AidC, a Novel *N*-Acylhomoserine Lactonase from the Potato Root-Associated *Cytophaga-Flavobacteria-Bacteroides* (CFB) Group Bacterium *Chryseobacterium* sp. Strain StRB126

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N-Acylhomoserine lactones (AHLs) are used as quorum-sensing (QS) signal molecules by many Gram-negative bacteria. We have reported that *Chryseobacterium* sp. strain StRB126, which was isolated from the root surface of potato, has AHL-degrading activity. In this study, we cloned and characterized the *aidC* gene from the genomic library of StRB126. AidC has AHL-degrading activity and shows homology to several metallo-β-lactamase proteins from *Bacteroidetes*, although not to any known AHL-degrading enzymes. Purified AidC, as a maltose-binding fusion protein, showed high degrading activity against all tested AHLs, whether short- or long-chain forms, with or without substitution at carbon 3. High-performance liquid chromatography (HPLC) analysis revealed that AidC functions as an AHL lactonase catalyzing AHL ring opening by hydrolyzing lactones. An assay to determine the effects of covalent and ionic bonding showed that Zn^{2+} is important to AidC activity both *in vitro* and *in vivo*. In addition, the *aidC* gene could also be PCR amplified from several other *Chryseobacterium* strains. In conclusion, this study indicated that the *aidC* gene, encoding a novel AHL lactonase, may be widespread throughout the genus *Chryseobacterium*. Our results extend the diversity and known bacterial hosts of AHL-degrading enzymes.

Quorum sensing (QS) is a cell-cell communication mechanism that allows bacterial populations to coordinate gene expression in response to cell density. It is believed that QS constitutes a central element in the social life of bacteria, conferring to the members of a bacterial community the ability to behave as an organized multicellular organism (2, 3). The majority of the QS systems thus far described in Gram-negative bacteria rely on *N*-acyl-L-homoserine lactones (AHLs) as signaling molecules; these QS systems have been found in more than 100 bacterial species, including many pathogens (10). AHL-mediated QS regulates the expression of many genes and is responsible for bioluminescence, production of pigments and antibiotics, biofilm formation, and many other factors that are important for pathogenicity (6, 24). In general, AHL-negative mutants show defects in pathogenicity; hence, it is expected that disrupting or manipulating the QS signals could inhibit the expression of virulence and infection of host cells (26).

Many AHL-degrading genes have been cloned and characterized from bacteria, fungi, mammalian cells, and metagenomic libraries constructed from environmental soil samples (20, 22). AHL-degrading enzymes have been divided into two functional groups: AHL lactonases, which catalyze AHL ring opening by hydrolyzing lactones, and AHL acylases, which hydrolyze the amide bond of AHL (22). The first demonstrated and best-studied AHL-degrading enzyme is AiiA lactonase, which belongs to the metallo-β-lactamase superfamily and was identified from *Bacillus* sp. strain 240B1 (8). Expression of *aiiA* in the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum* significantly attenuates pathogenicity on some crops (7). Transgenic plants expressing AHL lactonase exhibited significantly enhanced resistance to the infection of *P. carotovorum* subsp. *carotovorum* (7). The AHL lactonases, which also belong to the metallo-lactamase superfamily, were called "AiiA-type lactonases," with respect to being first identified as AiiAs. AiiA-type lactonases have been identified from

a vast range of bacterial original hosts, including *Firmicutes*, *Acidobacteria*, and *Proteobacteria* (22, 28). Several AiiA-type lactonases have been studied in depth and hold potential for application in the biocontrol of plant diseases (22, 28).

In previous studies, we have reported the putative AHL lactonase activities in the potato root-associated strains of *Chryseobacterium* sp., which is a member of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group (16). Recently, it was reported that the fish pathogen *Tenacibaculum maritimum*, also a member of the CFB group, produces and degrades AHLs (18). However, to our knowledge, there are no reports of the isolation and characterization of any AHL-degrading gene from the CFB group bacteria. In this study, we report the cloning of a gene that encodes a novel AiiA-type lactonase from the potato root-associated *Chryseobacterium* sp. strain StRB126 and characterization of the enzymatic kinetics of the novel AHL lactonase.

MATERIALS AND METHODS

Bacterial strains, plasmids, compounds, and growth conditions. Selected bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) medium (19). All other bacteria were grown at 30°C in tryptic soy broth (TSB; Nippon Becton, Dickinson, Tokyo, Japan). Solid bacterial media were made by adding agar at a final concentration of 1.5% to the liquid media. Antibiotics were added at final concentrations of 100 μg/ml ampicillin, 100 μg/ml chloramphenicol, 50 μg/ml kanamycin, and 10 μg/ml gentamicin

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source
Strains		
E. coli		
DH5α	F⁻ *supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Nippon Gene
C. violaceum		
CV026	ATCC 31532 derivative, *cviI*::Tn5*xylE* Km^r, Sm^r	11
VIR07	ATCC 12472 derivative, *cviI*::Km^r, Ap^r	13
Chryseobacterium sp.		
StRB126	AHL-degrading strain isolated from potato root surface	16
StRB340	AHL-degrading strain isolated from potato root surface	16
StRB341	AHL-degrading strain isolated from potato root surface	16
StRB342	AHL-degrading strain isolated from potato root surface	16
C. gleum		
NBRC 15054	Clinical isolate, type strain	NBRC
Plasmids		
pUC118	Cloning vector; Ap^r	TaKaRa Bio
pST126-E1	8.8-kb Sau3AI fragment from StRB126 genomic DNA in pUC118	This study
pHSG398	Cloning vector; Cm^r	TaKaRa Bio
pHSG-aidC126	pHSG398 containing *aidC* gene from StRB126	This study
pLux28	8.8-kb SalI fragment from pHV200 in pSTV28 vector; Cm^r	26
pUC118-aidC340	pUC118 containing *aidC* gene from StRB340	This study
pUC118-aidC341	pUC118 containing *aidC* gene from StRB341	This study
pUC118-aidC342	pUC118 containing *aidC* gene from StRB342	This study
pUC118-aidC15054	pUC118 containing *aidC* gene from NBRC15054	This study
pGEM-T easy	Cloning vector; Ap^r	Promega
pMAL-c2X	Cloning vector to make MBP fusions; Ap^r	New England Biolabs
pMAL-aidC	pMAL-c2X containing *aidC* gene from StRB126	This study

as required. AHLs used in this study, *N*-hexanoyl-L-homoserine 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 (3OC$_6$-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (3OC$_8$-HSL), *N*-(3-oxode-canoyl)-L-homoserine lactone (3OC$_{10}$-HSL), and *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC$_{12}$-HSL), were synthesized by a previously described method (4).

Cloning of an AHL-degrading gene from StRB126. A standard protocol for genetic manipulation was used as described previously (19). The AHL-degrading gene of StRB126 was cloned by a previously described method (26). Briefly, both the genomic library of StRB126 and the AHL-responsive plasmid pLux28 were transformed into *E. coli* DH5α. The transformants were grown on LB agar plates containing ampicillin and chloramphenicol. The resulting colonies were inoculated into 200 μl of fresh LB medium containing ampicillin, chloramphenicol, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in 96-well plates. After incubation at 30°C for 20 h with gentle shaking, the cell cultures in each well were evaluated for luminescence activities using a Luminescenser JNR-II (Atto, Tokyo, Japan). Positive clones were sequenced using a BigDye Terminator version 3.1 sequencing kit and an ABI Prism 3100 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Cloning of the *aidC* gene and its homolog. The *aidC*-coding region on the positive clone pST126-E1 was excised by EcoRV and PstI digestion and inserted into the SmaI-PstI sites of the pHSG398 cloning vector for construction of pHSG-aidC126. The *aidC* gene homologs from the root-associated *Chryseobacterium* sp. strains StRB340, StRB341, and StRB342 were amplified with GoTaq DNA polymerase (Promega, Tokyo, Japan) and the following primer set: 5′-CTC AGC TGG CAT TAG CAT GGG TCT TGA ATC-3′ and 5′-GCA GAC AGC TAT TCT GTT AGT TTT CAG CAG C-3′. The *aidC* gene homolog from *Chryseobacterium gleum* strain

NBRC 15054 was also amplified with the same cycling parameters and the following primer set: 5′-AGC TTG CGC TAG CTT GGG TAT TGA ACC AGG-3′ and 5′-CTA CCT GTT ACC TAT CAT CTG CCT CTG TC-3′. PCR was performed using the following cycling parameters: 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 30 cycles. The PCR products were cloned into the pGEM-T easy cloning vector (Promega), excised by SphI and PstI digestion, and inserted into the SphI-PstI sites of pUC118 for construction of pUC118-aidC340, pUC118-aidC341, pUC118-aidC342, and pUC118-aidC15054.

Purification of AidC as an MBP fusion. The *aidC*-coding region in the genome of StRB126 was amplified with GoTaq DNA polymerase and the following primers, containing BamHI and PstI restriction sites (underlined), respectively: 5′-GGA TCC ATG AAT AGA AAG CGG GTT ATT GGC-3′ and 5′-CTG CAG AGC TAT TCT GTT AGT TTC AGC AGC-3′. PCR was performed using the following cycling parameters: 95°C for 30 s, 57°C for 30 s, and 72°C for 3 min for 30 cycles. The PCR products were cloned into the pGEM-T easy cloning vector, excised by BamHI and PstI digestion, and inserted into the BamHI-PstI sites of pMAL-c2X for construction of pMAL-aidC. For expression and purification of the maltose-binding protein (MBP)-AidC fusion protein, the complete preculture of *E. coli* DH5α harboring pMAL-aidC was inoculated into 300 ml of fresh LB medium and incubated for 2 h at 37°C, with shaking. Expression of the recombinant MBP-AidC fusion was induced upon the addition of 0.1 mM IPTG after 2 h, and expression was continued for an additional 8 h at 37°C. After incubation, cells were harvested by centrifugation and resuspended with 3 ml of BugBuster protein extraction reagent (Novagen, Inc., Madison, WI) and then incubated for 10 min at room temperature, with gentle shaking. Next, the suspension was sonicated, centrifuged at 10,000 × *g* for 5 min to remove the cell debris, and filtrated. Protein purification was performed using the same method described previously (26). As a negative control, we also purified an MBP-

LacZα fusion from *E. coli* DH5α harboring pMAL-c2X. Expression and purification of recombinant MBP-AidC and MBP-LacZα were checked by SDS-PAGE analysis.

Detection of AHL-degrading activity of AidC. The residual AHLs were detected using AHL biosensors *Chromobacterium violaceum* CV026 and VIR07, which respond to exogenous AHLs by producing the purple pigment violacein (11, 13). For detecting the AHL-degrading activity of AidC *in vivo* and *in vitro*, we used a previously described method (26). To determine the chemical structures of products from the reaction between AidC and AHLs, 50 μl of purified protein solution was mixed with 49 μl of column buffer [20 mM Tris-HCl buffer and 200 mM NaCl (pH 7.4)] and 1 μl of 200 mM C10-HSL stock solution (in methanol). After incubation at 30°C for 10 min, reactions were stopped with an equal volume of acetonitrile, and the mixture was vortexed and centrifuged to pellet the precipitated protein. The hydrolyzed C10-HSL, as a control, was made by incubating C10-HSL in 10 mM NaOH at room temperature for 30 min. Samples (25 μl) were analyzed by high-performance liquid chromatography (HPLC) according to a previously described method (26).

Characterization of enzymatic activity of AidC. To determine the enzyme kinetics of AidC, purified MBP-AidC (final concentration, 1 μM) was added to a substrate solution in the column buffer, with a final volume of 40 μl γ-butyrolactone (γ-BL). L-Homoserine lactone (HSL), C_6-HSL, C_8-HSL, C_{10}-HSL, C_{12}-HSL, $3OC_6$-HSL, $3OC_8$-HSL, and $3OC_{12}$-HSL were selected as the substrates for an enzyme specificity analysis. The reaction mixtures were incubated at 30°C, stopped by adding 40 μl acetonitrile, and subjected to HPLC analysis. The residual AHL and its hydrolysis product were quantified by HPLC. All experiments were performed in triplicate, and all velocities were determined at time points at which no more than 20% of the substrate had been consumed. The k_{cat} and K_m values were calculated based on Michaelis-Menten equation. The effects of various metal ions and a metal-chelating reagent (EDTA) on AidC activity were examined both *in vitro* and *in vivo*. For the *in vitro* assay, 1 mM EDTA and 1 mM metal ions comprising Cu^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, and Co^{2+} were mixed with 1 μM purified AidC protein in column buffer (pH 7.4). After incubation at 30°C for 10 min, the remaining activity was measured under the standard conditions described above. For the *in vivo* assay, the full preculture of *E. coli* harboring the plasmid pMAL-aidC was diluted into fresh LB medium with 1 mM EDTA and 1 mM metal ions. IPTG was then added to induce AidC overexpression after 4 h cultivation. After an additional 8-h cultivation, crude cell extracts were prepared and adjusted to the same concentration. The AHL-degrading activities of crude cell extracts were examined by the same procedure as used in the *in vitro* assay.

Nucleotide sequence accession number. The nucleotide sequences of *aidC* and its flanking open reading frames (ORFs) from *Chryseobacterium* sp. StRB126 have been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB733663.

RESULTS AND DISCUSSION

Identification of AHL-degrading gene from the *Chryseobacterium* sp. StRB126. For cloning the AHL degradation gene, a pUC118-based StRB126 genomic library was constructed and used to transform *E. coli* DH5α harboring pLux28 (26). We then measured the luminescence activities of the resulting colonies on fresh LB medium. When approximately 2,300 transformants were screened, three clones expressed a low level of luminescence. To elucidate whether the reduction in luminescence resulted from degradation of AHL, *E. coli* DH5α cells harboring the plasmids from the positive clones were inoculated into LB medium containing 10 μM C_6-HSL or C_{10}-HSL. After incubation for 6 h, the residual AHL in the culture supernatant was detected by *Chromobacterium violaceum* reporters, which produced the purple pigment violacein in response to the presence of AHLs. Consequently, *E. coli* DH5α harboring a single plasmid, designated
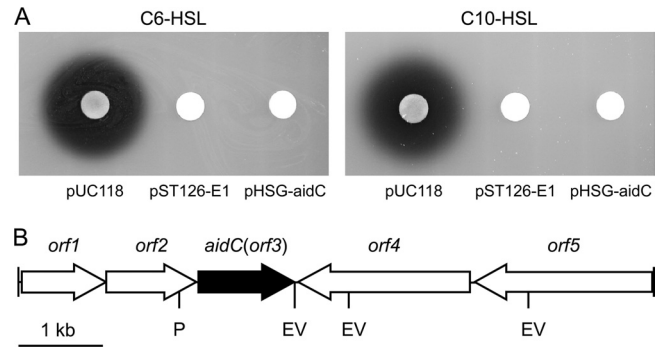

FIG 1 (A) AHL-degrading activity of *E. coli* DH5α harboring pUC118, pST126-E1, and pHSG-aidC. Subcultures of *E. coli* DH5α harboring these plasmids were mixed with 10 μM C_6-HSL or C_{10}-HSL and incubated at 37°C for 2 h. The residual AHL was detected using *C. violaceum* strain CV026 or VIR07. (B) Arrangement of the predicted ORFs on the original genomic clone pST126-E1. The scale represents a 1-kb length of nucleotides. The filled arrow indicates the *aidC* gene, and the open arrows indicate the other ORFs. EV, EcoRV; P, PstI.

pST126-E1, showed clear AHL-degrading activity against both C_6-HSL and C_{10}-HSL (Fig. 1A).

The sequence of the genomic DNA fragment (8,784 bp), which was inserted into pST126-E1, contained five complete open reading frames (ORFs) (Fig. 1B). The third ORF (*orf3*) encoded a putative metallo-β-lactamase of 330 amino acids. The Orf3 amino acid sequence showed low-level similarity (13% identity) to the known AHL lactonases, including AiiA from *Bacillus* sp. 240B1 (7, 8). To test whether *orf3* encodes an AHL-degrading enzyme, the complete *orf3* was amplified by PCR and subcloned into the pHSG398 easy vector. *E. coli* DH5α harboring the *orf3*-expressing plasmid showed clear AHL-degrading activity at the same level as *E. coli* DH5α harboring pST126-E1 (Fig. 1A). These results suggested that *orf3* encodes an AHL-degrading enzyme. Therefore, we named *orf3* the autoinducer degrading gene from *Chryseobacterium* sp. (*aidC*) as being the first AHL-degrading gene identified from the genus *Chryseobacterium*.

AidC encodes an AHL lactonase. We purified AidC as an MBP fusion in an *in vitro* AHL-degrading assay. The MBP-AidC fusion protein was overproduced in *E. coli* DH5α and purified by maltose affinity chromatography, and the purified proteins were analyzed by 10% SDS-PAGE. The results revealed that the overexpressed protein was approximately 80 kDa in size (Fig. 2A), which was congruent with the predicted molecular weight of MBP-AidC, based on its amino acid sequence. When the AHL-degrading activity of the purified protein was examined, purified MBP-AidC completely degraded 100 μM C_6-HSL and C_{10}-HSL within 2 h, whereas MBP-LacZα did not (Fig. 2B). In our previous study, putative AHL lactonase activities were detected from StRB126 (16). Therefore, to determine whether AidC functions as an AHL lactonase, the structure of C_{10}-HSL treated with MBP-AidC was analyzed by HPLC. Fractionation of the C_{10}-HSL standard revealed a single major HPLC peak, with a retention time of approximately 6.3 min (Fig. 3A). To prepare the lactone ring-opened C_{10}-HSL, C_{10}-HSL was hydrolyzed by 10 mM NaOH. Fractionation of the hydrolyzed C_{10}-HSL revealed one major HPLC peak with a retention time of approximately 3.9 min (Fig. 3B). To examine the enzymatic properties of AidC, solutions of MBP-LacZα and MBP-AidC were mixed with C_{10}-HSL and incubated at 30°C
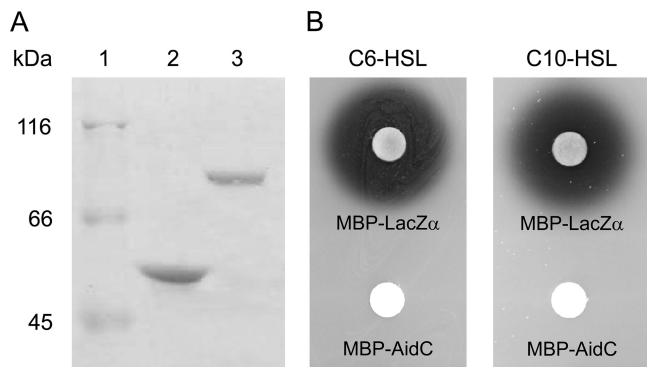
FIG 2 (A) Purification of MBP-LacZα and MBP-AidC fusion proteins. Lane 1, protein molecular mass marker (Fermentas, Hanover, MD); lane 2, purified MBP-LacZα; and lane 3, purified MBP-AidC. Samples were analyzed by SDS-PAGE using a 10% polyacrylamide gel. (B) AHL-degrading activity of MBP-LacZα and MBP-AidC. Solutions of purified MBP-LacZα and MBP-AidC were mixed with an equal volume of 1 mM C_6-HSL or C_{10}-HSL solutions and were then incubated at 30°C for 15 min. The residual AHL was detected using *C. violaceum* CV026 or VIR07.

for 10 min. Fractionation of MBP-LacZα-treated C_{10}-HSL revealed a major HPLC peak, which corresponded to that of the C_{10}-HSL standard (Fig. 3C). This result indicated that the MBP domain did not contain AHL-degrading activity. In contrast, fractionation of MBP-AidC-treated C_{10}-HSL revealed two HPLC peaks, which corresponded to those of the C_{10}-HSL standard and the lactone ring-opened C_{10}-HSL (Fig. 3D). These results indicated that AidC functions as an AHL lactonase that catalyzes the opening of the AHL ring by hydrolyzing lactones.

AidC is a new member of the AiiA-type AHL lactonases. AiiA-type AHL lactonases, which belong to the metallo-β-lactamase superfamily, have been identified and characterized from various bacteria (8, 9, 14, 17, 22, 29). However, AidC has less than 13%

identity to each of the known AiiA-type AHL lactonases, including 13% identity across the entire length of AiiA from *Bacillus* sp. 240B1, 12% identity across AttM from *Agrobacterium tumefaciens* strain C58 (29) and AhlD from *Arthrobacter* sp. strain IBN110 (15), 10% identity across AhlS from *Solibacillus silvestris* strain StLB046 (14), and 8% identity across QlcA from the soil metagenome (17). When the amino acid sequence of AidC was compared with those of the known AiiA-type lactonases (Fig. 4), the zinc-binding motifs HXHXDH~H, which are commonly conserved sequences of the metallo-β-lactamase superfamily, were found in the known AiiA-type lactonases and in AidC ([145]HAHP DH[150]~H[239]) (Fig. 4). Interestingly, D191 of AiiA, which has been proven to be essential for enzymatic activity by site-directed mutagenesis (8) and is shared by all the known AiiA-type lactonases (8, 14, 15, 17, 29), was replaced by L261 in AidC (Fig. 4).

The AHL lactonases can be grouped into three groups: the metallo-β-lactamase superfamily, the α/β-hydrolase-fold family (12, 26), and the phosphotriesterase (PTE) family (23). Among these, members from the metallo-β-lactamase superfamily have garnered more attention due to their wide distribution across the phyla of *Firmicutes*, *Proteobacteria*, and *Acidobacteria* (22, 28). To evaluate the novelty of AidC, we employed the amino acid sequence of AidC to determine the phylogenetic relationship between AidC and the known representative AHL lactonases from various bacteria using the neighbor-joining method (Fig. 5). Although AidC belongs to the same protein family as the known AiiA-type lactonases, we found that it is distant from the other lactonase clusters represented by the five other AiiA-type lactonases.

The substrate specificity and properties of AidC. To assess the AHL substrate range of AidC, we assessed its ability to degrade various AHLs by HPLC. We found that MBP-AidC exhibited high relative activities toward all the tested AHLs, with or without 3-oxo substitution (Fig. 6). The 3-oxo substitution of AHLs is
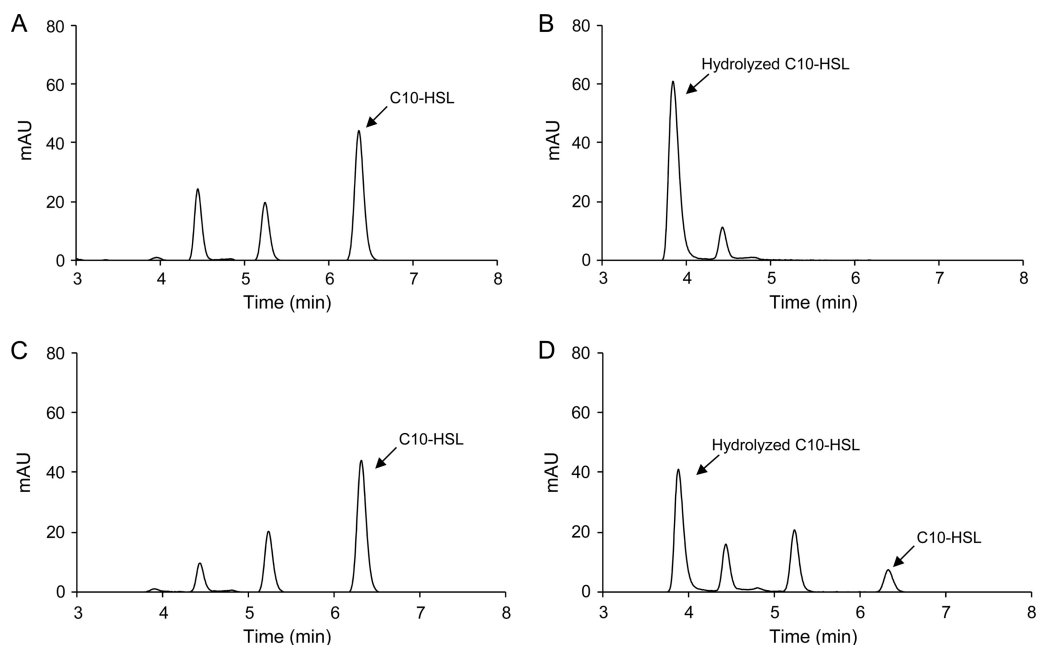

FIG 3 HPLC profiles of C_{10}-HSL (A), C_{10}-HSL hydrolyzed by 10 mM NaOH (B), C_{10}-HSL treated with MBP-LacZα (C), and C_{10}-HSL treated with MBP-AidC (D). The peaks corresponding to C_{10}-HSL (a retention time of approximately 6.3 min) and hydrolyzed C_{10}-HSL (3.9 min) are indicated by arrows.
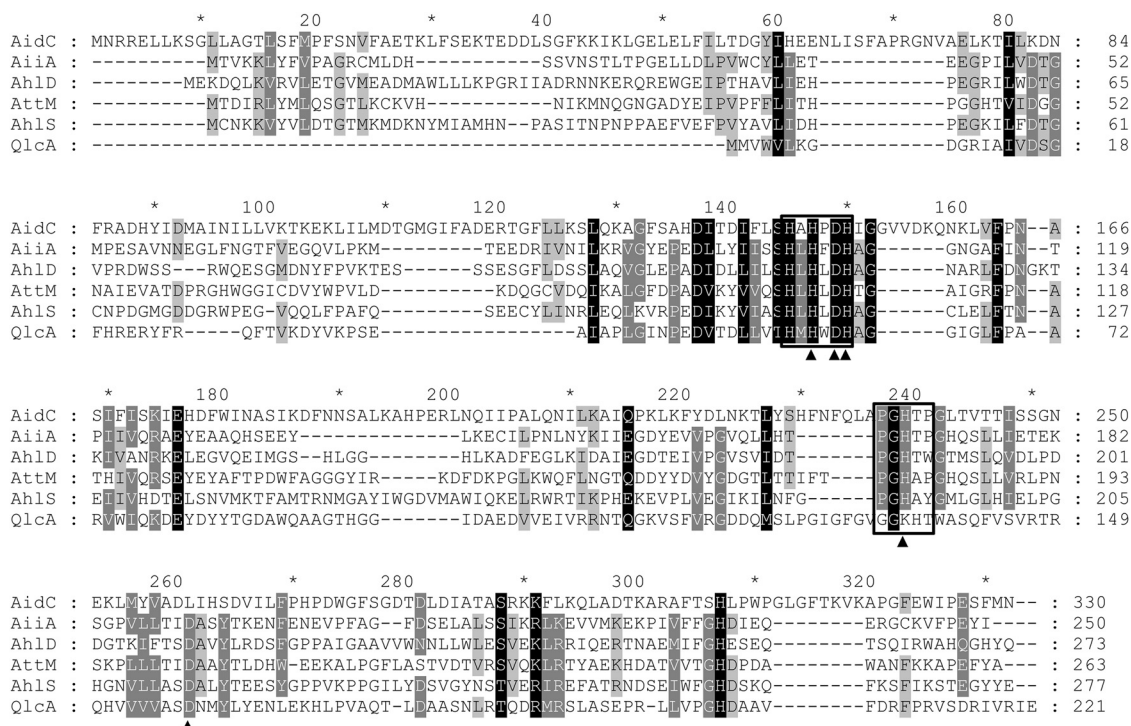
FIG 4 Comparison of amino acid sequences of AidC and five known AiiA-type lactonases. The amino acid sequence of AidC was compared with those of AiiA from *Bacillus* sp. 240B1, AhlD from *Arthrobacter* sp. strain IBN110, AttM from *A. tumefaciens* strain A6, AhlS from *S. silvestris*, and QlcA from the soil metagenome. Sequences were aligned using the ClustalW program (21) and shaded using the Genedoc program (http://www.nrbsc.org/gfx/genedoc/). The two zinc-binding motifs are boxed with rectangles. The amino acid residues that are essential for enzymatic activity of the known AHL lactonases are indicated by solid triangles.

known to negatively affect the enzymatic activity of AiiA from *Bacillus* sp. 240B1 (25, 26). AidC, on the other hand, showed slightly higher degrading activity against 3-oxo-substituted AHLs than against unsubstituted AHLs (Fig. 6). In addition, although differences in acyl acid chain length did not significantly affect the enzymatic activity of AiiA (25), AidC was somewhat more effective at degrading C_6-HSL and C_8-HSL than C_{10}-HSL and C_{12}-HSL (Fig. 6). AidC did not show any degrading activity toward L-homoserine lactone or γ-butyrolactone, similar to AiiA (25).

The optimal pH for AHL-degrading activity of MBP-AidC was examined using C_{10}-HSL as the substrate at 30°C. AHL-degrading activity was enhanced as pH increased and reached a maximum at pH 8, but no or little activity was detected when pH was adjusted

to pH 5 or below (Fig. 7A). The optimal temperature for the AHL-degrading activity of MBP-AidC was also examined using C_{10}-HSL as the substrate. MBP-AidC displayed more than 80% of its maximum activity at 20°C to 50°C, but the relative activity was greatly reduced when temperature exceeded 50°C (Fig. 7B). Correspondingly, strain StRB126 could grow at temperatures ranging from 20°C to 50°C, reaching its optimum temperature at 30°C (data not shown). To determine the thermostability of AidC, purified MBP-AidC was preincubated at various temperatures for 2 h and the residual enzymatic activity was then determined. More
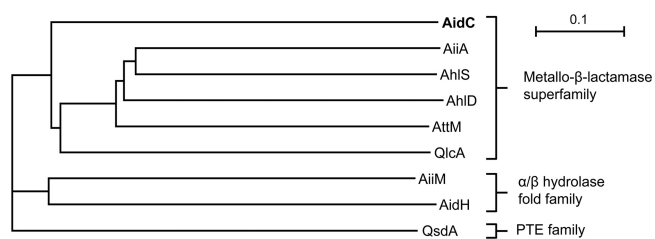

FIG 5 Phylogenetic tree based on amino acid sequences of AidC, AiiA, AhlD, AttM, AhlS, QlcA, and AiiM from *Microbacterium testaceum* strain StLB037, AidH from *Ochrobactrum* sp. strain T63, and QsdA from *Rhodococcus erythropolis* strain W2. The dendrogram was constructed by the neighbor-joining method using the ClustalW program that is included with NJplot software. The scale bar represents 0.1 substitutions per amino acid position.
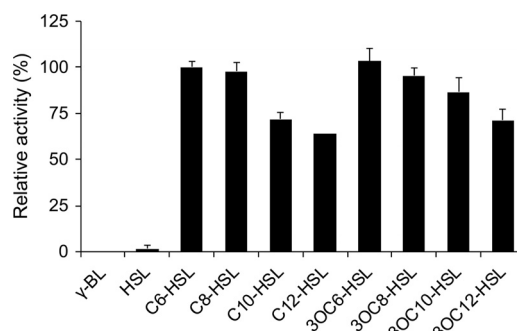

FIG 6 Substrate specificity of purified MBP-AidC. Purified MBP-AidC was mixed with substrate solutions in the column buffer (pH 7.4). After incubation at 30°C for 10 min, the residual AHL and its hydrolysis products were quantified by HPLC. We defined 100% relative activity as the activity toward C_6-HSL. The data were reproduced at least three times, and error bars indicate standard deviations.
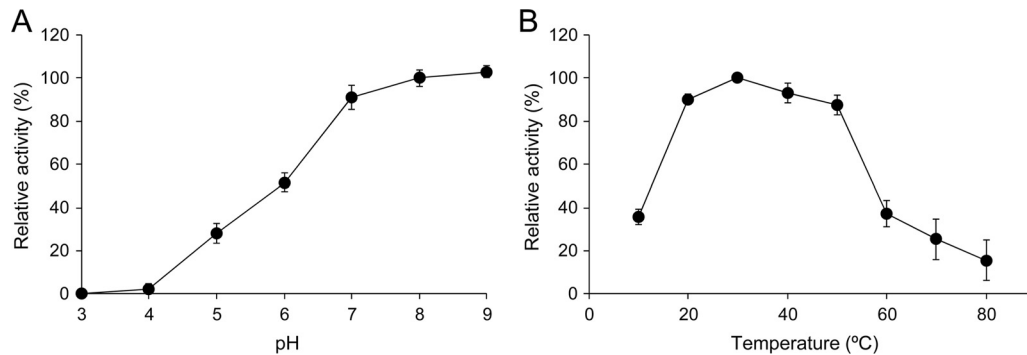
FIG 7 (A) Optimal pH of AHL-degrading activity of purified MBP-AidC. Purified MBP-AiiM was mixed with C_{10}-HSL in reaction buffer with a pH ranging from pH 3 to pH 8 and was then incubated at 30°C. The data were reproduced at least three times, and error bars indicate standard deviations. (B) Optimal temperature of AHL-degrading activity of purified MBP-AidC. Purified MBP-AiiM was mixed with C_{10}-HSL in the reaction buffer (pH 7.4) and incubated at temperatures ranging from 10 to 80°C. After incubation for 10 min, the residual substrate was quantified by HPLC. We defined 100% relative activity as the activity in the column buffer (pH 7.4) at 30°C. Control samples, which had been prepared under the same conditions but without AidC, were used to exclude the influence of autodegradation at high pH or high temperature. The data were reproduced at least three times, and error bars indicate standard deviations.

than 90% of the activity remained after incubation at temperatures of 50°C or less, but the enzymatic activity was markedly reduced after incubation at temperatures over 60°C (data not shown).

Kinetic analysis of AHL lactonase activity of AidC. AHL hydrolysis kinetics of AidC was determined by a plot of the substrate concentration and velocity. The k_{cat} and K_m values were calculated by fitting the data to the Michaelis-Menten equation (Table 2). The AHL lactonase activity of AidC showed comparable catalytic activities against a range of structurally different AHLs, with k_{cat} and K_m values in the range of 1.97 to 2.35 s^{-1} and 0.046 to 0.072 mM, respectively. Within these narrow ranges, the enzyme showed higher affinities (K_m), faster hydrolysis rates (k_{cat}), and stronger catalytic efficiencies (k_{cat}/K_m) toward the AHLs with short acyl side chains than toward those with the longer derivatives. Additionally, AidC displayed somewhat lower k_{cat} and k_{cat}/K_m values against the unsubstituted AHLs than against the 3-oxo-substituted AHLs (Table 2). Hydrolysis kinetics of the AiiA from *Bacillus* sp. 240B1 have been determined by plotting velocity versus substrate concentration (25). Compared with AiiA from *Bacillus* sp. 240B1, AidC showed lower K_m and k_{cat} values but higher catalytic efficiencies (k_{cat}/K_m) toward the AHLs (data not shown). In addition, K_m values of AidC against AHLs are at levels of tens of micromolar and even higher for several other AHL lactonases (1, 5, 25), but AHLs can function as signals at multiple

orders of magnitude below that level. Thus, in natural environments, the reaction rate should depend on the AidC concentration and the AHL concentration. The AidC/AHL reaction resembles a bimolecular reaction with a corresponding catalytic efficiency (k_{cat}/K_m). These results suggested that AidC may function much more effectively at quenching the bacterial QS systems which employ higher concentrations of AHLs than those employing lower concentrations of AHLs.

Zinc is essential for AHL-degrading activity of AidC. Sequence alignment of AidC and the known AiiA-type lactonases revealed that they share the zinc-binding motifs HXHXDH~H in the central region (Fig. 4). To verify whether AidC requires any cofactor for its enzymatic activity, the effects of several divalent metal ions on the AHL-degrading activity of AidC were evaluated. For the *in vitro* assay, purified MBP-AidC was mixed with C_{10}-HSL in column buffer containing EDTA and divalent metal ions. When 1 mM EDTA was added to this reaction mixture, the AHL-degrading activity of AidC was decreased by approximately 50% (Fig. 8A). However, AidC activity was successfully restored to 82% by the addition of Zn^{2+}, but not by any other of the metal ions tested (Fig. 8A). For *in vivo* assays, *E. coli* harboring pMAL-aidC was cultivated in the LB medium containing EDTA and divalent metal ions, and the AHL-degrading activity of AidC in the crude cell extracts was then determined. The AHL-degrading activity of AidC was abolished by the addition of 1 mM EDTA but was restored by the addition of Zn^{2+} (Fig. 8B). These results suggested that Zn^{2+} is essential for the AHL-degrading activity of AiiA and the known AiiA-type lactonases. In addition, the AHL-degrading activity of AidC activity was markedly inhibited by the addition of Cu^{2+} (data not shown). This feature of AidC also corresponded to that of the known AiiA-type lactonases (25).

***aidC* is widely conserved in the genus *Chryseobacterium*.** In a previous study, we isolated 34 AHL-degrading *Chryseobacterium* strains from the roots of potato plants and divided them into eight related species groups (16). We now attempted to amplify by PCR procedures the *aidC*-homologous genes from the three AHL-degrading *Chryseobacterium* strains which belong to group VI, as well as StRB126 (16). The group VI strains, which were StLB126, StLB340, StLB341, and StLB342, had higher AHL-degrading activity than the other groups (16). Specific primers were designed

TABLE 2 Kinetic parameters of AidC against AHLs[a]

AHL	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
C_6-HSL	2.31 ± 0.16	0.055 ± 0.0043	42.0
$3OC_6$-HSL	2.35 ± 0.29	0.046 ± 0.0021	51.1
C_8-HSL	2.01 ± 0.31*	0.064 ± 0.0049*	31.4
$3OC_8$-HSL	2.12 ± 0.24*	0.064 ± 0.0037*	33.1
C_{10}-HSL	ND	ND	ND
$3OC_{10}$-HSL	1.97 ± 0.11*	0.072 ± 0.0053*	27.4
C_{12}-HSL	ND	ND	ND
$3OC_{12}$-HSL	ND	ND	ND

[a] The data are the means from triplicate experiments. The significance was determined by Student's *t* test (*, $P < 0.05$). ND, not determined due to poor solubility of the substrate in the column buffer.
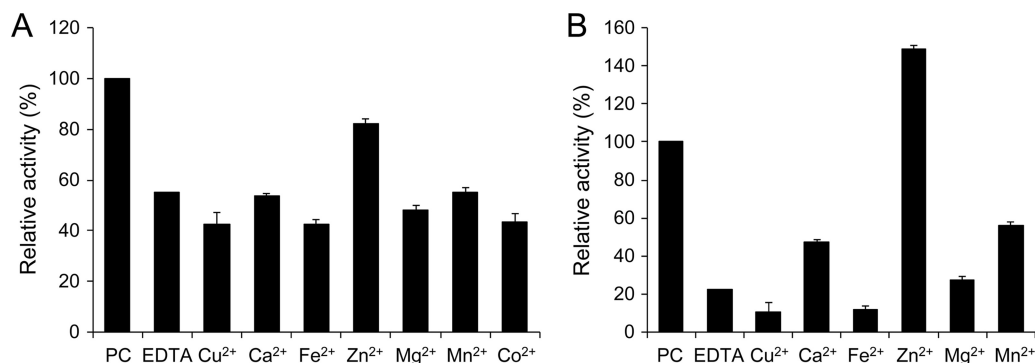
FIG 8 The effects of EDTA and metal ions on AHL-degrading activity of AidC by *in vitro* (A) and *in vivo* (B) assays. For the *in vitro* assay, purified MBP-AidC was incubated in reaction buffer containing substrate and 1 mM each metal ion. After incubation at 30°C for 10 min, the residual activity of AidC was analyzed by HPLC. For the *in vivo* assay, *E. coli* harboring pMAL-aidC was cultivated with 1 mM EDTA and 1 mM each metal ion. Crude cell extracts were prepared and used for the AHL-degrading assay. We defined 100% relative activity as the activity in the absence of EDTA or metal ions; this was represented as PC (positive control). The data were reproduced at least three times, and error bars indicate standard deviations.

based on the DNA sequences of *aidC* from StRB126. Following PCR amplification and DNA sequencing, *aidC* gene homologs were successfully cloned from all group VI strains. AidC from StLB126, StLB340, StLB341, and StLB342 had highly similar DNA sequences (more than 99% identity). In addition, *E. coli* DH5α harboring these *aidC* homologs showed significant AHL-degrading activity against various AHLs (data not shown). These results suggested that *aidC* sequences are highly conserved among group VI strains.

Based on BLAST search results, an *aidC* homologous gene was found in the whole-genome shotgun sequence of *C. gleum* NBRC 15054 (accession no. ACKQ00000000). However, we have previously reported that *C. gleum* NBRC 15054 did not show any AHL-degrading activity (16). To assess whether the AidC homolog ($aidC_{15054}$) from NBRC 15054 shows AHL lactonase activity, $aidC_{15054}$ was amplified from the chromosome of NBRC 15054 and cloned into the pUC118 cloning vector. *E. coli* DH5α harboring pUC118-aidC15054 showed a clear AHL-degrading activity level similar to AidC from StRB126. These results may suggest that the *aidC* gene homolog was not expressed in NBRC 15054 cells. NBRC 15054 appears distant from StRB126 in the phylogenetic analysis. Therefore, these results suggested that the *aidC* gene homolog may be widespread throughout *Chryseobacterium* strains, with or without AHL-degrading activity. In addition, in our previous study, *aiiM* homologous genes (encoding a group of AHL lactonases, AiiM) were only found from the potato leaf-associated *Microbacterium* isolates, but not found in strains from other resources (27). Correspondingly, although the *aiiM* homologous genes were highly similar to each other, their neighboring genes showed high diversity (27). When comparing the neighboring genes of *aidC* and $aidC_{15054}$, we found it interesting that, although their downstream genes showed no identity, the nearest three upstream genes of *aidC* shared high identities with the corresponding genes located upstream of $aidC_{15054}$ (data not shown). These results also suggested that the *aidC* gene may be widespread in the genus *Chryseobacterium*.

In summary, in this study, a novel AHL lactonase, AidC, was identified and characterized from a CFB group. Although AidC showed conserved zinc-binding motif sequences, as did the known AiiA-type lactonases, it showed little identity to known AHL lactonases; thus, we classified it into a novel group. In our

previous study, AHL-degrading *Chryseobacterium* strains could be isolated from potato roots, which were collected from various areas (16). Our results suggested that CFB bacteria may also retain functional AHL lactonases, extending the diversity of AiiA-type lactonases and again highlighting the relationship between AHL lactonase and metallo-β-lactamases.

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