the most dominant AHL-degrading isolates were assigned to the genus *Acinetobacter* (12). One of the AHL-degrading *Acinetobacter* strains, *Acinetobacter* sp. strain Ooi24, had the strongest AHL-degrading activity and showed putative AHL acylase activity (12). Although AHL lactonase activity has been detected in *Acinetobacter* sp. strain GG2, isolated from the rhizosphere of ginger (13), no reports of the cloning of AHL acylase genes from the genus *Acinetobacter* have been published. In this study, we report the cloning and characterization of a gene that encodes a novel AHL acylase from *Acinetobacter* sp. Ooi24.

MATERIALS AND METHODS

Bacterial strains, plasmids, compounds, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α and *Pseudomonas aeruginosa* PAO1 (14) were grown at 37°C in Luria-Bertani (LB) medium (15). AHL biosensor strains *Chromobacterium violaceum* CV026 and VIR07, which respond to short-chain and long-chain AHLs, respectively, were grown at 30°C in LB medium (16, 17). Solid bacterial media were prepared by adding agar to the liquid medium to a final concentration of 1.5%. Antibiotics were added as required at final concentrations of 100 μg/ml for ampicillin, 10 μg/ml for chloramphenicol, and 50 μg/ml for gentamicin. The AHLs used in this study, *N*-hexanoyl-L-homoserine lactone (C_4-HSL), *N*-hexanoyl-L-ho-

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
Escherichia coli DH5α	F$^-$ *supE44* Δ*(lacZYA-argF)U169* ϕ80d*lacZ*ΔM15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Nippon Gene
Chromobacterium violaceum		
CV026	ATCC 31532 derivative, *cviI*::Tn*5 xylE* Km^r Sm^r	16
VIR07	ATCC 12472 derivative, *cviI*::Km^r Ap^r	17
Acinetobacter sp. Ooi24	AHL-degrading strain isolated from activated sludge	12
Pseudomonas aeruginosa PAO1	Wild-type strain	14
Plasmids		
pUC118	Cloning vector, Ap^r	TaKaRa Bio
pAO24-1	5,356-bp Sau3AI fragment from Ooi24 genomic DNA in pUC118	This study
pGEM-T Easy	Cloning vector, Ap^r	Promega
pGEM-amiE	pGEM-T Easy containing *amiE* from Ooi24	This study
pLas28	pSTV28 vector containing *lasI* from PAO1, Cm^r	26
pBBR1MCS5	Broad-host-range cloning vector, Gm^r	22
pBBR1-ahlS	pBBR1MCS5 containing *ahlS* from *Solibacillus silvestris* StLB046	23
pBBR1-amiE	pBBR1MCS5 containing *amiE* from Ooi24	This study

moserine lactone (C_6-HSL), *N*-octanoyl-L-homoserine lactone (C_8-HSL), *N*-decanoyl-L-homoserine lactone (C_{10}-HSL), *N*-dodecanoyl-L-homoserine lactone (C_{12}-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone, *N*-(3-oxooctanoyl)-L-homoserine lactone, *N*-(3-oxodecanoyl)-L-homoserine lactone ($3OC_{10}$-HSL), and *N*-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL), were synthesized using a previously described method (18). The identities and purities of all compounds were verified by ^{1}H nuclear magnetic resonance. AHLs were dissolved in dimethyl sulfoxide (DMSO) to prepare 10 mM stock solutions.

Identification of the AHL-degradative gene from *Acinetobacter* sp. Ooi24. For the cloning of the AHL-degradative gene, a pUC118-based genomic library of Ooi24 was constructed according to a previously described method (19). Briefly, chromosomal DNA of Ooi24 was partially digested with Sau3AI, and the fragments were inserted into the BamHI site of cloning vector pUC118 that had been dephosphorylated by bacterial alkaline phosphatase (TaKaRa Bio, Shiga, Japan). The genomic library of Ooi24 and the pLas28 plasmid were transformed into *E. coli*. Then, *E. coli* harboring the genomic library and pLas28 was inoculated into LB agar medium prepared in 96-well plates. After incubation at 30°C for 24 h, VIR07 was also inoculated into the lower position of the well. After incubation at 30°C for 24 h, the colonies that did not induce the production of purple pigments by VIR07 were selected as AHL-degradative clones. Positive clones were sequenced using a BigDye Terminator (version 3.1) sequencing kit and an ABI 3500 genetic analyzer (Applied Biosystems, Tokyo, Japan).

To amplify the upstream regions of the genomic fragment in the positive clone, we carried out inverse PCR. The chromosomal DNA of Ooi24 was digested with EcoRV and then self-ligated. The inverse primers 5′-TAA AAG AGG TCC TGG AGT GAG CAG CAC TGC TGC G-3′ and 5′-AAC TAA GTC GAT TGT CCA TGT CGG CAT CTA CGC C-3′ were designed in accordance with the nucleotide sequences of the genomic fragment in the positive clone. PCR was performed with KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) using the following cycling parameters: 98°C for 10 s, 60°C for 30 s, and 68°C for 5 min for 30 cycles.

Detection of the AHL-degrading activity of *amiE*. The *amiE*-coding region on the Ooi24 genome was amplified with Blend *Taq* DNA polymerase (Toyobo) and the following primers: 5′-TAC TTG TTC GC AAT GTG TGA TGG CAC GC-3′ and 5′-CCA CGT TTA TTG AGC AAT GTC CAA ACA ATG GG-3′. PCR was performed with the following cycling parameters: 94°C for 30 s, 60°C for 30 s, and 74°C for 3 min for 30 cycles. The PCR products were cloned into the pGEM-T Easy cloning vector (Promega, Tokyo, Japan) for construction of pGEM-amiE. The full-grown culture of *E. coli* harboring pGEM-amiE was inoculated into fresh LB medium (a 1% inoculum) with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultivated for 16 h. Cells were collected via centrifugation, washed with 50 mM phosphate buffer (pH 7.4), and resuspended in 1 ml phosphate buffer. Then, 2 μl of AHL stock solution was mixed with the cell suspension at a final concentration of 20 μM. For the control, 2 μl of DMSO was mixed with the cell suspension. After incubation at 30°C with shaking, the cells were removed via centrifugation for preparation of the supernatant. The residual AHLs were detected using the AHL biosensors CV026 and VIR07. An overnight culture of CV026 or VIR07 was mixed with 25 ml LB agar medium, and the mixture was poured onto the plates. AHL samples were applied to paper discs with 8-mm diameters (Advantec, Tokyo, Japan), and the discs were placed onto LB agar plates containing CV026 or VIR07. The assay plates were incubated overnight at 30°C, and the appearance of pigment was assessed. The residual amounts of AHL were calculated using relationship equations based on the sizes of the purple zones and known amounts of AHLs (20).

Identification of AHL acylase activity. Detection of AHL acylase activity was performed according to a previously described method with modification (20, 21). The full-grown culture of *E. coli* harboring pGEM-amiE was inoculated into fresh LB medium (a 1% inoculum) with 1 mM IPTG and cultivated for 16 h. Cells were collected via centrifugation, washed with 50 mM phosphate buffer (pH 7.4), and resuspended in 1 ml of phosphate buffer containing 500 μM $3OC_{10}$-HSL. The reaction mixture was incubated at 30°C for 6 h and centrifuged to remove the cells. Then, 150 μl of supernatant was mixed with 150 μl of saturated borax solution and 300 μl of dansyl chloride (10 mg/ml in acetone) at 40°C for 1 h. As a control, phosphate buffer containing 500 μM L-homoserine lactone (HSL) was dansylated in parallel with the same reagents.

Twenty microliters of sample was chromatographed on a high-performance liquid chromatography (HPLC) system (Jasco, Tokyo, Japan) with a UV-visible detector set at 270 nm and a Mightysil RP-18GP column (250 mm by 4.6 mm, 5-μm particle diameter; Kanto Kagaku, Tokyo, Japan). The initial mobile phase was water-acetonitrile-acetic acid (75:25:0.05 [vol/vol/vol]), and the elution was created with 100% acetonitrile running at 2 ml/min according to the following profile: 0 to 2.5 min, 0%; 2.5 to 7.5 min, 0 to 100%; and 7.5 to 10 min, 100%.

Effect of AmiE on production of AHLs and virulence factors in *P. aeruginosa*. The *amiE*-coding region on the Ooi24 genome was amplified with Blend *Taq* DNA polymerase and the primers 5′-TCT AAG CTT CCG ATC ATG AGC TTC AAT ATT GCA CC-3′ and 5′-TCT GGA TCC TCG TCA ATC AAT TGA TTT CTA GTC GG-3′ containing the HindIII and

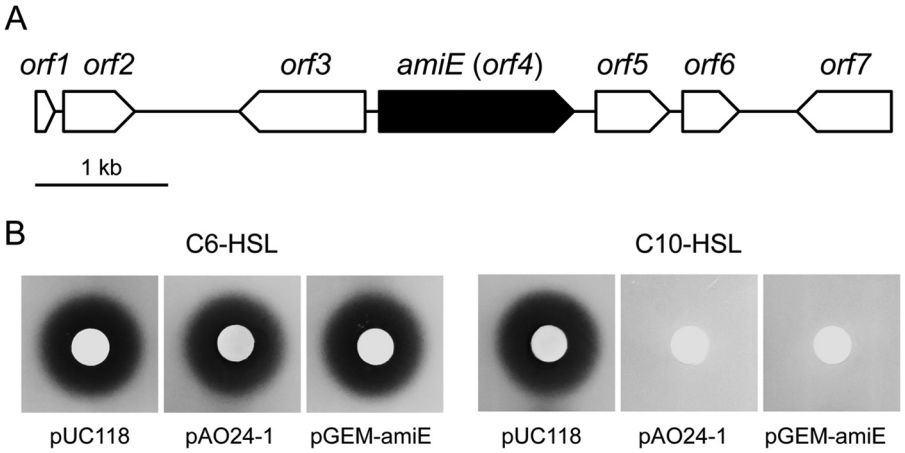

FIG 1 (A) Arrangement of the predicted ORFs on the original genomic clone pAO24-1. The scale represents a 1-kb length of nucleotides. Filled arrow, the *amiE* gene; open arrows, the other ORFs. (B) AHL-degrading activity of *Escherichia coli* harboring pUC118, pAO24-1, and pGEM-amiE. Subcultures of *E. coli* harboring these plasmids were mixed with 10 μM C_6-HSL or C_{10}-HSL and incubated at 37°C for 8 h. The residual AHL was detected using *Chromobacterium violaceum* CV026 (for C_6-HSL) or VIR07 (for C_{10}-HSL).

BamHI restriction sites (underlined), respectively. PCR was performed with the following cycling parameters: 94°C for 30 s, 60°C for 30 s, and 74°C for 1.5 min for 30 cycles. The PCR products were cut with HindIII and BamHI digestion and inserted into the HindIII and BamHI sites of the broad-host-range vector pBBR1MCS5 (22) for construction of pBBR1-amiE. The pBBR1-ahlS plasmid, which contains the AHL lactonase gene, *ahlS*, from *Solibacillus silvestris* StLB046, was used as a positive control (23). The plasmids pBBR1MCS5, pBBR1-amiE, and pBBR1-ahlS were transformed into *P. aeruginosa* PAO1 via electroporation (24). The full-grown culture of PAO1 harboring plasmids was inoculated into fresh LB medium (a 1% inoculum) with 1 mM IPTG, cultivated for 15 h, and centrifuged to remove the cells.

For the extraction of AHLs, 600 μl of culture supernatant was mixed with an equal volume of ethyl acetate and vortexed for 10 min. The ethyl acetate layer was evaporated to dryness and dissolved in 100 μl of dimethyl sulfoxide. C_4-HSL and $3OC_{12}$-HSL were detected using the AHL biosensors, as described above. The elastase activity in the culture supernatant of PAO1 harboring plasmids was quantified with an elastin-Congo red assay using a previously described method (25).

Nucleotide sequence accession numbers. The nucleotide sequence of 16S rRNA from *Acinetobacter* sp. Ooi24 was deposited in the DNA Data Bank of Japan (DDBJ)/EMBL/GenBank databases under accession no. AB933637. The nucleotide sequences of *amiE* and its flanking open reading frames (ORFs) from *Acinetobacter* sp. Ooi24 were deposited under accession no. AB933638.

RESULTS

An AHL-degradative gene is present in the genome of *Acinetobacter* sp. Ooi24. *Acinetobacter* sp. Ooi24 showed degrading activity against C_{10}-HSL but poor activity against C_6-HSL (12). Since most AHL acylases degrade AHLs that have an acyl chain longer than eight carbons (4), we used the *E. coli*/pLas28 reporter system to clone the AHL-degradative gene. The pLas28 reporter plasmid carried the *las* quorum-sensing system genes of *P. aeruginosa* PAO1, which contains the AHL synthase gene (*lasI*) (26). Ooi24 is also able to degrade $3OC_{12}$-HSL as well as C_{10}-HSL (data not shown). *E. coli* harboring pLas28 produced $3OC_{12}$-HSL and induced violacein production by the VIR07 reporter strain. The prepared pUC118-based genomic library of Ooi24 was transformed into *E. coli* harboring pLas28. The formed colonies were transferred to LB agar medium prepared in 96-well plates, and the released

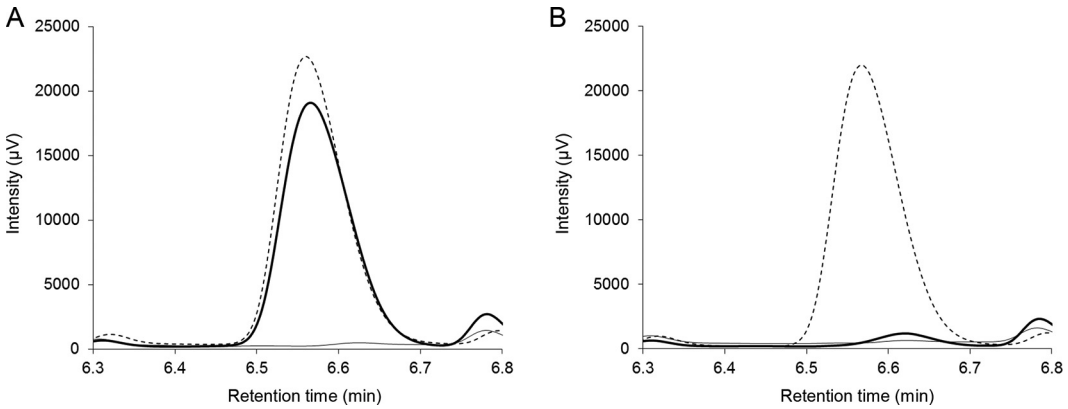

FIG 2 HPLC analysis of the products of $3OC_{10}$-HSL treated with *E. coli* harboring pGEM-amiE (A) and pGEM-T Easy (B). $3OC_{10}$-HSL (500 μM) was incubated for 6 h at 30°C with *E. coli* harboring each plasmid. HSL liberated from $3OC_{10}$-HSL was dansylated and fractionated with HPLC (bold line). Dotted and gray lines, the specific peak of 500 μM HSL and the control without $3OC_{10}$-HSL, respectively.

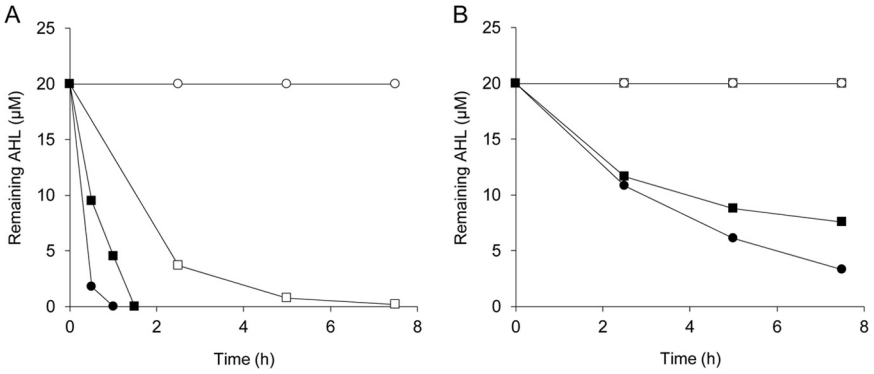

FIG 3 AHL-degrading activity of *E. coli* harboring pGEM-amiE. The cell suspension of *E. coli* harboring pGEM-amiE was mixed with 20 μM 3-oxo-unsubstituted (A) or 3-oxo-substituted (B) AHLs with acyl chain lengths of 6 (open circles), 8 (open squares), 10 (filled circles), and 12 (filled squares) carbons and incubated at 30°C with shaking. The remaining AHLs in the culture supernatant were visualized by spotting the supernatant onto plates mixed with *C. violaceum* CV026 or VIR07. Residual amounts of AHLs were calculated using relationship equations based on the size of the purple zones.

$3OC_{12}$-HSL was visualized through violacein production by VIR07.

When approximately 10,000 transformants were screened, one positive clone, designated pAO24-1, did not stimulate violacein production by VIR07. pAO24-1 contained the 5,356-bp genomic DNA fragment in the BamHI site of pUC118. After amplification of the upstream region of the genomic DNA fragment by inverse PCR, the sequence of the extended genomic DNA fragment (6,445 bp) contained two incomplete ORFs and five complete ORFs (Fig. 1A). The incomplete ORFs, *orf1* and *orf7*, showed similarities to endoribonuclease and single-stranded DNA-specific exonuclease, respectively. The complete ORFs *orf2*, *orf3*, and *orf4* showed similarities to FMN reductase, an AraC-family transcriptional regulator, and amidase, respectively. The two complete ORFs *orf5* and *orf6* were predicted to encode an IS*4*-family transposase.

Because sequencing analysis revealed that only *orf4*, designated *amiE*, encoded a member of the hydrolase family, we next determined whether *amiE* works as an AHL-degradative gene. We amplified *amiE* by PCR and subcloned it into the pGEM-T Easy vector for construction of pGEM-amiE. *E. coli* harboring pAO24-1 or pGEM-amiE was inoculated into LB medium containing 10 μM C_6-HSL or C_{10}-HSL. After incubation for 9 h, residual C_6-HSL and C_{10}-HSL in the culture supernatant were detected using CV026 and VIR07, respectively. Compared with the activity of the control, *E. coli* harboring pAO24-1 and pGEM-amiE showed obvious C_{10}-HSL-degrading activity because of the vanishing of violacein production (Fig. 1B). These results suggested that the *amiE* gene product has AHL-degrading activity. In contrast, *E. coli* harboring each plasmid showed no degrading activity with C_6-HSL (Fig. 1B). In contrast to *amiE*, the other four complete ORFs did not show any degrading activity against C_6-HSL and C_{10}-HSL (data not shown).

AmiE encodes a long-chain AHL acylase. To determine whether AmiE functions as an AHL acylase, we used HPLC to analyze the structure of $3OC_{10}$-HSL digested by AmiE. The phosphate buffer containing 500 μM $3OC_{10}$-HSL was incubated for 6 h at 30°C with *E. coli* harboring pGEM-T Easy or pGEM-amiE. The detection of HSL liberated from $3OC_{10}$-HSL by AHL acylase activity was performed via dansylation of the free amine of HSL. The results of HPLC analysis are shown in Fig. 2. Fractionation of $3OC_{10}$-HSL treated with *E. coli* harboring pGEM-amiE revealed one specific peak at a retention time of 6.56 min. The specific peak of the HSL standard was also detected at a retention time of 6.56 min. In contrast, $3OC_{10}$-HSL treated with *E. coli* harboring pGEM-T Easy displayed no distinct peak at this retention time. The reaction mixture with *E. coli* harboring each plasmid which was not mixed with $3OC_{10}$-HSL did not display the specific peak of the HSL standard. These results demonstrate that AmiE works as an AHL acylase that hydrolyzes the amide bond of $3OC_{10}$-HSL. To analyze the substrate specificity of AmiE, we mixed a cell suspension of *E. coli* harboring pGEM-amiE with a wide range of AHLs. AmiE degraded C_8-HSL, C_{10}-HSL, and C_{12}-HSL and showed the highest degrading activity against C_{10}-HSL, but it did not degrade C_6-HSL (Fig. 3A). In addition, *E. coli* harboring pGEM-amiE did not degrade C_4-HSL, which has the shortest acyl chain (data not shown). AmiE showed higher degradation activity toward the 3-oxo-unsubstituted AHLs than toward the 3-oxo-substituted AHLs (Fig. 3B).

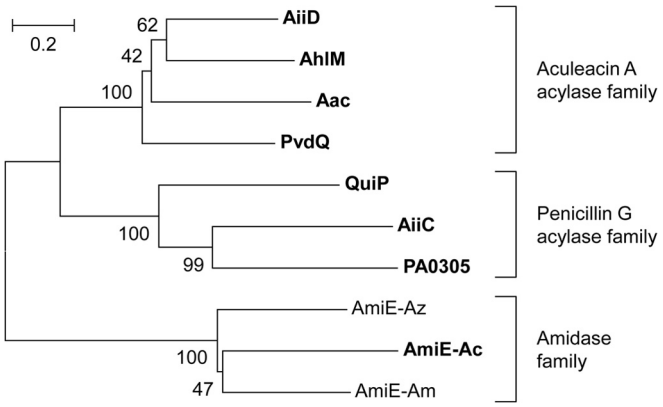

FIG 4 Phylogenetic tree based on the amino acid sequences of AiiD, AhlM, PvdQ, Aac, PA0305, AiiC, QuiP, and AmiE from *Acinetobacter* sp. Ooi24 (AmiE-Ac), *Azospirillum* sp. B510 (AmiE-Az), and *A. orientalis* HCCB10007 (AmiE-Am). The phylogenetic tree was constructed using the neighbor-joining method with the ClustalW program of the MEGA (version 6) package (36). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The scale bar represents 0.2 substitution per amino acid position. The known AHL acylases and *Acinetobacter* sp. Ooi24 AmiE are shown in bold.

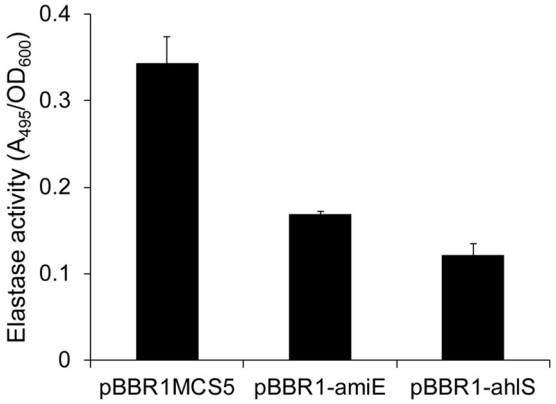

FIG 5 The elastase activities in the culture supernatants of PAO1 harboring pBBR1MCS5, pBBR1-ahlS, and pBBR1-amiE were measured using the elastin-Congo red assay. The absorbance of the supernatants measured at 495 nm was divided by the optical density at 600 nm (OD_{600}) of the culture. The results were reproduced in triplicate, and the error bars indicate the standard deviations.

AmiE is a new AHL acylase. To evaluate the novelty of AmiE, we determined the phylogenetic relationship between AmiE and known AHL acylases from various bacteria using the neighbor-joining method with the ClustalW program (Fig. 4) (27). AiiD from *Ralstonia* sp. strain XJ12B (21), AhlM from *Streptomyces* sp. strain M664 (28), PvdQ from *P. aeruginosa* PAO1 (29), and Aac from *Shewanella* sp. strain MIB015 (20) belong to the aculeacin A acylase protein family (EC 3.5.1.70). PA0305 and QuiP from *P. aeruginosa* PAO1 (25, 30) and AiiC from *Anabaena* sp. strain PCC 7120 (31) belong to the penicillin G acylase protein family (EC 3.5.1.11). However, AmiE showed no significant similarity with these protein families. A Basic Local Alignment Search Tool (BLAST) search revealed that AmiE showed homology to known amidases (EC 3.5.1.4), which are enzymes that catalyze the hydrolysis of monocarboxylic acid amide to produce ammonia and monocarboxylate (32). The putative gene product of *amiE* from Ooi24 was a 490-amino-acid protein and showed 36.9% and 37.1% sequence identities with AmiE from *Azospirillum* sp. strain B510 (UniProt accession no. D3NY90) and *Amycolatopsis orientalis* HCCB10007 (UniProt accession no. R4SWV0), respectively (Fig. 4). The BLAST search revealed that AmiE from Ooi24 showed 100% sequence identity with an uncharacterized protein from *Acinetobacter ursingii* CIP 107286 (UniProt accession no. N9BV73) and 99.8% sequence identity with a protein of *Acinetobacter* sp. strain CIP 102129 (UniProt accession no. N8UB59).

AmiE reduces the production of AHLs and virulence factors in *P. aeruginosa*. To evaluate the anti-quorum-sensing activity of AmiE, we evaluated the potential use of the heterologous expression of *amiE* to interfere with quorum sensing in *P. aeruginosa* PAO1. In *P. aeruginosa* PAO1, LasI and RhlI are responsible for the synthesis of the *las* and *rhl* signals $3OC_{12}$-HSL and C_4-HSL, respectively (33). The production of C_4-HSL and $3OC_{12}$-HSL was detected in PAO1 harboring pBBR1MCS5 but not in PAO1 harboring pBBR1-ahlS (data not shown). Although AmiE does not have C_4-HSL-degrading activity, neither C_4-HSL nor $3OC_{12}$-HSL was detected in the supernatant of PAO1 harboring pBBR1-amiE (data not shown). Because the *las* and *rhl* quorum-sensing systems cross-regulate each other and C_4-HSL is not detected in the *lasI* mutant of PAO1 (34), it was assumed that the degradation of $3OC_{12}$-HSL by AmiE affected the production of C_4-HSL in PAO1. One of them, $3OC_{12}$-HSL, regulates the production of the elastase enzyme as a virulence-enhancing factor in *P. aeruginosa* (33). The elastase activities of PAO1 harboring pBBR1-ahlS and pBBR1-amiE were drastically decreased (more than 50%) compared with the elastase activity of PAO1 harboring pBBR1MCS5 (Fig. 5). In a previous study, the overexpression of another AHL acylase gene, *pa0305*, resulted in an approximately 50% reduction in elastase activity in *P. aeruginosa* PAO1 (25). Neither PA0305 nor AmiE had C_4-HSL-degrading activity (25). These results indicate that the expression of *amiE* in PAO1 contributes to the self-degradation of $3OC_{12}$-HSL and reduces the AHL-mediated expression of elastase.

DISCUSSION

In this study, we found the novel AHL acylase gene *amiE* from the genome of *Acinetobacter* sp. Ooi24. In our previous study, *Acinetobacter* sp. Ooi24 showed high degrading activity against C_{10}-HSL but very weak degrading activity against C_6-HSL (12). Similarly, *E. coli* harboring the AmiE-expressing plasmid degraded AHLs with acyl chains longer than six carbons and showed the highest degrading activity against C_{10}-HSL, but it did not degrade

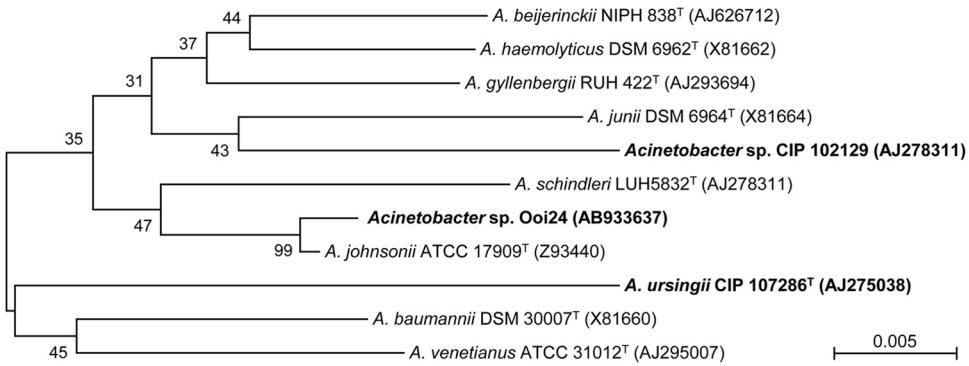

FIG 6 Neighbor-joining trees of the 16S rRNA gene sequences obtained for *Acinetobacter* strains. For phylogenetic analysis with the 16S rRNA gene, nine type strains of the genus *Acinetobacter* and two non-type *Acinetobacter* strains were used. DDBJ/EMBL/GenBank accession numbers appear in parentheses. *Acinetobacter* strains that contain *amiE* gene homologs in their genomes are shown in bold. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The scale bar represents 0.005 substitution per nucleotide position. Phylogenetic analyses were conducted with the MEGA (version 6) program (36).

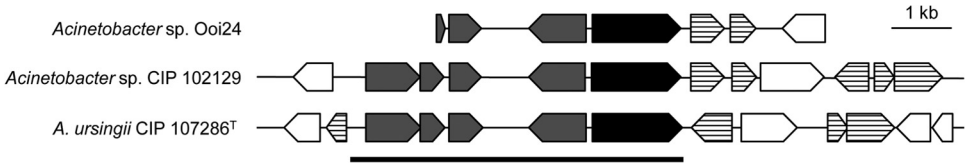

FIG 7 Arrangement of the predicted ORFs in the upstream and downstream regions of the *amiE* genes in the genomes of *Acinetobacter* sp. Ooi24 and CIP 102129 and *A. ursingii* CIP 107286. The scale bar represents a 1-kb length of nucleotides. Filled, gray, and lined arrows, the *amiE* gene, conserved genes, and putative transposon genes, respectively.

C_6-HSL (Fig. 3A). These results demonstrate that the AHL-degrading activity of *E. coli* harboring the AmiE-expressing plasmid corresponds to that of Ooi24. Previous studies have reported that most AHL acylases degrade long-chain AHLs more efficiently than short-chain forms (4). Especially, some AHL acylases, Aac, PvdQ, AhlM, and QuiP, are unable to degrade AHLs with acyl chains shorter than eight carbons (4). These results suggest that AmiE has activity similar to the activities of known AHL acylases. In general, enzymes belonging to this protein family (EC 3.5.1) catalyze the hydrolysis of carbon-nitrogen bonds other than peptide bonds. On the other hand, some EC 3.5.1-family enzymes, aculeacin A acylase and penicillin G acylase, are able to catalyze the hydrolysis of the amide bond of AHLs. To the best of our knowledge, the present study is the first to report that AmiE, which belongs to the amidase protein family, has AHL acylase activity as well as activity against other EC 3.5.1-family enzymes.

The *amiE* gene homolog was also found in the genome sequences of two other *Acinetobacter* strains, *A. ursingii* CIP 107286 and *Acinetobacter* sp. CIP 102129. Although these two *Acinetobacter* strains are phylogenetically different from Ooi24 (Fig. 6), the nucleotide sequences of *amiE* from these three strains show almost complete identity, as divergence is limited to one nucleotide position. The *amiE* gene homolog was not found in the draft or complete genome sequences of other *Acinetobacter* strains deposited in the DDBJ/EMBL/GenBank databases (data not shown). These results demonstrate that the *amiE* gene homolog is not conserved in a wide range of *Acinetobacter* strains but is encoded by the genomes of specific *Acinetobacter* strains.

To identify the chromosomal locus of *amiE*, we compared the upstream and downstream regions of the *amiE* genes in *Acinetobacter* strains. Those in *A. ursingii* CIP 107286 and *Acinetobacter* sp. CIP 102129 were obtained from the whole-genome shotgun sequences deposited in the DDBJ/EMBL/GenBank databases under accession no. APQA01000004 and APPA01000039, respectively. Interestingly, the nucleotide sequences of the upstream regions of *amiE* from these strains, which contain genes encoding phytanoyl coenzyme A dioxygenase, endoribonuclease, FMN reductase, and the AraC family transcriptional regulator, showed almost complete identity, as divergence is limited to less than two nucleotide positions (Fig. 7). In addition, many transposase-like genes were found around these highly conserved sequences (Fig. 7). The amino acid sequence of AmiE showed no similarity with any amidase-family proteins from *Acinetobacter* strains but showed 46.0% and 39.9% identities with amidases from the gammaproteobacteria *Thalassolituus oleivorans* MIL-1 (UniProt accession no. M5E2I7) and *Marinobacter algicola* DG893 (UniProt accession no. A6F504), respectively. These results strongly suggest that *amiE* is transferred by a putative transposon carrying acylhomoserine lactone acylase.

In summary, our study reports that AmiE from *Acinetobacter* sp. Ooi24, which belongs to the amidase family of proteins, has AHL acylase activity. The putative transposon, including *amiE*, was found in the chromosome of three *Acinetobacter* strains. It is possible that the horizontal transfer of *amiE* among *Acinetobacter* strains is observed and influences the quorum-sensing system in activated sludge. Quorum sensing is reportedly associated with the formation of a biofouling layer on the membrane surface of membrane bioreactor (MBR) wastewater treatment systems (7). Lee et al. (35) demonstrated that cross-linked AHL acylase molecules in magnetically separable mesoporous silica work together as an antifouling material. The AHL acylase activity of *Acinetobacter* strains might also perform as an antifouling agent in MBR systems.

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