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MomL, a Novel Marine-Derived *N*-Acyl Homoserine Lactonase from *Muricauda olearia*

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Gram-negative bacteria use *N*-acyl homoserine lactones (AHLs) as quorum sensing (QS) signaling molecules for interspecies communication, and AHL-dependent QS is related with virulence factor production in many bacterial pathogens. Quorum quenching, the enzymatic degradation of the signaling molecule, would attenuate virulence rather than kill the pathogens, and thereby reduce the potential for evolution of drug resistance. In a previous study, we showed that *Muricauda olearia* Th120, belonging to the class *Flavobacteriia*, has strong AHL degradative activity. In this study, an AHL lactonase (designated MomL), which could degrade both short- and long-chain AHLs with or without a substitution of *oxo*-group at the C-3 position, was identified from Th120. Liquid chromatography-mass spectrometry analysis demonstrated that MomL functions as an AHL lactonase catalyzing AHL degradation through lactone hydrolysis. MomL is an AHL lactonase belonging to the metallo-β-lactamase superfamily that harbors an N-terminal signal peptide. The overall catalytic efficiency of MomL for C_6-HSL is $\sim 2.9 \times 10^5$ s^{-1} M^{-1}. Metal analysis and site-directed mutagenesis showed that, compared to AiiA, MomL has a different metal-binding capability and requires the histidine and aspartic acid residues for activity, while it shares the "HXHXDH" motif with other AHL lactonases belonging to the metallo-β-lactamase superfamily. This suggests that MomL is a representative of a novel type of secretory AHL lactonase. Furthermore, MomL significantly attenuated the virulence of *Pseudomonas aeruginosa* in a *Caenorhabditis elegans* infection model, which suggests that MomL has the potential to be used as a therapeutic agent.

N-Acyl homoserine lactones (AHLs) are quorum-sensing (QS) signaling molecules that are used by many Gram-negative bacteria to communicate within species, to regulate gene expression and to synchronize social behaviors, such as biofilm formation, bioluminescence, and secretion of virulence factors (1, 2). An AHL molecule typically consists of a homoserine lactone and an acyl chain with an even number of carbons, with an occasional modification (hydroxy or olefinic double bond) at the C-3 position (1). It has been well established that AHL-dependent QS regulates virulence factor production in many bacterial pathogens, such as *Pseudomonas aeruginosa*, *Erwinia carotovora*, *Vibrio* spp., and *Burkholderia* spp. (1). Interference with QS has been recognized as a promising antivirulence therapy. Disturbing the QS systems in these pathogens would attenuate virulence rather than kill the bacteria and thereby weaken the selective pressure imposed on the pathogens and reduce the potential for evolution of drug resistance (3). QS inhibitors (QSIs; small molecules) and quorum-quenching (QQ) enzymes can both be used to interfere with QS. QSIs generally act to inactivate autoinducer synthases or receptors through competitive binding, whereas QQ enzymes switch off signal transmission through degradation of the signaling molecules. It has been demonstrated that QSIs and QQ enzymes can effectively reduce the production of virulence factors of bacterial pathogens *in vitro* and *in vivo* (4).

AHL-degrading enzymes have been extensively studied and were found in mammals, plants, fungi, achaea, and bacteria (4, 5). These enzymes may be classified in three major types according to their enzymatic mechanisms: AHL lactonases (lactone hydrolysis), AHL acylases (amidohydrolysis), and AHL oxidases and reductases (oxidoreduction). AHL lactonases hydrolyze the lactone ring of AHL, yielding the corresponding *N*-acyl-homoserine. This hydrolysis may also occur spontaneously at alkaline pH and can be reversed at lower pH (6). All lactonases identified thus far mainly belong to the metallo-β-lactamase superfamily, phosphotriesterase family and alpha/beta hydrolase family. The first identified AHL lactonase, AiiA from *Bacillus* sp. strain 240B1, is a member of the metallo-β-lactamase family (7). AiiA has a broad substrate specificity and a preference for substrates with long-acyl-chain AHLs (8). The amino acid sequence of AiiA contains a "^{104}HXHXDH^{109}~60aa~H^{169}" motif, which is common in metallo-β-lactamases. AiiA is a metalloprotein binding two equivalents of zinc, which is necessary for its activity (9). Although zinc was found in native AiiA, dicobalt-, dimanganese-, or dicadmium-substituted AiiA exhibits hyperactivity compared to that of the dizinc-substituted enzyme (10, 11). Heterologous expression of *aiiA* in numerous pathogenic bacteria, including *P. aeruginosa*, *Burkholderia thailandensis*, and *E. carotovora*, reduced the accumulation of AHLs and production of virulence factors (12).

Members of the phylum *Bacteroidetes* harbor many enzymes for degrading high-molecular-weight organic matter, and are commonly assumed to play a key role in the marine carbon cycle (13). Within the *Bacteroidetes*, seven species of QQ bacteria all belonging to the class *Flavobacteriia* have been identified (14).

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TABLE 1 Primers used in cloning *momL* and QuikChange mutagenesis[a]

Primer	Sequence (5′–3′)[b]	Restriction site or length (nt)
Murol1831F-1	GGAATTCCATATGAATATGAAAAAGCTACTTG	NdeI
Murol1831F-2	GGAATTCCATATGAAAAAGGAAGCTGCAG*	NdeI
Murol1831F-3	GGTGGTTGCTCTTCCAACAAAAAGGAAGCTG*	SapI
Murol1831R-1	CCGCTCGAGTTGTAAATAGTTGGGTG	XhoI
Murol1831R-2	CGCGGATCCTTATTGTAAATAGTTGGGTGC	BamHI
H117S-Antisense	ATGGCCAATATGATCAAAATGGGTAgaGGACAATGCGATAAAATCAATATCA	52
H117S	TGATATTGATTTTATCGCATTGTCCtcTACCCATTTTGATCATATTGGCCAT	52
H119S-Antisense	CGCATGGCCAATATGATCAAAAgaGGTATGGGACAATGCGATAAAA	46
H119S	TTTTATCGCATTGTCCCATACCtcTTTTGATCATATTGGCCATGCG	46
D121S/H122S-Antisense	AACACGTTCGCATGGCCAATAgaAgaAAAATGGGTATGGGACAATGCGATAAAATCAATATCA	63
D121S/H122S	TGATATTGATTTTATCGCATTGTCCCATACCCATTTTtcTtcTATTGGCCATGCGAACGTGTT	63
H189S-Antisense	CACTTGATGGCCAGGGGTAgaGCCTGGCATAAATTTCATT	40
H189S	AATGAAATTTATGCCAGGCtcTACCCCTGGCCATCAAGTG	40
D211S-Antisense	TCTCGTAAAAATGGTACATGgaCCCAGAAAGCATCAACGGTC	42
D211S	GACCGTTGATGCTTTCTGGGtcCATGTACCATTTTTACGAGA	42
H214G-Antisense	CTCCCGGTTCTCGTAAAAAccGTACATGTCCCCAGAAAGC	40
H214G	GCTTTCTGGGGACATGTACggTTTTTACGAGAACCGGGAG	40

[a] Primers were designed using the QuikChange primer design tool (Agilent).

[b] Mutagenic codons are underlined, and the mutagenic nucleotides are represented in lowercase. *, amplified genes with labeled primers lack signal peptide sequences.

Most of these QQ bacteria were isolated from the marine environment (14–17), and only one was derived from soil (the potato root-associated *Chryseobacterium* sp. strain StRB126) (18). The AHL lactonase AidC from this *Chryseobacterium* sp. strain represents a novel AiiA-type lactonase and shares only 13% identity to AiiA from *Bacillus* sp. strain 240B1 across the entire length, although it contains the "HXHXDH~H" motif and belongs to the metallo-β-lactamase superfamily (18). However, to our knowledge, the responsible QQ enzyme-encoding genes in the marine isolates have not been determined.

In a previous study, we demonstrated that the flounder mucus-derived strain *Muricauda olearia* Th120 has strong AHL degradative activity (14) and could decrease biofilm formation and virulence in *P. aeruginosa* PAO1 (19). In the present study, we identified the *momL* gene that encodes a novel secretory AHL lactonase from Th120. The enzymatic kinetics and metal content of MomL, as well as the effects of the novel AHL lactonase on *in vitro* virulence factor production and *in vivo* virulence of *P. aeruginosa*, were determined.

MATERIALS AND METHODS

Bacterial strains, media, growth conditions, and chemicals. *M. olearia* Th120 was cultured in marine broth 2216 (MB; Becton Dickinson) at 28°C. *Escherichia coli* strain BL21(DE3) was cultured on Luria-Bertani (LB) agar at 37°C and used as a host for expressing proteins whose encoding genes were cloned into pET24a(+) (Novagen) or pTWIN1 (New England BioLabs [NEB]). The AHL biosensor *Agrobacterium tumefaciens* A136(pCF218)(pCF372) (20) was maintained on LB agar and grown in AT minimal medium (21) containing 0.5% (wt/vol) glucose for detecting AHLs (C_6 to C_{14}) in liquid X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) assay. The AHL biosensor *Chromobacterium violaceum* CV026 (22) and VIR24 (23) were maintained on LB agar at 28°C, and were used to detect short-chain (C_4 to C_8) and long-chain (C_8 to C_{14}) AHLs, respectively. *P. aeruginosa* PAO1 was cultured on tryptic soy agar (Oxoid) at 37°C. Appropriate antibiotics were added at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}; and tetracycline, 4.5 μg ml^{-1}. *Caenorhabditis elegans* N2 (*glp-4*; *sek-1*) was propagated under standard conditions, synchronized by hypochlorite bleaching, and cultured on nematode growth medium using *Escherichia coli* OP50 as a food source (24, 25). C_4-HSL, C_6-HSL, 3-oxo-C_6-HSL, and C_8-HSL were purchased from Cayman Chemical Company (Ann Arbor, MI); 3-oxo-C_8-HSL, C_{10}-HSL, 3-oxo-C_{10}-HSL, C_{12}-HSL, 3-oxo-C_{12}-HSL, C_{14}-HSL, and 3-oxo-C_{14}-HSL were purchased from Sigma-Aldrich (St. Louis, MO). All of the AHL stock solutions (10 to 500 mM) were prepared in dimethyl sulfoxide (DMSO).

Characterization of the AHL degradative activity of *M. olearia* Th120. The C_6- and C_{12}-HSL degradative activities of the whole culture, cells, and supernatant of Th120 were determined. Briefly, Th120 was inoculated in MB and incubated with shaking (170 rpm) at 28°C for 24 h. The supernatant of the culture was collected after centrifugation at 13,000 × *g* for 10 min at 4°C and filtered through a 0.22-μm-pore-size filter. The cells were harvested and washed three times with MB and resuspended in MB. The cell suspension was sonicated on ice, and the supernatant and cell content were collected after centrifugation and filtration. The C_6- and C_{12}-HSL degradative activities of these sample were measured using the A136 liquid X-Gal assay, which applies 1,4-piperazinediethanesulfonic acid (PIPES) buffer (pH 6.7) to prevent alkaline hydrolysis (14). In addition, the acidification test was used to demonstrate whether the AHL degradation was due to lactonase activity (6). Briefly, the mixture was boiled for 5 min and acidified with HCl to pH 2, followed by further incubation at 28°C for 24 h. The recovery of C_6-HSL or C_{12}-HSL were measured by the A136 liquid X-Gal assay and further confirmed by the CV026 or VIR24 plate assay (22, 23). In order to determine whether the QQ activity is due to enzymatic degradation, these three samples were preboiled for 5 min or pretreated with proteinase K (final concentration, 200 μg ml^{-1}) at 37°C for 3 h, after which their AHL degradative activities were measured.

Expression and purification of QQ enzyme of Th120. Putative QQ enzyme-encoding genes were searched from whole-genome data of Th120 (K. Tang and X.-H. Zhang, unpublished data) through local BLASTP against a known QQ enzyme database. In order to obtain recombinant proteins with or without signal peptide, the putative gene was amplified with the primer pairs AHL1831F-1/AHL1831R-1 and AHL1831F-2/AHL1831R-1 and cloned into pET24a(+) or with the primer pair AHL1831F-3/AHL1831R-2 and then cloned into pTWIN1 (Table 1). The resulting vectors were transformed into *E. coli* BL21(DE3). *E. coli* strains harboring different vectors were grown in LB broth at 37°C until cells reached an optical density at 600 nm (OD_{600}) of 0.5 to 0.7. Protein expression was induced by the addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cultures were further grown at 16°C with moderate shaking (150 rpm) for 12 h.

Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C and resuspended in 50 mM Tris-HCl buffer with 10 mM imidazole–0.5 M NaCl (pH 8; for cells harboring pET plasmid) or in 20 mM Na-HEPES with 0.5 M NaCl, 10% glycerol, and 0.1% Triton X-100 (pH 8.5; for cells harboring pTWIN1). The suspensions were sonicated on ice, and the cell debris was removed by centrifugation at 13,000 × *g* for 10 min at 4°C. The supernatants were filtered through a 0.22-μm-pore-size filter and loaded onto NTA-Ni (Qiagen) or chitin columns (NEB). Proteins were purified according to the manufacturer's recommendations. For the purification of MomL without the signal peptide sequence, the on-column cleavage of the intein tag was induced by equilibrating the chitin resin in 20 mM Na-HEPES with 0.5 M NaCl (pH 6.5) at room temperature for 24 h. The purified proteins were stored at −20°C with 25% glycerol. The AHL degradative activities of the expressed proteins were tested using the A136 liquid X-Gal assay.

Analysis of metal content of the expressed protein. The MomL protein was expressed and purified as described above using *E. coli* BL21(DE3) harboring pTWIN1-*momL*(-SP), and alternatively $ZnCl_2$ or $CoCl_2$ was added, along with IPTG, to a final concentration of 200 or 50 μM, respectively. Protein concentration was measured spectrophotometrically by GeneQuant 100 (GE Healthcare Life Science). The metal concentrations of the purified proteins were analyzed by Agilent 7500c inductively coupled plasma mass spectrometry (ICP-MS).

In-gel QQ activity assay. Protein samples were characterized by native-PAGE using a 4% stacking gel and 12% separating gel at 4°C as described previously (26), except that sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) were omitted. One lane slice was washed three times in distilled water and then placed on a plate, which was overlaid with the soft agar. The ready-to-use soft agar was prepared as follows: AT minimal glucose medium containing 0.5% agarose, 200 mM PIPES (pH 6.7), 250 μg of X-Gal ml^{-1}, and 2 mM C_6-HSL was inoculated with A136 overnight culture with an inoculum size of 3%. The plate was further incubated for 12 to 24 h at 28°C. The other duplicate lane slice loaded with an identical sample was stained with Coomassie brilliant blue.

Liquid chromatography-mass spectrometry (LC-MS) analysis of products formed by MomL catalysis. A 10-μl (~10-μg) portion purified MomL was mixed with 50 μl of PIPES buffer (1 M, pH 6.7), AHLs (C_6- or C_{12}-HSL with a final concentration of 2 mM), and deionized water was added up to 500 μl. The hydrolysis was carried out at 28°C for 12 h, and the mixture was extracted with ethyl acetate three times. After evaporation, the samples were analyzed by LC using a SunFire C_{18} reversed-phase column (3.5 μm; 4.6 by 50 mm) with a mobile phase of acetonitrile-water (0.01% trifluoroacetic acid; a linear gradient [vol/vol] of acetonitrile from 5 to 95% over 1.5 min at a flow rate of 1.8 ml min^{-1} for C_6-HSL or 2.1 ml min^{-1} for C_{12}-HSL). The separated fractions were further analyzed by electrospray ionization mass spectrometry (ESI-MS).

Kinetic assay of MomL activity. The catalytic activity of MomL was measured by a pH sensitive colorimetric assay (27). Briefly, in a typical morpholinepropanesulfonic acid (MOPS)/bromothymol blue (BTB) system, 100 μl of reaction mixture contains 3.5 nM enzyme, 100 μM BTB, and 0 to 5 mM AHL substrate in 2.5 mM MOPS buffer (pH 7.1) with 1% DMSO. In this MOPS/BTB system, protons released from the hydrolysis of AHLs would alter the pH of the weakly buffered solution and thereby change the protonation state of BTB, a pH-sensitive dye, resulting in a color change. The color change was spectrophotometrically measured at 630 nm over time using the Tecan Sunrise microplate absorbance reader at 25°C. Initial reaction velocities were calculated after subtracting the background hydrolysis rates of AHLs in the absence of enzyme and loaded into the GraphPad Prism software (version 5.01) for calculating the K_m and k_{cat} values. A standard curve representing the relationship between the absorbance change and the proton concentration was generated using HCl. In this MOPS/BTB system, the value of OD_{630} would decrease by 0.18 with the addition of 100 nmol of HCl.

Physical and chemical parameters that affect MomL activity. The optimal temperature of MomL activity was measured based on the kinetic assay with slight modifications. Briefly, 10 mM C_6-HSL was used as the substrate. Hydrolysis velocities of MomL at 10 to 90°C (10 degrees intervals) were determined by start-endpoint detection during 20 min of incubation. To determine the thermostability of MomL, the purified MomL protein was preincubated at 40, 60, 80, or 100°C for 30 min, after which the residual activity was evaluated based on the kinetic assay. Likewise, the pH stability of MomL was determined after the purified MomL was preincubated at 4°C and different pHs (Na_2HPO_4/citric acid for pH 2.0 to 7.0, Tris-HCl for pH 8.0 to 9.0, and Na_2CO_3/$NaHCO_3$ for pH 10.0 to 11.0) for 3 h. Furthermore, to determine the effect of metal ion on MomL, the residual activity was evaluated after the purified MomL protein was preincubated with 0.1 mM concentrations of different metal ions (Ni^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Fe^{2+}) or with 1 mM EDTA at 4°C for 1 h. Subsequently, the EDTA-treated MomL protein was reincubated with a 1.1 mM concentration of each metal ion, and the residual activity was measured. The MOPS/BTB system was supplemented with a 0.1 mM concentration of each corresponding metal ion. For each assay, three independent replicates were performed, and the initial velocity was calculated.

Site-directed mutagenesis of *momL*. Site-directed mutagenesis of *momL* was carried out by using a QuikChange Lightning site-directed mutagenesis kit (Agilent) according to the instruction manual. The plasmid pTWIN1-*momL*(-SP) was used as a template. Primers used to obtain each mutant are listed in Table 1. Each mutated protein was expressed and purified as described above. The kinetic constant for hydrolysis of C_6-HSL by each mutated enzyme was measured as described above.

Effect of MomL on virulence factor production in *P. aeruginosa* PAO1. One unit of enzyme was defined as the amount of protein catalyzing 1 μmol of C_6-HSL in 1 min. The extracellular proteolytic activity of *P. aeruginosa* PAO1 cultured with different concentrations of MomL (0, 0.05, 0.5, or 5 U ml^{-1}) was determined according to the method of Ayora and Giötz (28) with slight modifications. The cell density of PAO1 and the amount of digested azocasein were spectrophotometrically measured at 590 and 415 nm, respectively. The proteolytic activity was expressed as OD_{415}/OD_{590}. Moreover, the pyocyanin production of *P. aeruginosa* PAO1 was measured as described by Essar et al. (29).

***C. elegans* survival assay.** The *C. elegans* survival after infection with *P. aeruginosa* PAO1 was determined as described previously (30) with minor modifications. In brief, synchronized worms (L4 stage) were suspended in a medium containing 95% M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, and 1 ml of 1 M $MgSO_4 \cdot 7H_2O$ in 1 liter of water), 5% brain heart infusion broth (Oxoid), and 10 μg of cholesterol (Sigma-Aldrich)/ml. Then, 50 μl of this suspension of nematodes was transferred to the wells of a 96-well microtiter plate. An overnight *P. aeruginosa* PAO1 culture was centrifuged, resuspended in the assay medium, and 1/1,000 diluted. Next, 50-μl aliquots of this standardized suspension were added to each well, while 50 μl of sterile medium was added to the positive control. The purified MomL protein was lyophilized with sucrose and added to the test wells at a final concentration of 0.5, 0.1, 0.01, or 0.001 U ml^{-1}, respectively. The assay plates were incubated at 25°C for up to 2 days. The fraction of dead worms was determined by counting the number of dead worms and the total number of worms in each well using a dissecting microscope. Samples were tested at least four times in each assay, and each assay was repeated at least three times ($n \geq 12$).

Nucleotide sequence accession number. The nucleotide sequence of *murol1831* (*momL*) from *M. olearia* Th120 has been deposited in the GenBank database under accession no. KJ756328.

RESULTS

***M. olearia* Th120 possesses extracellular AHL lactonase activity.** Heat treatment completely abolished the activity of the supernatant, whereas it had little effect on that of the whole culture and cell content of Th120 (Fig. 1). Likewise, no activity could be detected in the supernatant after treatment with proteinase K. In addition, complete recovery of C_6- and C_{12}-HSL by acidification was only observed in the supernatant of Th120. These results suggest that Th120 may produce an extracellular AHL lactonase and an intra-

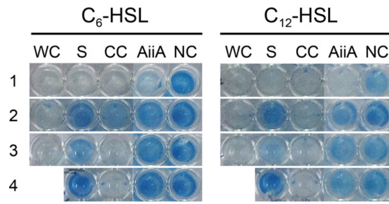

FIG 1 Characterization of the AHL degradative activity of *M. olearia* Th120. AHL degradative activity of Th120 was analyzed by the A136 liquid X-Gal assay. AiiA and MB medium were used as the positive and negative controls, respectively. WC, whole culture; S, supernatant; CC, cell content; NC, negative control. In consideration of incomplete degradation of proteins, no proteinase K-treated WC was included. Four arabic numbers represent different pretreatments of each sample: untreated (row 1), recovery of AHLs by acidification (row 2), heat treated (row 3), and proteinase K treated (row 4).

cellular acylase (or other intracellular enzymes that can further hydrolyze ring-opened AHLs).

Identification of the extracellular QQ enzyme in *M. olearia* Th120. Local BLASTP against known QQ enzymes database was performed to search for possible QQ enzymes, and a protein (Murol1831) composed of 293 amino acid residues showed relatively high identities to the known AHL lactonases. Murol1831 belongs to the metallo-β-lactamase superfamily. The amino acid sequence of Murol1831 showed <25% identity to each of the known AiiA-type QQ enzyme across the entire length, and the best hit is AiiA from *Bacillus* sp. strain 240B1 (identity, 24.5%; similarity, 37.2%). Further BLASTP sequence similarity searching against the NR protein database showed that its homologues mainly occur in marine bacteria of the family *Flavobacteriaceae*. Murol1831 may represent a novel marine-derived AHL lactonase and thus was termed MomL (*M*uricauda *o*learia *m*arine AHL *l*actonase). In contrast to other AHL lactonases of this superfamily, MomL was predicted to be extracellular with an N-terminal signal peptide of 21 amino acid residues based on SignalP analysis (31) (Fig. 2). Further *in silico* analysis of MomL showed a "HXHXDH" zinc-binding motif that is conserved in several groups of metallohydrolases. Two conserved Phe residues were found in the sequence of MomL (Fig. 2).

The recombinant Murol1831 expressed by each system efficiently degraded both short- and long-chain AHLs (see Fig. S1 in the supplemental material). However, most of the recombinant

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MomL          1  MNMKKLLVLLLVVGAFSCKQAKKEAAESETAVTEAAKP..........EIKLYAFSGGTVNANMLELFSQDTTYT
WP_019670967  1  ..MKKYITLLVIFSLFACKEVKKEVVSASEIKEINSRP..........EVKLYTFDGGKVVVNTLGLFAQDETYK
AidC          1  MNRRELLKSGLLAGTLSFMPFSNVFAETKLFSEKTEDDLSGFKKIKLGELELFILTDGYIHEENLISFAPRGNVA
AiiA          1  MTVK............................................KLYFVPAGRCMLDHSSVNSTLTP..
AttM          1  ..............................................MLQSGTLKCKVHNIKMNQGN..
AiiB          1  ..............................M..........GNKLFVLDLGEIRVDENFIIANSTFVT
QlcA             ...........................................................................

MomL         66  .......GQSK.....EFADAFYVIVHPKGTLMWDAGLPESLVGLPEPFTSP...DGAFT.......VSRKDSVA
WP_019670967 64  .......GQSK.....EFADAFYVIKHPKGNIMWDAGLPESLVGLPEPYTSP...DGAFT.......VSRKDSVL
AidC         76  ELKTILKDNFRADHYIDMAINILLVKTKEKLILMDTGM.......................GIFA.......DERTGFLL
AiiA         28  .......GELL.....DLPVWCYLLETEEGPILVDTGMPESAVNNEGLF......NGTFVEGQVLPKMTEEDRIV
AttM         21  .......GADY.....EIPVPFFLITHPAGHTVIDGGNAIEVATDPRGH......WGGICD.VYWPVLDKDQGCV
AiiB         29  PQKPTVSSRLI.....DIPVSAYLIQCTDATVLYDTGCHPECMGTNGRWPAQSQLNAPYI.......GASECNLP
QlcA          1  ..................MMVWVLKGDGRIAIVDSGFHRE.....RY......FRQFT.......VKDYVKPS

MomL        119  NQLASIDMTVDDIDFIALSHTHFDHIGHAN......VFAGSTWLVQEKEYDFV..TSEDNQKSN.......PDIY
WP_019670967 117 NQLKQIGMTPDDFQYISLSHTHFDHSGHAN......LFKNSTWLVQKEEYDFV..TGKELKESN.......PDMY
AidC        126  KSLQKAGFSAHDITDIFLSHAHPDHIGGVVDKQNKLVFPNASIFISKIEHDFW..INASIKDFNNSALKAHPERL
AiiA         85  NILKRVGYEPEDLLYIISSHLHFDHAGGNG......AFINTPIIVQRAEY.......EAAQHSE.......EYLK
AttM         77  DQIKALGFDPADVKYVVQSHLHLDHTGAIG......RFPNATHIVQRSEYEYA..FTPDWFAGG.......GYIR
AiiB         92  ERLRQLGLSPDDISTVVLSHLHNDHAGCVE......YFGKSRLIAHEDEFATAVRYFATGDHSS.......PYIV
QlcA         38  EAIAPLGINPEDVTDLLVTHMHWDHAGGIG......LFPAARVWIQKDEYDYY..TGDAWQAAGTHGGIDAEDVV

MomL        179  NSI....KELTKVKKINGD....YDVFGDGSVVMKF..MPG..HTPGHQVLYLDMVEHGPLMLSGDMYHFYENR.
WP_019670967 177 NAI....KDLKKIKKIDGD....FDVFNDGTVILKY..MPG..HTPGHQVLFLDLKEHGPLLLSGDLYHFYENR.
AidC        199  NQII...PALQNILKAIQPKLKFYDLNKTLYSHFNFQLAPG..HTPGLTVTTISSGNEK.LMYVADLIHSDVILF
AiiA        140  ECI....LPNLNYKIIEGD....YEVVPG..VQLLH..TPG..HTPGHQSLLIETEKSGPVLLTIDASYTKENF.
AttM        137  KDFD...KPGLKWQFLNGAQDDYYDVYGDGTLTTIF..TPG..HAPGHQSFLVRLPNSKPLLLTIDAAYTLDHW.
AiiB        154  KDIEAWLATPRNWDLVGRDERE.RELAPGVNL.LNF..GTG..HASGMLGLAVRLEKQPGFLLVSDACYTATNYG
QlcA        105  EIVRR..NTQGKVSFVRGDD...QMSLPG....IGF..GVGGKHTWASQFVSVRTRQHV.VVVASDNMYLYENL.

MomL        241  EFRRVPIFN.YDVALTKKSMGEFEAFAEEKGAKVY........LQHSKEDFEKLPQAPN.YLQ..
WP_019670967 239 EYKRIPIFN.YDVSQTRESMKNFEAFADEKKAKVY........LQHQKDDFNKMPKAPQ.YL...
AidC        268  PHPDWGFSGDTDLDIATASRKKFLKQLADTKARAFTSHLPWPGLGFTKVKAPGFEWIPESFMN..
AiiA        200  .ENEVPFAG.FDSELALSSIKRLKEVVMKEKPIVF........FGHDIEQERGCKVFPE.YI...
AttM        204  EEKALPGFL.ASTVDTVRSVQKLRTYAEKHDATVV........TGHDPDAWANFKKAPE.FYA..
AiiB        223  PPARRAGVL.HDTIGYDRTVSHIRQYAESRSLTVL........FGHDREQFASLIKSTDGFYE..
QlcA        167  .EKHLPVAQTLDAASNLRTQDRMRSLASEPRLLV.........PGHDAAVFDRFPRVSDRIVRIE
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FIG 2 Multiple-sequence alignment of amino acid sequences of MomL, putative homologues, and other representative AHL lactonases. Sequence alignment was performed by the MUSCLE program in the MEGA software package and enhanced by ESPript 3.0. MomL homologue from *Eudoraea adriatica* (WP_019670967) showed highest score when BLASTP searching nonredundant (NR) databases. Other sequences of AHL lactonase are AiiA from *Bacillus* sp. strain 240B1 (AAF62398), AidC from *Chryseobacterium* sp. strain StRB126 (BAM28988), QlcA from unculturable soil bacteria, and AttM (AAD43990) and AiiB (NP 396590) from *Agrobacterium fabrum* C58. These lactonases share the "HXHXDH" motif. MomL harbors an N-terminal signal peptide, and the predicted cleavage site was labeled by arrow. Empty circles indicate the conserved two Phe residues in some lactonases. Filled triangles show the amino acid residues chosen for site-directed mutagenesis.

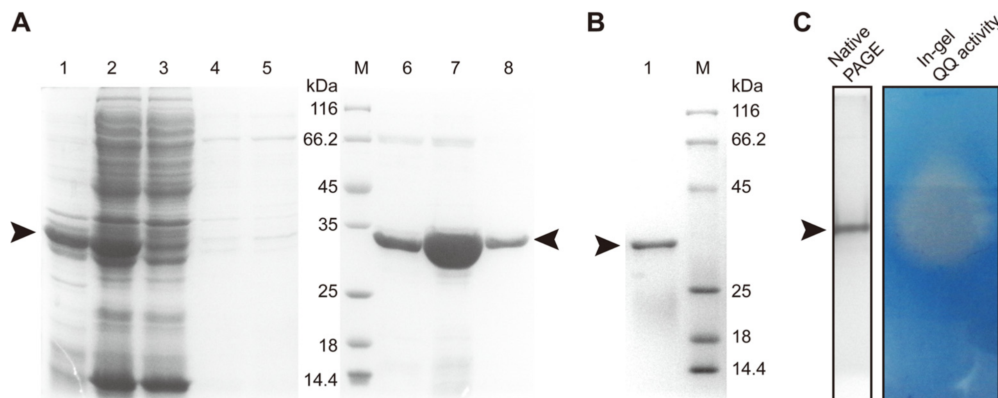

FIG 3 PAGE analysis of expressed MomL lacking the signal peptide sequence. MomL protein bands stained by Coomassie brilliant blue are indicated by arrows. (A) SDS-PAGE analysis of MomL-6×His-tagged protein. M, molecular mass markers; lane 1, insoluble fraction of induced *E. coli* BL21(DE3)/pET 24a(+)-*momL*(-SP); lane 2, soluble fraction; lane 3, soluble fraction after passage over NTA-Ni column; lanes 4 and 5, 20 mM imidazole wash; lanes 6, 7, and 8, 250 mM imidazole elution. (B) SDS-PAGE analysis of the purified MomL protein without signal peptide from *E. coli* BL21(DE3)/pTWIN1-*momL*(-SP). (C) In-gel QQ activity assay of the purified MomL protein from *E. coli* BL21(DE3)/pTWIN1-*momL*(-SP).

MomL protein was produced as inclusion bodies when it was expressed in a pET24a(+)/BL21(DE3) system with a full-length amino acid sequence, probably because MomL contains an N-terminal signal peptide. Therefore, MomL lacking the signal peptide sequence was expressed in a pET24a(+)/BL21(DE3) system, and most of the recombinant MomL protein was in a soluble form (Fig. 3A). SDS-PAGE analysis showed a band of purified recombinant MomL with a molecular mass of nearly 31 kDa, which was consistent with the predicted molecular mass of MomL lacking the signal peptide (31.6 kDa, including a 6×His tag). In order to obtain MomL protein without vector-derived amino acid residues, the MomL protein lacking the signal peptide was expressed in pTWIN1/BL21(DE3) system (Fig. 3B); the purified MomL showed strong degradative activity against C_6-HSL by in-gel QQ activity analysis (Fig. 3C). Moreover, hydrolyzed AHLs by MomL could be recovered by acidification, suggesting that MomL is an AHL lactonase (Fig. 1).

MomL is an *N*-acyl homoserine lactonase. To further determine how MomL degrades AHLs, C_6- and C_{12}-HSL were digested by MomL, and the reaction products were analyzed by LC-MS. The enzymatic digestion of C_6-HSL resulted in one product with a retention time of 1.58 min, as determined by the LC analysis (Fig. 4). ESI-MS analysis of the product revealed a strong quasimolecule (M-H) ion at an *m/z* (mass-to-charge ratio) of 218.0, suggesting that the enzymatic action with C_6-HSL (M-H ion *m/z* of 200) resulted in a mass increase of 18, corresponding to a water molecule. This is consistent with the M-H ion *m/z* of the lactone-opened C_6-HSL, namely, *N*-hexanoyl-L-homoserine (C_6-HS, M-H ion *m/z* of 218). ESI-MS analysis revealed that the composition of the LC peak with a retention time of 1.71 min was undigested C_6-HSL. Likewise, MomL could degrade C_{12}-HSL to generate C_{12}-HS (see Fig. S2 in the supplemental material). These results strongly suggest that MomL is an AHL lactonase that hydrolyzes the ester bond of the homoserine lactone ring of AHL.

Kinetic characterization of MomL. To investigate the substrate specificity of MomL, kinetic constants for hydrolysis of AHL substrates differing in the length of the aliphatic *N*-acyl side chain or in the substitution of the C-3 carbon were determined (Table 2). The k_{cat} values for all substrates were in the range of 135 to 293 s^{-1}. The K_m values for long-chain AHLs were lower than those for short-chain AHLs. Likewise, the overall catalytic efficiencies (k_{cat}/K_m) gradually increased as the acyl chain lengthened, suggesting a preference of MomL for long-chain AHLs.

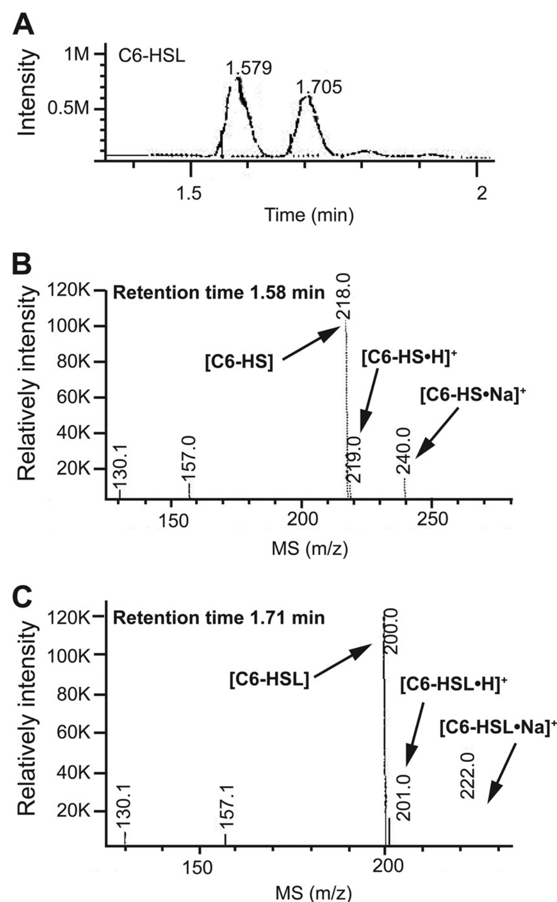

FIG 4 LC-MS analysis of the MomL-hydrolyzed C_6-HSL product. (A) LC profile of the MomL-hydrolyzed C_6-HSL product. (B and C) ESI-MS analysis of LC fractions containing the 1.58-min product (B) and 1.71-min undigested C_6-HSL (C).

TABLE 2 Kinetic constants for hydrolysis of AHLs by MomL[a]

Substrate	Mean ± SD		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
C_4-HSL	135 ± 5	0.85 ± 0.09	1.6×10^5
C_6-HSL	226 ± 8	0.79 ± 0.08	2.9×10^5
3-Oxo-C_6-HSL	293 ± 17	0.95 ± 0.16	3.1×10^5
C_8-HSL	158 ± 17	0.44 ± 0.16	3.6×10^5
3-Oxo-C_8-HSL	218 ± 14	0.49 ± 0.11	4.5×10^5
3-Oxo-C_{10}-HSL	224 ± 12	0.44 ± 0.10	5.1×10^5

[a] Reactions were carried out at pH 7.1 and 25°C. The kinetic constants for the hydrolysis of C_{10}-, C_{12}-, 3-oxo-C_{12}-, C_{14}-, and 3-oxo-C_{14}-HSL were not determined due to poor solubility in MOPS buffer.

Physical and chemical parameters that affect MomL activity. The effect of several physical and chemical parameters, i.e., temperature, pH, EDTA, and metal ions, that may affect MomL activity was investigated. MomL exhibited relatively high activity at temperatures ranging from 20 to 50°C, reaching its optimum activity at 40°C (Fig. 5A). MomL retained ca. 30% activity after being heated to 60°C for 30 min and still possessed weak activity after being boiled for 30 min (Fig. 5B and see Fig. S3 in the supplemental material). In addition, incubation at pH lower than 7 for 3 h abolished most of the MomL activity (Fig. 5C). At 100 μM, Ni^{2+}, Cu^{2+}, and Fe^{2+} inactivated MomL, whereas Mg^{2+}, Ca^{2+}, and Co^{2+} enhanced MomL activity (Fig. 5D). Moreover, most of the activity was lost after MomL was treated with 1 mM EDTA, while MomL activity was partially recovered by reincubation with Mg^{2+}, Zn^{2+}, and Co^{2+} (Fig. 5D). These results indicated that MomL is a metallohydrolase, and we speculate that the effect of low pH on MomL might be due to the metal chelating agent citric acid used in the buffer.

Metal analysis and kinetic constants of MomL. After *E. coli* was grown in LB medium with different metal supplements, the metal content of MomL was determined by ICP-MS analysis (Table 3). The purified MomL contained 0.57 equivalents of magnesium when the medium was not supplemented with extra metal ions. When the growth medium was supplemented with $ZnCl_2$ (0.2 mM), the molar ratios of purified MomL to zinc and magnesium were determined to be 1.67 and 0.23 equivalents, respectively. When 0.05 mM $CoCl_2$ was added in the medium, no cobalt was detected; however, nearly 0.1 equivalents of beryllium and magnesium were determined in the purified MomL. Moreover, the overall catalytic efficiencies of MomL purified from medium with extra metal supplement for C_6-HSL were higher than that purified from LB medium (Table 3).

The conserved histidine and aspartic acid residues are necessary for MomL activity. Site-directed mutagenesis was used to identify the role of several conserved histidine and aspartic acid residues in MomL. In AiiA, it has been shown that the "^{104}HXHXDH^{109}~60aa~H^{169}" motif is necessary for metal binding and catalysis and that Tyr194 plays an important role in both substrate binding and catalysis by forming a hydrogen bond between the phenol side chain of Tyr194 and the lactone carbonyl of the substrate (7, 8, 32–34). The conserved histidine and aspartic acid residues in the MomL "HXHXDH~H" motif were replaced by non-metal-binding serine residues (Fig. 2; see also Table S1 in the supplemental material). The MomL residue corresponding to Tyr194 in AiiA is His214 (Fig. 2). A hydrogen bond was assumed to exist between the N–H of His214 and the C=O group of the substrate. His214 was therefore replaced by glycine, a neutral residue, with the consideration to remove the possible hydrogen bond, regardless of its contribution to protein conformation (see Table S1 in the supplemental material). All of the mutations resulted in a nonfunctional MomL enzyme (see Table S1 in the supplemental material), suggesting that these conserved histidine and aspartic acid residues are absolutely required for MomL activity.

MomL reduces the *in vitro* production of *P. aeruginosa* PAO1 virulence factors. The effect of MomL on the virulence factor production in *P. aeruginosa* PAO1 was evaluated *in vitro*. At 0.05 U ml^{-1}, MomL reduced the extracellular protease activity of *P. aeruginosa* PAO1 with ca. 50% (Fig. 6A). Similarly, adding MomL greatly reduced pyocyanin production by *P. aeruginosa* PAO1 in a concentration-dependent way (Fig. 6B). The growth of PAO1 was not affected obviously, whereas AHL accumulations in the cultures were significantly reduced (see Fig. S4 in the supple-

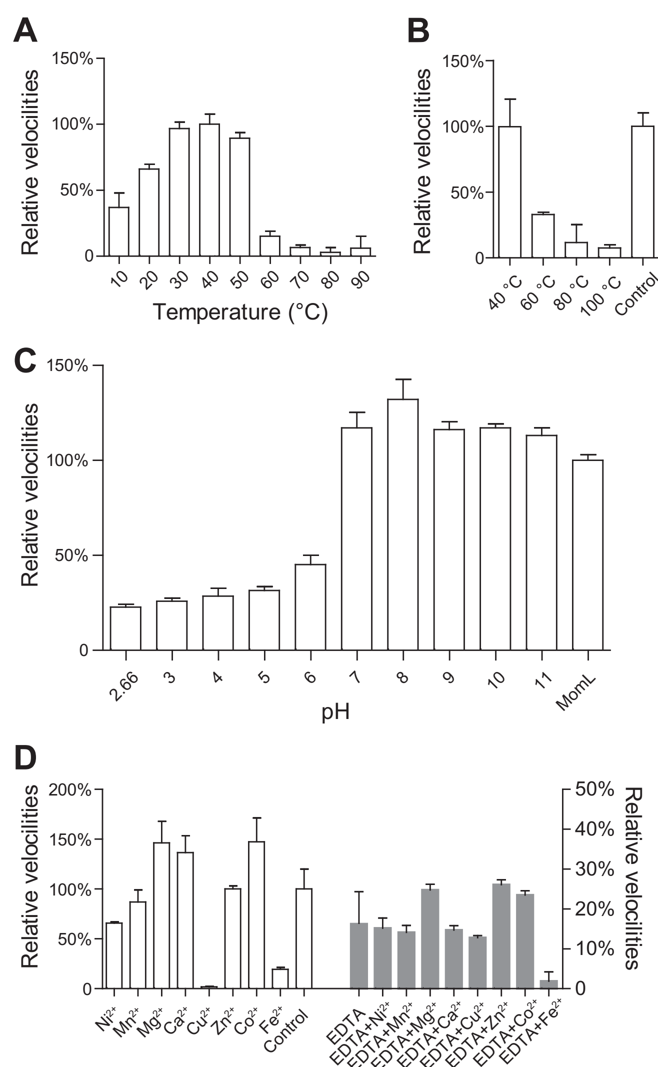

FIG 5 Physical and chemical parameters that affect MomL activity. The optimal temperature (A), temperature stability (B), and pH stability (C) of MomL activity and the effects of metal ions on MomL activity (D) were determined. The data were shown as means ± the standard deviation (SD).

TABLE 3 Metal contents and kinetic constants of MomL purified from LB medium with different metal supplements

Enzyme	Metal contents (equivalent)[a]						Kinetic constants[b]		
	Be	Mg	Cr	Fe	Zn	Mo	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
MomL (LB)[c]	0.04	0.57	0.00	0.01	0.00	0.00	226 ± 8	0.79 ± 0.08	2.9×10^5
MomL (LB+$ZnCl_2$)	0.00	0.23	0.01	0.01	1.64	0.01	454 ± 21	0.74 ± 0.18	6.1×10^5
MomL (LB+$CoCl_2$)	0.08	0.09	0.00	0.00	0.00	0.00	407 ± 27	0.45 ± 0.11	9.0×10^5

[a] The ICP-MS analysis also showed that MomL did not contain calcium, vanadium, manganese, cobalt, nickel, copper, arsenic, selenium, and cadmium. Be, beryllium; Mg, magnesium; Cr, chromium; Fe, iron; Zn, zinc; Mo, molybdenum.

[b] That is, the kinetic constants for hydrolysis of C_6-HSL were measured. k_{cat} and K_m values are expressed as means ± the standard deviations.

[c] The kinetic constants for hydrolysis of C_6-HSL by MomL purified from medium without extra metal supplement are from Table 2.

mental material), suggesting the reduction of virulence factor production was due to AHL degradation.

Administration of MomL increases the survival of infected nematodes. MomL did not display any toxic activity against *C. elegans*, since the survival of uninfected nematodes was not different from that of uninfected nematodes exposed to MomL in concentrations up to 0.5 U ml^{-1} (Fig. 6C). Addition of MomL (0.01, 0.1, or 0.5 U ml^{-1}) significantly increased the survival of infected nematodes, both after 24 and 48 h of infection, in a concentration-dependent way (Fig. 6C). Addition of the highest concentrations (0.1 and 0.5 U ml^{-1}) of recombinant MomL protein even restored survival to the levels of the uninfected controls. No effect was observed when MomL was added in a low concentration (0.001 U ml^{-1}).

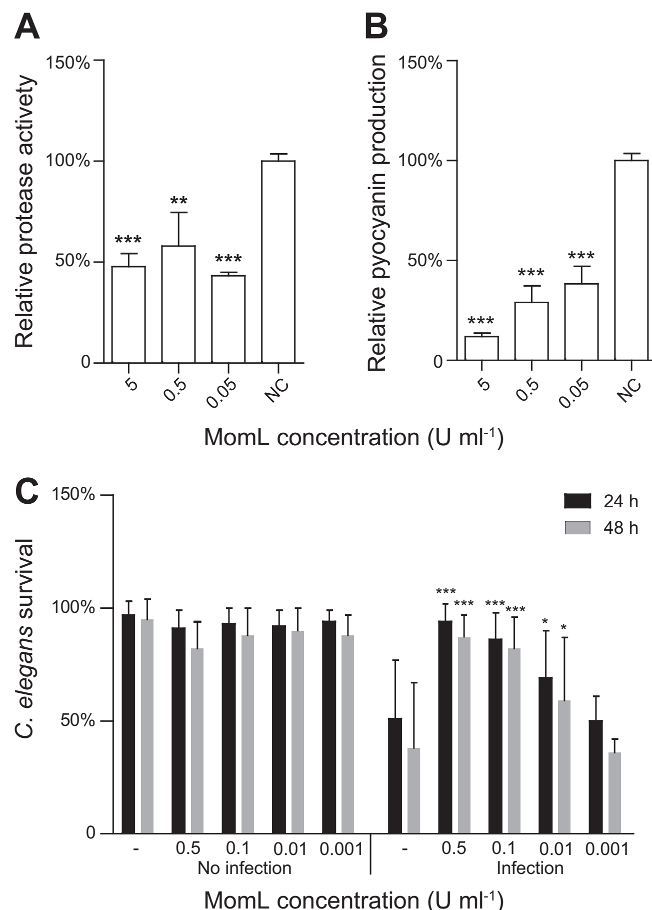

FIG 6 Effect of MomL on virulence factor production. The extracellular protease activity (A) and pyocyanin production (B) of *P. aeruginosa* PAO1 and *C. elegans* survival (C) were determined. NC, negative control. The data are shown as means ± the SD. One-way analysis of variance, followed by Dunnett's multiple-comparison test (GraphPad Prism v5.01), was performed to detect significant differences among treated groups and controls (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

DISCUSSION

MomL represents a novel class of extracellular AHL lactonases. MomL homologues are widespread in marine bacteria of the family *Flavobacteriaceae* (Fig. 7). Based on the phylogenetic analysis, MomL and its homologues are distinct from the AiiA and AidC clusters, although all of these enzymes belong to the same metallo-β-lactamase superfamily (Fig. 7). Compared to other members in this superfamily, MomL displayed some unique characteristics.

MomL is a novel AHL lactonase belonging to the metallo-β-lactamase superfamily that possesses an N-terminal signal peptide. Although there is no direct evidence to demonstrate the cleavage site of signal peptide, it was speculated that MomL may be responsible for the extracellular lactonase activity of *M. olearia*

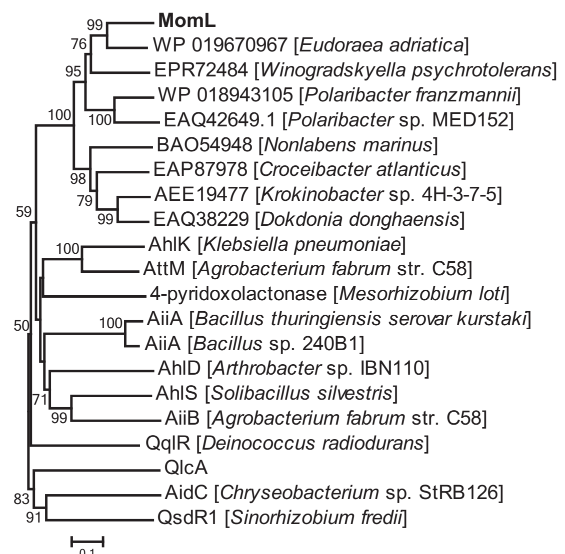

FIG 7 Neighbor-joining tree of AHL lactonases belonging to the metallo-β-lactamase superfamily based on amino acid sequences. Each of these AHL lactonases was experimentally identified, except the proteins designated with GenBank accession numbers. The dendrogram was constructed by neighbor-joining method with the MUSCLE program in the MEGA software package (1,000 bootstrap replicates). Bootstrap coefficients below 50% were not shown. Scale bar, 0.1 substitutions per amino acid position.

Th120 according to the *in silico* prediction and subcellular locations of the expressed MomL proteins. Among the AHL lactonases, QsdH, one member of the GDSL hydrolase family, is the only one that is predicted to possess a signal peptide by SignalP (35). However, no signal peptide was predicted by SignalP in all of the known AHL lactonases belonging to the metallo-β-lactamase superfamily. The original studies did not explore the subcellular location of AHL lactonases in these bacteria, except that the AhlD of *Arthrobacter* sp. was demonstrated to be an intracellular protein (36).

MomL may have a different AHL-degrading mechanism compared to other lactonases. Although MomL shares the "HXHXDH" motif with other AHL lactonases of the metallo-β-lactamase superfamily, it showed a different metal-binding capability and required all of the histidine and aspartic acid residues in the conserved motif. AiiA could bind two zinc ions per protein molecule even when the *E. coli* host was grown in medium without extra metal supplement (9). AiiA and AiiB could bind nearly two equivalents of the corresponding metal ions when the growth medium was supplemented with $CoCl_2$, $MnSO_4$, $CdCl_2$, etc. (11, 37). Nonetheless, it seems that MomL preferably binds magnesium, and the binding of beryllium, magnesium, and zinc may contribute differently to MomL activity according to the metal contents and kinetic constants of MomL (Table 3). Moreover, His117, His11,9 and His189 are necessary for MomL activity, whereas replacement of the corresponding amino acid residues (His104, His106, and His169) in AiiA did not abolish (or only partially abolished) AiiA activity (12). Further metal content analysis of MomL with different metal supplements or crystal study may lead to the discovery of the AHL degradative mechanism of MomL.

The in-gel QQ activity assay may be used for identifying QQ enzyme. The in-gel activity assay, combined with tandem mass spectrometry, can rapidly identify the target enzyme presented in a proteome. Although this has been successfully used to identify various types of enzymes, the in-gel activity assay for detecting QQ enzyme has not been reported before. Most of the known QQ enzymes were identified by constructing genomic libraries along with screening for positive clones. However, measuring AHL degrading activities on thousands of clones is time- and labor-consuming. Our method was effective to detect the in-gel QQ activity of a proteome fractionated by native-PAGE rather than SDS-PAGE, which might be due to the influence of SDS. The in-gel QQ activity assay was used to identify the QQ enzymes of Th120 in our study. Two and one inhibitory zones were observed by this assay in the cell content and extracellular product of Th120, respectively (see Fig. S5 in the supplemental material), suggesting that Th120 possesses at least two QQ enzymes. However, it should be further analyzed whether MomL is the protein responsible for causing inhibitory zones in the gel.

MomL as a potential therapeutic agent. MomL has the potential to be used in disease control. The recombinant MomL showed moderate thermostability, which is similar with that of AidC, derived from a terrestrial bacterium *Chryseobacterium* sp. StRB126 of the family *Flavobacteriaceae* (18). In comparison, the AiiA activity was abolished after being treated at 45°C for 2 h (38). Compared to other QQ enzymes, MomL has a high activity against a wide range of AHLs, decreases virulence factor production in *P. aeruginosa in vitro*, and protects *C. elegans* from killing by *P. aeruginosa* PAO1, even at low concentrations.

QQ already has attracted considerable attention as a new antivirulence therapy. Since AiiA was discovered, dozens of QQ enzymes have been reported. In most reports, it was demonstrated that heterologous expression of QQ enzymes in some pathogenic bacteria could significantly decrease virulence. Furthermore, in other reports, the administration of purified QQ enzyme to animals showed a similar effect. Oral administration of purified $AiiA_{AI96}$ protein protected zebrafish from killing by *Aeromonas hydrophila* (39). Likewise, coinjection of $AiiA_{B546}$ with *A. hydrophila* decreased the mortality rate in common carp (40), and exogenous addition of PvdQ protein in rearing medium attenuated the virulence of *P. aeruginosa* PAO1 to *C. elegans* (41). Issues that still need to be resolved include the production and delivery of these protein drugs and the low bioavailability. Recently, Wahjudi et al. (42) tried to produce a PvdQ powder by spray-freeze drying, intending to administer it directly to the lungs of the patients. It was found that the excipient of trehalose or inulin stabilized PvdQ powder during storage. In our study, lyophilized MomL powder incorporated with sucrose protected *C. elegans* from killing by *P. aeruginosa* PAO1, although the specific activity of dried MomL decreased by ~17-fold after lyophilization (data not shown). Further research is needed before QQ enzymes can be used in a clinical setting.

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