AhID, an N-acetylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria

Sun-Yang Park,1,3 Sang Jun Lee,1† Tae-Kwang Oh,2 Jong-Won Oh,3 Bon-Tag Koo,1 Do-Young Yum1 and Jung-Kee Lee1‡

1R&D Center, inBioNET Corporation, Daejeon 305-390, Korea
2Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea
3Department of Biotechnology, Yonsei University, Seoul 120-749, Korea

Quorum sensing is a signalling mechanism that controls diverse biological functions, including virulence, via *N*-acylhomoserine lactone (AHL) signal molecules in Gram-negative bacteria. With the aim of isolating strains or enzymes capable of blocking quorum sensing by inactivating AHL, bacteria were screened for AHL degradation by their ability to utilize *N*-3-oxohexanoyl-L-homoserine lactone (OHHL) as the sole carbon source. Among four isolates, strain IBN110, identified as *Arthrobacter* sp., was found to grow rapidly on OHHL and to degrade various AHLs with different lengths and acyl side-chain substitutions. Co-culture of *Arthrobacter* sp. IBN110 and the plant pathogen *Erwinia carotovora* significantly reduced both the AHL amount and pectate lyase activity in co-culture medium, suggesting the possibility of applying *Arthrobacter* sp. IBN110 in the control of AHL-producing pathogenic bacteria. The *ahlD* gene from *Arthrobacter* sp. IBN110 encoding the enzyme catalysing AHL degradation was cloned, and found to encode a protein of 273 amino acids. A mass spectrometry analysis showed that AhID probably hydrolysers the lactone ring of *N*-3-hexanoyl-L-homoserine lactone, indicating that AhID is an *N*-acylhomoserine lactonase (AHLase). A comparison of AhID with other known AHL-degrading enzymes, *Bacillus* sp. 240B1 AiiA, a *Bacillus thuringiensis* subsp. *kyushuensis* AiiA homologue and *Agrobacterium tumefaciens* AttM, revealed 25, 26 and 21 % overall identities, respectively, in the deduced amino acid sequences. Although these identities were relatively low, the HXDH=H=D motif was conserved in all the AHLases, suggesting that this motif is essential for AHLase activity. From a genome database search based on the conserved motif, putative AhID-like lactonase genes were found in several other bacteria, and AHL-degrading activities were observed in *Klebsiella pneumoniae* and *Bacillus stearothermophilus*. Furthermore, it was verified that *ahlK*, an *ahlD* homologue, encodes an AHL-degrading enzyme in *K. pneumoniae*. Accordingly, the current results suggest the possibility that AhID-like AHLases could exist in many other micro-organisms.

INTRODUCTION

Many Gram-negative bacteria monitor their population density and control the expression of specialized gene sets in response to bacterial cell density based on a mechanism referred to as quorum sensing (Salmond et al., 1995; Swift et al., 1996). This type of signalling mechanism depends on small diffusible signal molecules, *N*-acylhomoserine lactones (AHLs), which share an identical homoserine lactone ring, although the acyl side-chain length and substitutions in the side-chain differ. At a high population density, the accumulated AHLs bind to specific regulators and trigger the expression of a variety of target genes, including genes for bioluminescence (Hastings & Nealson, 1977; Nealson &
Hastings, 1979), swarming (Eberl et al., 1996), antibiotic biosynthesis (Bainton et al., 1992; Pierson et al., 1994), and biofilm differentiation (Davies et al., 1998). In particular, a quorum-sensing system plays an important role in the control of virulence gene expression in pathogenic bacteria, including Erwinia carotovora (De Kievit & Iglewski, 2000; Jones et al., 1993) and Pseudomonas aeruginosa (Pearson et al., 1997).

Since AHL-mediated signalling mechanisms are widespread and highly conserved in many pathogenic bacteria, they can be attractive targets for novel anti-infective therapies (Finch et al., 1998). Whitehead et al. (2001) suggested that the inactivation of AHL itself is the most obvious strategy among intervention strategies for disrupting quorum sensing in bacteria. Recently, it was found that various Bacillus cereus groups are able to disrupt a quorum signal system through the enzymatic degradation of the AHL molecules (Dong et al., 2000, 2002). Also, transgenic plants expressing AiiA, an N-aclyhomoserine lactone (AHLase) from Bacillus sp. 240B1, are protected from infection from the plant pathogen Erwinia carotovora (Dong et al., 2001). In addition, our previous studies revealed that many subspecies of Bacillus thuringiensis have aiiA homologue genes, and a recombinant microbe producing the AiiA-like protein of B. thuringiensis can reduce the plant pathogenicity of Er. carotovora (Lee et al., 2002). Similarly, the Gram-negative Variovorax paradoxus has been reported to metabolize AHL as the sole carbon and nitrogen source using an aminoclylase, whose AHL-degrading mechanism was apparently different from that of B. cereus groups (Leadbetter & Greenberg, 2000). Interestingly, a recent report showed that an Arthrobacter isolate could utilize two signal breakdown products, homoserine lactone and acyl homoserine, rather than AHL as the sole carbon and nitrogen source (Flagan et al., 2003).

As a source of AHL-degrading enzymes, micro-organisms are increasingly being investigated, and we have also been interested in isolating novel AHL-degrading bacteria for the purpose of disrupting and manipulating quorum-sensing signalling in agricultural pathogenic bacteria. In this study, we isolated an Arthrobacter strain that can utilize AHL as the sole carbon and nitrogen source, and also cloned a novel ahlD gene encoding AHLase from this strain. The existence of putative AhdD-like AHLases in several other bacteria is discussed.

**METHODS**

**Bacterial strains and culture media.** AHL-degrading bacteria were screened on a modified minimal medium (Leadbetter & Greenberg, 2000) adjusted to pH 6.5, containing N-3-octanoyl-L-homoserine lactone (OHHL) as the carbon source and NH₄Cl as the nitrogen source. Several Arthrobacter strains were isolated from different soil samples and cultivated in nutrient broth or Luria-Bertani (LB) medium at 30 °C. Erwinia carotovora N98 isolated from pepper leaves with soft rot symptoms was kindly provided by Dr K.-S. Han (Chung-Nam Agricultural Research & Extension Services, Korea), and grown in LB medium at 30 °C. Chromobacterium violaceum CV026 (Latifi et al., 1995; Throup et al., 1995) and Agrobacterium tumefaciens NT1(pDCI41E33) (Cook et al., 1997), reporter strains for the bioassay, were cultivated in LB medium and defined minimal medium (Zhang et al., 1993), respectively, at 30 °C. For the plate bioassay, A. tumefaciens NT1(pDCI41E33) was cultivated overnight in minimal medium, then added to a minimal agar medium at an OD₆₅₀ of 0.1. Then 5 ml of the mixture was overlaid on the surface of a minimal agar plate containing 40 μg X-Gal ml⁻¹. Bacillus stearothermophilus and Klebsiella pneumoniae strains were obtained from the Korean Collection for Type Culture (KCTC), and cultivated in LB medium at 55 °C and 30 °C, respectively. If necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg ml⁻¹ for Escherichia coli, carbenicillin, 100 μg ml⁻¹ for A. tumefaciens NT1(pDCI41E33).

**Screening and isolation.** Fifty milligrams of soil was added to 2 ml of the minimal medium containing 2.5 mM OHHL. After 48 h cultivation at 30 °C, a 5% (v/v) transfer was made to 200 μl of the minimal medium in a 96-well plate. After the third round of cultivation, turbid samples were spread on nutrient agar (NA) plates. To isolate bacteria growing on OHHL minimal medium, different types of colonies on NA plates were cultivated repeatedly in the minimal medium with and without OHHL in the 96-well plate. After 24 h cultivation, the optical densities and residual OHHL of the samples were measured using a SPECTRAMAX190 (Molecular Devices) and a bioassay plate, respectively.

**Strain identification.** The 16S rDNA gene of strain IBN110 was PCR-amplified from a colony using the primers 9f (5'AGAGTTT GACTTGCAGCAG-3') and 926r (5'CGCTGAATTCCTTGA/ GAGTGT-3'). The PCR conditions involved denaturation at 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 60 s with a PCR Master (Roche). The PCR product of 890 bp was sequenced by an ABI3700 automatic sequencer (Applied Biosystems). Sequence identification was performed by use of the BLAST facility of the National Center for Biotechnology Information and the sequence match facility of the Ribosomal Database Project (Maidak et al., 1999).

**Growth experiments.** Growth with OHHL as the sole carbon source was determined in minimal medium containing 2.5 mM OHHL. Cell growth was monitored based on OD₆₅₀. To measure the residual OHHL in the growth medium, duplicate culture supernatants (100 μl) were collected, and incubated at 95 °C for 5 min to stop the reaction. Appropriately diluted culture supernatants were loaded into a well in a plate overlaid with A. tumefaciens NT1(pDCI41E33), and the diameter of the blue zone on the bioassay plate was measured after incubation at 30 °C for 24 h. The relative amounts of OHHL were calculated using the following equation, obtained by Microsoft Excel regression analysis program: OHHL (pmol) = 0.0335e⁻⁰.₃₆⁹x (R²=0.9952), where x is the diameter of the colour zone. The relationship was established by adding 0.15–10 pmol OHHL to the bioassay plate and measuring the diameter of the resulting colour zone after incubation at 30 °C for 24 h.

**Bioassay of AHL-degrading activity.** The AHL substrates N-butanoyl-L-homoserine lactone (BHL), N-octanoyl-L-homoserine lactone (OHL), N-decanoyl-L-homoserine lactone (DHL) and N-3-oxododecanoyl-L-homoserine lactone (OdBHL) were all purchased from Quorum Sciences Inc. and Fluka. N-Hexanoyl-L-homoserine lactone (HHL) and N-3-oxohexanoyl-L-homoserine lactone (OHHL) were synthesized in the authors' laboratory, as previously described (Zhang et al., 1993). For the whole-cell assay, Arthrobacter sp. IBN110 cultured overnight was harvested and resuspended in 100 mM Tris/HCl (pH 7.0). Then, 50 μl of the cell resuspension (OD₆₅₀ 1.0) and an equal volume of 40 μM AHL were mixed and incubated at 30 °C with gentle agitation. After boiling at 95 °C for
5 min to stop the reaction, the reaction mixtures were diluted to the appropriate concentration, and loaded into the well of plates overlaid with *A. tumefaciens* NT1(pDC41E33) for OHHL, OHL, DHL and OaDHL detection, or of plates overlaid with *C. violaceum* CV026 for BHL and HHL detection. The residual amounts of AHLs were calculated using relationship equations based on the color zone size and known amounts of AHLS.

**Co-culture of Er. carotovora N98 and Arthrobacter sp. IBN110.** *Er. carotovora* N98 and *Arthrobacter* sp. IBN110 were precultured separately in LB medium at 30 °C for 15 h. For co-culture, *Er. carotovora* N98 was inoculated in LB medium at 2 × 10^9^ c.f.u. ml^-1^ whereas initial levels of Arthrobacter sp. IBN110 were 2 × 10^6^ and 6 × 10^6^ c.f.u. ml^-1^ Co-culture experiments of all combinations were done in triplicate. To determine the growth rate, viable cells were enumerated as c.f.u. by duplicate plating of serially diluted samples. After 10 h incubation (early stationary phase) at 30 °C, samples were withdrawn for determination of OHHL amounts and pectate lyase activities. Five-microlitre samples of cell-free culture supernatants were loaded into wells in plates overlaid with *A. tumefaciens* NT1(pDC41E33) for detecting the amount of OHHL present during the co-culture. Extracellular pectate lyase activity was determined as described by Laurent et al. (2000). One unit was defined as a 0.01 increase of A530 min^-1^ (ml supernatant)^-1^.

**Partial purification of the AHL-degrading enzyme.** *Arthrobacter* sp. IBN110 cells harvested from the culture broth were resuspended in 10 mM Tris/HCl (pH 7.0) containing 0.1 M PMSF, then disrupted three times using a French pressure cell (model FA-030; Aminco) at 260 MPa. The precipitated proteins were dissolved in 10 mM Tris/HCl (pH 7.0) and dialysed against the same buffer. The dialysate was applied to a DEAE-Sepharose CL-6B column (Pharmacia) and eluted with an NaCl gradient (0–1 M), whereas initial levels of Arthrobacter sp. IBN110 were 2 × 10^6^ and 6 × 10^6^ c.f.u. ml^-1^ Co-culture experiments of all combinations were done in triplicate. To determine the growth rate, viable cells were enumerated as c.f.u. by duplicate plating of serially diluted samples. After 10 h incubation (early stationary phase) at 30 °C, samples were withdrawn for determination of OHHL amounts and pectate lyase activities. Five-microlitre samples of cell-free culture supernatants were loaded into wells in plates overlaid with *A. tumefaciens* NT1(pDC41E33) for detecting the amount of OHHL present during the co-culture. Extracellular pectate lyase activity was determined as described by Laurent et al. (2000). One unit was defined as a 0.01 increase of A530 min^-1^ (ml supernatant)^-1^.

**Determiniation of amino-terminal amino acid sequence.** Due to the instability of the enzyme, we failed to fully purify the AHL-degrading enzyme by column chromatography. To determine the amino-terminal sequence of the AHL-degrading enzyme, the enzyme from the collected active fractions was first identified as follows. A mixed with 20 μL OHL, and incubated at 30 °C with gentle shaking. After incubation, the mixture was extracted three times with ethyl acetate, and evaporated. For the HPLC analysis, the samples were dissolved in 0 M) containing 2 M Tris/HCl (pH 7.0) and dialysed against the same buffer. The dialysate was applied to a DEAE-Sepharose CL-6B column (Pharmacia) and eluted with a NaCl gradient (0–1 M). The fractions exhibiting AHL-degrading activity were collected, concentrated using Centriprep-10 tubes (Millipore), and injected onto a 5 ml Hitrap Q-Sepharose column (Pharmacia). Finally, the proteins were eluted with a NaCl gradient (0–1 M) and the active fractions were collected. Determination of amino-terminal amino acid sequence. Due to the instability of the enzyme, we failed to fully purify the AHL-degrading enzyme by column chromatography. To determine the amino-terminal sequence of the AHL-degrading enzyme, the enzyme from the collected active fractions was first identified as follows. A partially purified sample was mixed with native gel electrophoresis sample buffer and separated by SDS-12% PAGE. After electrophoresis, the duplicate gels were either stained with Coomasie brilliant blue R250 or renatured by washing three times with 10 mM Tris/HCl (pH 7.0) containing 2.5% Triton X-100 for 15 min at room temperature with gentle agitation. The renatured gel was then sliced according to the protein bands on the stained gel. The slices were mixed with 20 μL OHL, and incubated at 30 °C with gentle agitation for 3 h. Thirty microlitres (3 pmol OHL) of the reaction mixtures diluted 200-fold was loaded into the well of a plate overlaid with *A. tumefaciens* NT1(pDC41E33). The protein band with AHL-degrading activity was then transferred to a PVDF membrane (Bio-Rad) after SDS-PAGE, and sequenced by automatic Edman degradation (Applied Biosystems).

**Cloning of the ahiD gene.** The total DNA of *Arthrobacter* sp. IBN110 was used as the template for the PCR. The PCR primers were designed based on the most conserved amino acid sequences of AHLSases, AiiA and Atm (see Fig. 6). The sequences of the degenerate primers were 5′-GAAGATCCCA(C/T)CTICA(C/T)TG(T/C)CTGGA (C/T)CA-3′ and 5′-ATGATCCTGAGCTGTA (A/G)GTGCCNGNTG-3′ (the BamHI and EcoRI sites are underlined). The PCR amplification was performed for 30 cycles consisting of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s. The PCR products were then subcloned into a pT7Blue T-vector (TaKaRa), sequenced, and used as a probe for colony hybridization, as described below.

To construct the genomic library of *Arthrobacter* sp. IBN110, the genomic DNA was digested with KpnI. The 3–6 kb DNA fragments were then ligated to the dephosphorylated KpnI site of pUC19, and transformed into *E. coli* DH5α. The library was screened by colony hybridization using a DIG DNA Labelling and Detection Kit (Boehringer Mannheim). The candidate showing a positive signal in the colony hybridization was sequenced. The nucleotide sequence was analysed using the BLASTX facility (NCBI) and aligned using the cluster alignment algorithm in Vector NTI (Informax).

**Expression of recombinant AhlD and its homologue in *E. coli.*** The ORF of the *ahiD* gene was amplified by a PCR using the following primers: EN1, 5′-ATGGAGTCATATGGAAAAAGAT-CA-3′; EN2, 5′-ATGGAGTGGCATGGAAAAAGATCA-3′; and EC, 5′-TGATAGAAACCTCCTGATGCGC-3′ (the *Ndel*, *NcoI* and *HindIII* sites are underlined). The PCR products amplified by the EN1 and EC primers were digested with *Ndel* and *HindIII*, and subcloned into the 6×His tagging expression vector pET22b(+) (Novagen) between the *Ndel* and *HindIII* sites to give pAhlD. The PCR products amplified by the EN2 and EC primers were cloned into the *Ndel* and *HindIII* sites of pTrc99A (Amersham Pharmacia Biotech), resulting in pEahID. The expression vectors pAhlD and pEahID were then transformed into *E. coli* BL21(DE3) and *E. coli* JM109, respectively. The *E. coli* cells carrying the recombinant AhlD were cultivated for 3 h after IPTG induction at 30 °C. For the SDS-PAGE analysis, the cell extracts were sonicated in 10 mM Tris/HCl (pH 7.0) and loaded onto a polyacrylamide gel. The AHL-degrading activity of the IPTG-induced recombinant cells was measured using the bioassay described above.

To investigate whether the *ahlK* from *K. pneumoniae* KCT2241 encodes an AHL-degrading enzyme, the gene containing promoter was amplified by PCR using the following primers: K1, 5′-CTGAAATTCTGAGCTGACGGAATCAGGAGGAATCGATG-3′ (the EcoRI sites are underlined). The PCR product of 855 bp was subcloned into the EcoRI site of pUC19, and sequenced. The resulting plasmid was named pUAhlK. The AHL-degrading activity of the *E. coli* JM109 harbouring pUAhlK was determined by whole-cell assay.

**HPLC and ESI-MS analysis.** HHL digestion by AhlD was analysed by using HPLC and electrospray ionization-mass spectrometry (ESI-MS). A 10 ml sample of the IPTG-induced recombinant cells was harvested and sonicated. Next, 490 μl of cell extract of *E. coli* carrying the recombinant pEahID was mixed with 10 μl HHL (2 mM final concentration), and incubated at 30 °C for 1 h with gentle shaking. After incubation, the mixture was extracted three times with ethyl acetate, and evaporated. For the HPLC analysis, the sample was dissolved in 0.1 M 50:50 (v/v) methanol/water and analysed using a C18 reverse-phase column. The fractions were separated by eluting isocratically with 70:30 (v/v) methanol/water at a flow rate of 0.3 ml min^-1^ The ESI-MS was performed using a Mariner instrument (Perspective Biosystems) in the Korea Basic Science Institute (KBSI), where the sample was dissolved in 50:50 (v/v) methanol/water and ionized by a positive ion electrospray.

**RESULTS**

**Isolation of AHL-degrading bacteria and growth characteriization.** AHL-degrading bacteria were screened from 430 soil samples collected in Korea using a minimal medium containing

---

http://mic.sgmjournals.org

---

1543

IP: 54.191.40.80
OHHL as the sole carbon source and NH₄Cl as the nitrogen source. Among four isolates, strain IBN110 exhibited the most rapid growth rate and consumption of OHHL from OHHL-minimal medium. Strain IBN110 was Gram-positive and formed yellow colonies on LB plate. The 16S rDNA sequence of the strain had a 97–99% identity with the Gram-positive Arthrobacter species, and was phylogenetically clustered with Arthrobacter group I (Koch et al., 1995). Arthrobacter histidinolovorans and A. nicotinovorans. Particularly, the 16S rDNA of IBN110 shared 100% sequence identities with those of A. histidinolovorans HAMBI strains. Therefore, the AHL-degrading isolate IBN110 was named Arthrobacter sp. IBN110.

With OHHL as the sole carbon source, the doubling time of Arthrobacter sp. IBN110 was 2.1 h. As the cell density increased, the residual amount of OHHL in the culture medium significantly decreased. Most of the OHHL in the culture medium was consumed after 10 h of cultivation (Fig. 1). No growth was observed in an OHHL-depleted minimal medium. It has been previously reported that the half-life of an AHL is <3 h under alkaline conditions (pH 8.0) (Leadbetter & Greenberg, 2000; Yates et al., 2002). During 10 h of cultivation, the pH of the culture medium changed from 6.5 to 6.8. Thus, the dramatic decrease of OHHL in the medium is unlikely to have been due to alkaline pH. In minimal medium containing 0.2% glucose as the carbon source and 0.5 mM OHHL as the sole nitrogen source, Arthrobacter sp. IBN110 grew exponentially with a doubling time of 1.6 h, and utilized most of the OHHL during the exponential phase (data not shown).

Thus Arthrobacter sp. IBN110 was able to utilize OHHL as both the sole carbon source and the sole nitrogen source.

**Degradation of various AHLs by Arthrobacter sp. IBN110**

To assess the AHL substrate range of Arthrobacter sp. IBN110, its degrading activities against various AHLs were determined using a whole-cell assay. The strain was cultivated overnight in LB medium, then harvested and reacted with different AHLs. It effectively degraded OHHL, OHL and OdDHL, which have different lengths and acyl side-chain substitutions (Fig. 2). In particular, OHL was completely eliminated within 30 min. The strain also degraded BHL, HHL and DHL (data not shown). Boiled Arthrobacter sp. IBN110 failed to degrade OHHL. These results suggest that the AHL degradation of Arthrobacter sp. IBN110 occurs by an enzymic inactivation mechanism. Furthermore, the fact that Arthrobacter sp. IBN110 grown in LB medium can degrade various AHLs implies that the strain might express an AHL-degrading enzyme constitutively.

**Co-culture of Er. carotovora N98 and Arthrobacter sp. IBN110**

To investigate whether Arthrobacter sp. IBN110 could decrease virulence factors controlled by the quorum signal molecule, a co-culture experiment with an AHL producer and an AHL degrader was designed. The soft-rot causative pathogen Er. carotovora N98 was used as a test organism. Er. carotovora N98 and Arthrobacter sp. IBN110 were mixed at different c.f.u. ratios and co-cultured in LB medium at 30°C. The growth rate of Er. carotovora N98 was not

![Fig. 1. Growth of Arthrobacter sp. IBN110 in minimal medium containing OHHL as sole carbon source. Growth in the medium with (●) and without (○) OHHL was assessed based on the OD₆₀₀. The residual OHHL concentration in the culture (◇) was measured using a bioassay. The experiments were repeated three times; the results are means ± SE (not shown where smaller than symbols).](image1)

![Fig. 2. Degradation of various AHLs by Arthrobacter sp. IBN110. Equal volumes of a cell suspension (OD₆₀₀ 1.0) and a solution containing 40 µM OHHL (●), OHL (■) or OdDHL (▲) were mixed and incubated at 30°C. A boiled culture suspension mixed with 40 µM OHHL was used as the control (◇). The experiments were repeated three times; the results are means ± SE.](image2)
Table 1. Decrease of AHL concentration and pectate lyase activity during co-culture of Er. carotovora N98 and Arthrobacter sp. IBN110

<table>
<thead>
<tr>
<th>Initial c.f.u. ratio</th>
<th>Specific growth rate (h⁻¹)</th>
<th>OHHL (µM)†</th>
<th>Pectate lyase activity (U ml⁻¹)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Er. carotovora N98</td>
<td>Arthrobacter sp. IBN110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>1-27*</td>
<td>1-1±0-2</td>
<td>552-2±10-1</td>
</tr>
<tr>
<td>1</td>
<td>0-57</td>
<td>0-2±0-04</td>
<td>184-4±10-1</td>
</tr>
<tr>
<td>3</td>
<td>0-65</td>
<td>0-1±0-02</td>
<td>52-2±1-2</td>
</tr>
</tbody>
</table>

*Growth rate of Er. carotovora N98 in pure culture.
†Measured after 10 h co-cultivation.

Influenced by the initial inoculum size of Arthrobacter sp. IBN110 during 10 h of co-culture (early stationary phase). However, the OHHL concentration and pectate lyase activity in the co-culture supernatant decreased according to the increase of initial c.f.u. ratio of Arthrobacter sp. IBN110 (Table 1). These results suggest that Arthrobacter sp. IBN110 is able to control the virulence of Er. carotovora N98 by reducing AHL concentration and pectate lyase activity.

**Amino-terminal amino acid sequence of the AHL-degrading enzyme**

The AHL-degrading activity of Arthrobacter sp. IBN110 was observed with the cell extract rather than the culture supernatant, indicating that the enzyme was a cellular protein. Thus, the purification of the AHL-degrading enzyme from the cell extract was carried out using DEAE-Sephacelose and Q-Sepharose chromatography. The active fractions eluted by a 0-2 M NaCl gradient were concentrated and separated by SDS-12% PAGE (Fig. 3a). Due to the enzyme instability, we failed to fully purify the AHL-degrading enzyme by column chromatography. The enzyme activities and protein band patterns for each eluted fraction from the Q-Sepharose chromatography were compared. The intensity of the proteins around 30 kDa coincided well with the AHL-degrading activity of the fractions. To identify the AHL-degrading enzyme from the partially purified proteins, an activity assay of the proteins separated by SDS-PAGE was performed. Among three candidate proteins, as indicated in Fig. 3(a), only one protein (band 2) of about 30 kDa exhibited AHL-degrading activity in the activity assay with renatured gel slices (Fig. 3b). The amino-terminal sequence of this protein was determined as NH₂-Met-Glu-Lys-Asp-Gln-Leu-Lys-Val.

**Cloning of the ahlD (acylhomoserine lactone degradation) gene**

While purification of the AHL-degrading enzyme was in progress, it was found that a zinc-binding motif is conserved in several groups of metallohydrolases including AHLases from Bacillus cereus group (Dong et al., 2000; Lee et al., 2002) and A. tumefaciens (Zhang et al., 2002). Based on these observations, a PCR was performed using degenerate primers of the conserved sequences to clone the AHL-degrading enzyme gene from Arthrobacter sp. IBN110. A single band of 0-2 kb fragments was amplified from the Arthrobacter sp. IBN110 total DNA and sequenced. A BLAST search revealed that the deduced amino acid sequences of the 0-2 kb DNA fragment had homologies with an AiiA homologue, a Zn-dependent hydrolase and a metallo β-lactamase that have a zinc-binding motif, although their amino acid sequence identities were relatively low. To determine whether the full ORF of the 0-2 kb fragment encodes an AHL-degrading enzyme, a KpnI-digested genomic DNA library of Arthrobacter sp. IBN110 was constructed, and screened by colony hybridization using the 0-2 kb DNA fragment as a probe. The positive clone was found to have a 2-3 kb insert, containing one ORF completely matching the probe sequences, which encoded a protein of 273 amino acids with a molecular mass of about 31 kDa, corresponding to that of the partially purified AHL-degrading enzyme (Fig. 3a). In addition, the deduced amino-terminal sequences were in agreement with the chemically determined sequences of the purified protein (see Fig. 6). Accordingly, the results indicate that...
the 2-3 kb DNA fragment contained the whole region of the ahld gene encoding the AHL-degrading enzyme.

In general, the genes that metabolize various organic compounds often appear as a gene cluster in Arthrobacter species. Part of a divergently transcribed ABC-transporter-like gene was identified 244 bp upstream of the ahld, while 435 bp downstream of ahld and transcribed in the opposite orientation to ahld, we identified a putative transcription regulator of the lacI type involved in carbon catabolite repression (data not shown).

Expression of recombinant AhlD in E. coli

The E. coli clone that contained the 2-3 kb DNA fragment showed relatively lower AHL-degrading activity than Arthrobacter sp. IBN110. To confirm that the ahld gene indeed encodes an AHL-degrading enzyme, it was expressed under control of the T7 promoter in E. coli. Upon induction of E. coli BL21(DE3)(pAhld) with IPTG, a His-tagged protein with a molecular mass of about 36 kDa was produced, yet most of the protein was expressed in an insoluble form (Fig. 4a). Although most of the AhlD protein was produced as inclusion bodies, 1 ml of the recombinant E. coli expressing AhlD effectively degraded the AHLS HHL, OHHL, OHL, DHL and OdDHL, while E. coli carrying pET22b, as the control, was unable to degrade any of the tested AHLS (Fig. 4b). It was also observed that the recombinant E. coli degraded less OHHL than OHL and OdDHL, which was in good agreement with the AHL degradation pattern of Arthrobacter sp. IBN110 (Fig. 2). Accordingly, these results reconfirm that the ahld gene of Arthrobacter sp. IBN110 encodes an AHL-degrading enzyme. In order to effectively express the AhlD enzyme, the ahld gene was cloned into a pTrc99A expression vector, resulting in pEAhlD. E. coli JM109(pEAhlD) induced with IPTG exhibited a higher AHL-degrading activity than that observed with E. coli BL21(DE3)(pAhld), although the expression level of AhlD was low (data not shown). Therefore, recombinant AhlD from E. coli JM109(pEAhlD) was used for an HHL product analysis.

ahlD encodes an AHLase

The AHL-utilizing V. paradoxus produces acylase to degrade BHL (Leadbetter & Greenberg, 2000). To determine whether AhlD of AHL-utilizing Arthrobacter sp. IBN110 is an acylase or an AHLase, HHL was digested with extracts of E. coli carrying the recombinant pEAhlD, and the reaction products were analysed by HPLC and ESI-MS. The enzyme digestion of HHL generated one product with a retention time of 10-1 min, as determined by the HPLC analysis (Fig. 5a). The ESI-MS analysis of the product revealed a quasimolecule M+H ion at m/z 218, suggesting that the enzymic action with HHL (M+H ion m/z 200) resulted in a mass increase of 18, corresponding to a water
molecule (Fig. 5b). In the case of cell extracts of *E. coli* carrying a pTrc99A vector as the control, only a peak corresponding to HHL was detected in the HPLC analysis (Fig. 5a). These results strongly suggested that *ahlD* encodes an AHLase that hydrolyses the ester bond of the homoserine lactone ring of AHLS.

**Sequence comparison of AhlD with AHLases and putative AHL-degrading enzymes**

The amino acid sequences of AhlD were compared with those of known AHLases. The sequence analysis of AhlD with *Bacillus* sp. 240B1 AiiA (Dong et al., 2000), a *B. thuringiensis* subsp. *kyushuensis* AiiA homologue (Lee et al., 2002) and *A. tumefaciens* AttM (Zhang et al., 2002) exhibited 25, 26 and 21% identities in the deduced amino acid sequences, respectively. AhlD shared the same HXHDXH=D pattern, referred to as the zinc metallohydrolase criterion (Vallee & Galdes, 1984), with known AHL-degrading enzymes, suggesting that AhlD may be a zinc metallohydrolase (Fig. 6). Furthermore, the sequence HXHDXH-65 amino acids-HXDH-21 amino acids-D, proved essential for AHLase activity by site-directed mutagenesis (Dong et al., 2000), was also identified in the AhlD sequence (Fig. 6). These results suggested that the conserved motif, HXDH<H, is necessary for AHLase activity, even though the overall identities among AiiA, AttM and AhlD were relatively low.

Consequently, a further search for AhlD-like enzymes was conducted using the microbial genome PEDANT (http://pedant.gsf.de/) and GenBank databases. Besides AiiA and AttM, six AhlD homologues were found, all of which included the conserved motif HXDH=D and exhibited 20–30% identity in their amino acid sequence with that of AhlD from *Arthrobacter* sp. IBN110. Among these homologues, two AhlD homologues, from *Bacillus stearothermophilus* 10 and *Klebsiella pneumoniae* MGH78578, were aligned with the other AHLases shown in Fig. 6. The Orf40 from the unfinished genome of *B. stearothermophilus* 10 exhibited 27% identity with AhlD, which was slightly higher than the identities with the other AHL-degrading enzymes. AhlD shared 20–23% identity with proteins of an unknown function, annotated as an AttM-like protein from *Bradyrhizobium japonicum*, *K. pneumoniae* MGH78578 and *Thermoplasma volcanium* GSS1. In particular, the Orf99 from the unfinished genome of *K. pneumoniae* MGH78578 exhibited 58.6% identity with the AttM of *A. tumefaciens* for the amino acid sequences, indicating that the strain may contain a putative AHL-degrading enzyme.

The AHL-degrading activity was tested using *B. stearothermophilus* KCTC3067 and *K. pneumoniae* KCTC2241. Indeed, AHL-degrading activities were observed in *B. stearothermophilus* KCTC3067 and *K. pneumoniae* KCTC2241 when using a whole-cell assay (data not shown). Recombinant *E. coli* expressing AhlK, an Orf99 homologue from *K. pneumoniae* KCTC2241, also exhibited AHL-degrading activity, indicating that *ahlK* and the Orf99 gene encode AHL-degrading enzymes (Fig. 7).

![Fig. 6. Alignment of amino acid sequences of AhlD, other AHLases, and putative homologues. The black and grey shading indicates identical and similar amino acids, respectively. The amino acid residues essential for AHLase activity are indicated by asterisks.](http://mic.sgmjournals.org)

**Legend:**

- **Ar_AhlD**: *Arthrobacter* sp. AHLCase
- **Bs_Orf40**: Orf40 of *Bacillus stearothermophilus* 10
- **Bt_AiiA**: AiiA homologue of *Bacillus thuringiensis* subsp. *kyushuensis*
- **Ba_AiiA**: AiiA of *Bacillus* sp. 240B1
- **At_AttM**: AttM of *Agrobacterium tumefaciens*
- **Kp_Orf99**: Orf99 of *Klebsiella pneumoniae* MGH78578
The AhlK was different from Orf99 in only two amino acid residues (Ala68→Thr; Leu196→Ile). Therefore, these results suggest the possibility that AhlD-like AHLases could exist in many other micro-organisms, including thermophilic bacteria.

DISCUSSION

It has been reported that about 5% of several hundred soil bacteria tested were able to inactivate AHLs (Dong et al., 2000). In the current study, it was found that a Gram-positive Arthrobacter isolate could degrade and utilize AHL as the sole carbon and nitrogen source. The Gram-negative V. paradoxus is known to metabolize AHLs (Leadbetter & Greenberg, 2000); however, to our knowledge, no previous examples of Gram-positive bacteria that can metabolize AHLs have been reported. Therefore, Arthrobacter strains offer a wider range of bacteria that utilize AHL in natural ecosystems. Recently, Flagan et al. (2003) reported that Arthrobacter strain VAI-A could grow with AHL degradation products, acylhomoserine and homoserine lactone, but it could not utilize AHL as the sole energy and nitrogen source, in contrast to Arthrobacter sp. IBN110, which apparently produces an AHLase. These facts indicate differences in the ability of Arthrobacter isolates to utilize AHLs as the energy and nitrogen source. Arthrobacter strains are considered as one of the major groups among aerobic soil bacteria (van Waasbergen et al., 2000). Several studies have already shown that members of the genus Arthrobacter are capable of degrading a variety of natural aromatic and aliphatic compounds, as well as synthetic compounds, such as organic pesticides (Chen & Tomasek, 1991; Sayler et al., 1990). The AHL-degrading activity of Arthrobacter species expands the list of diverse metabolic traits exhibited by members of this genus.

Although Arthrobacter sp. IBN110 was initially isolated based on its OHHL-utilizing ability (Fig. 1), the strain was also found to effectively degrade different kinds of AHLs that regulate the virulence factors in Erwinia (De Kievit & Iglewski, 2000; Jones et al., 1993), Agrobacterium (Piper et al., 1993; Zhang et al., 1993), and the human pathogen P. aeruginosa (Pearson et al., 1994, 1997) (Fig. 2). This ability to degrade various AHLs was also confirmed in recombinant E. coli expressing AhlD (Fig. 4). Furthermore, the co-culture of Arthrobacter sp. IBN110 and Er. carotovora resulted in a decreased OHHL concentration and OHHL-mediated pectate lyase activity by Er. carotovora in the medium without any change in the cell growth of Er. carotovora (Table 1), indicating the possibility of applying Arthrobacter sp. IBN110 and its ahlD gene in the control of AHL-producing Gram-negative bacteria.

For V. paradoxus, it has been reported that an aminoacylase may be involved in AHL metabolism by cleaving the intact lactone from the AHL acyl side-chain (Leadbetter & Greenberg, 2000). Recently, an AHL acylase was cloned from a Ralstonia isolate capable of using BHL and OdDHL as its carbon source (Lin et al., 2003). It was predicted that Arthrobacter sp. IBN110 growing on OHHL may also produce a putative aminoacylase for utilizing AHL. However, AhlD from Arthrobacter sp. IBN110 was found to contain the conserved motif, HXDH=D, the essential motif for AHLase activity (Dong et al., 2002) (Fig. 6). In addition, HPLC and MS analyses showed that AhlD produced acylhomoserine by hydrolysing AHL, indicating that AhlD is an AHLase (Fig. 5). Therefore, the current study shows that the quorum signal molecule AHL could be disrupted and utilized by the AHLase of Arthrobacter sp. IBN110, in contrast to the acylase of V. paradoxus and the Ralstonia isolate, suggesting that the metabolic pathway for utilizing AHL may be different between Arthrobacter sp. IBN110 and V. paradoxus. However, further study is required to determine the metabolic fate of AHL.

To date, two AHLase genes have been identified in A. tumefaciens and in B. cereus groups including B. thuringiensis. The identification of AhlD from Arthrobacter sp. IBN110 indicates that the conserved motif, HXDH=D, is required for AHLase activity, even though the overall identities among the AHLases AiiA, AttM and AhlD were <26% (Fig. 6). From a homology search based on the conserved motif, putative AhlD-like homologues were found in six different bacteria. Interestingly, AHL-degrading activity was detected in the thermophilic bacterium B. stearothermophilus (data not shown). Also, it was observed that recombinant E. coli expressing AhlK from K. pneumoniae KCTC2241 showed AHL-degrading activity (Fig. 7). Accordingly, the current findings may be helpful in uncovering AHLases in natural ecosystems. Putative AhlD-like homologues were distributed in diverse bacteria, including two Archaea, Thermoplasma volcanium GSS1 and Sulfolobus solfataricus P2, which grow optimally at 60 and 80 °C, respectively, and the symbiotic bacterium B. japonicum, where nodulation genes were regulated by
quorum sensing using a Bradyoxetin, rather than AHL (Loh et al., 2002). In addition, an AhlD-like homologue was also found in Burkholderia fungorum LB400 with quorum-sensing genes (data not shown), suggesting that it is involved in a signal turnover system, as in A. tumefaciens (Zhang et al., 2002). The actual roles of AhlD and putative AhlD-like homologues in bacteria and natural environment remain unclear. However, the current findings indicate that AhlD-like lactonases are widespread in diverse bacteria, although whether or not these homologues exhibit AHL-degrading enzyme activities remains to be elucidated.

ACKNOWLEDGEMENTS

The authors thank Kyung-Hwa Ryu and Su-Yeon Shin for their technical assistances. They also thank Ingyu Hwang (Seoul National University, Korea) for providing C. violaceum CV026 and A. tumefaciens NT1(pDC41E33). This work was supported by a grant from the Rural Development Administration of Korea (BioGreen21 Project).

REFERENCES


