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Genes Encoding the *N*-Acyl Homoserine Lactone-Degrading Enzyme Are Widespread in Many Subspecies of *Bacillus thuringiensis*

Sang Jun Lee, Sun-Yang Park, Jung-Ju Lee, Do-Young Yum, Bon-Tag Koo, and Jung-Kee Lee*

R&D Center, inBioNET Corporation, Daejeon 305-390, Korea

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Gram-negative bacteria can communicate with each other by *N*-acyl homoserine lactones (AHLs), which are quorum-sensing autoinducers. Recently, the *aiiA* gene (encoding an enzyme catalyzing the degradation of AHL) has been cloned from *Bacillus* sp. strain 240B1. During investigations in the course of the ongoing *Bacillus thuringiensis* subsp. *morrisoni* genome project, an *aiiA* homologue gene in the genome sequence was found. These results led to consideration of the possibility of the widespread existence of the gene in *B. thuringiensis*. *aiiA* homologue genes were found in 16 subspecies of *B. thuringiensis,* and their sequences were determined. Comparison of the *Bacillus* sp. strain 240B1 *aiiA* gene with the *B. thuringiensis aiiA* homologue genes showed high homologies of 89 to 95% and 90 to 96% in the nucleotide sequence and deduced amino acid sequence, respectively. Among the subspecies of *B. thuringiensis* having an *aiiA* gene, the subspecies *aizawai*, *galleriae*, *kurstaki*, *kyushuensis*, *ostriniae*, and *subtoxicus* were shown to degrade AHL. It was observed that recombinant *Escherichia coli* producing AiiA proteins also had AHL-degrading activity and could also attenuate the plant pathogenicity of *Erwinia carotovora*. These results indicate that insecticidal *B. thuringiensis* strains might have potential to compete with gram-negative bacteria in natural ecosystems by autoinducer-degrading activity.

Quorum sensing is a signaling mechanism used by bacteria for monitoring their population density and coordinating gene expression in response to changes in cell density (7). Many gram-negative bacteria secrete and accumulate signaling molecules, *N*-acyl homoserine lactones (AHLs), as the cells grow, and members of a bacterial strain can communicate with each other by these diffusible quorum-sensing autoinducers. AHLs consist of a lactone ring covalently linked to 4- to 14-carbon acyl side chains through an amide bond (18, 19, 28). These AHL molecules have critical roles in controlling a number of phenotypic traits, such as antibiotic production, biofilm formation, and hydrolytic enzyme production and, especially, the generation of virulence factors in some pathogenic bacteria (10, 18). It has been reported that pathogens use a quorum-sensing system to escape premature detection by host defenses and to overwhelm the host efficiently at the appropriate time (6, 13). In the case of *Erwinia carotovora*, which causes soft rot in a variety of plants, pathogenicity depends on the production of several exoenzymes which are involved in the maceration of plant tissue. The production of these exoenzymes is regulated by the accumulation of *N*-3-oxohexanoyl-L-homoserine lactone (10, 22). *Erwinia carotovora* does not produce plant tissue-degrading enzymes until sufficient bacterial density has been achieved for successful evasion of plant defenses.

Because many pathogenic bacteria use quorum sensing for the regulation of virulence, it is suggested that interfering with the bacterial communication system by disrupting quorum sensing is a way of treating or preventing infection. As one of the anti-quorum-sensing strategies, degradation of AHL-signaling molecules could have potential applications in attenuating plant disease. Signal-degrading enzymes from one bacterium interrupt the signaling among other pathogenic bacteria and can prevent plant disease such as soft rot caused by *E. carotovora* (5). Recently, autoinducer-degrading microbes or enzymes have been reported: Leadbetter and Greenberg (12) reported that some strains of *Variovorax paradoxus* could grow in a minimal medium containing an AHL as the sole energy and nitrogen source. *Variovorax paradoxus* cleaves the acyl-amide bond of AHLs. Also, Dong et al. (4, 5) cloned a novel lactonase gene from a *Bacillus* sp. that is closely related to the *Bacillus cereus* group. This gene encodes an enzyme that renders AHLs biologically inactive.

In our company, the microbial genome project of *Bacillus thuringiensis* subsp. *morrisoni* has been ongoing, and 90% of the genome has been sequenced. In the database of DNA sequences, an *aiiA* homologue gene was found in *B. thuringiensis* subsp. *morrisoni*. These findings led to consideration of the possibility of the existence of the *aiiA* gene in the species of *B. thuringiensis*, a spore-forming gram-positive bacterium. This bacterium produces various insecticidal crystal proteins encoded by *cry* genes (1, 24). *B. thuringiensis* has been developed and is presently used as a biological control agent against insect pests in the agriculture and forestry industries (3). In this paper, the distribution of *aiiA*-homologous genes in the insecticidal *B. thuringiensis* is reported and the possibility of biological control of plant-pathogenic bacteria by *B. thuringiensis* is discussed.

MATERIALS AND METHODS

Bacterial strains, culture media, and conditions. *Escherichia coli* DH5α and *E. coli* BL21(DE3) were used as a cloning host and an expression host, respectively. Subspecies of *B. thuringiensis* were kindly supplied by Institut Pasteur, Paris, France. *Bacillus* sp. strain IBN35 was isolated from a soil sample, and its partial 16S rDNA sequence was shown to be 99.0% identical to that of *B. cereus*. Red pepper soft-rot-causative *Erwinia carotovora* IBN98 was kindly supplied by K.-S. Han (Chung-Nam Agricultural Research and Extension Services, Daejon, Ko-

* Corresponding author. Mailing address: R&D Center, *in*BioNET Corporation, Jeonmin-dong 461-6, Yusong-Ku, Daejeon 305-390, Korea. Phone: 82-42-866-9194. Fax: 82-42-866-9111. E-mail: jklee@inbionet.com.

rea). *Chromobacterium violaceum* CV026 (11, 26) and *Agrobacterium tumefaciens* NT1 (pDCI41E33) (2) were used as reporter strains for bioassay. *Escherichia coli* and *Bacillus* spp. were cultivated in Luria-Bertani medium (1% bactotryptone, 0.5% yeast extract, 1% sodium chloride) at 37°C. If necessary, the medium was supplemented with 5 μg of ampicillin/ml. Cell growth was monitored by measurement of the optical densities at 600 nm (OD_{600}) of culture media. If needed, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM at an OD_{600} of 0.6.

DNA manipulations. Genomic DNAs were isolated from *B. thuringiensis* using a modification of a method by Schraft and Griffiths (25). Bacterial cells were washed with 0.85% (wt/vol) NaCl and then suspended in 400 μl of a 200 mM sucrose solution supplemented with lysozyme (400 μg) and RNase A (100 μg). After phenol-chloroform-isoamyl alcohol extractions, 2 volumes of absolute ethanol were added for DNA concentration. Then, the pelleted DNA was dissolved in 50 μl of distilled water. *aiiA* homologue genes were amplified using chromosomal DNAs of 16 strains of *B. thuringiensis* as a template and the oligonucleotide primers AIF (5′-TAAATGTAAAGGTGGATACATAATGACAGT [start codon underlined]) and AIR (5′-AGCTCATGACTTTTTGCACTATATATA [start codon underlined]). The PCR conditions involved denaturation at 94°C for 3 min followed by 28 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min with PCR Master Mix (Roche). The PCR product was ligated to the T7Blue T-vector (TaKaRa) and sequenced by an ABI3700 automatic sequencer (Applied Biosystems). The DNA sequences were analyzed using the Lasergene sequence analysis program (DNASTAR, Inc.). The *aiiA* genes were amplified using the chromosomes of *B. thuringiensis* subsp. *morrisoni* and *B. thuringiensis* subsp. *kyushuensis* as templates and the primer pair 5′-CCCCATATGACAGTAAAAAAGCTTTA and 5′-GGGCTCGAGTATATACTCCGGGAACA (the *Nde*I and *Xho*I restriction sites are underlined). The PCR products were digested with *Nde*I and *Xho*I, ligated with the *Nde*I-*Xho*I-digested pET22b(+) vector (Novagen), and introduced into *E. coli* BL21(DE3). The resulting *aiiA* expression vectors were named pETAIM and pETAIK, respectively.

SDS-PAGE analysis. Sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) was carried out by standard protocols (23). Molecular weight markers (M-3913; Sigma) were purchased from Sigma-Aldrich. Recombinant *E. coli* BL21(DE3) carrying pETAIM or pETAIK was cultivated for 3 h after IPTG induction, and the cells harvested from 10 ml of culture broth were resuspended in 1 ml of 50 mM Tris-HCl (pH 8.0). The resuspended cells were disrupted by sonication and centrifuged (5,000 × *g* for 5 min). Subsequently, 20 μl of supernatant was loaded in the polyacrylamide gel.

Bioassay of AHL-degrading activity. *N*-octanoyl-L-homoserine lactone (OHL), *N*-hexanoyl-L-homoserine lactone (HHL), and *N*-3-oxohexanoyl-L-homoserine lactone (OHHL) were purchased from Quorum Sciences, Inc. Whole cells of *B. thuringiensis* and *E. coli* were used for bioassay of AHL-degrading activity. Various strains of *B. thuringiensis* were cultivated, harvested, and resuspended in 200 mM morpholineethanesulfonic acid buffer (pH 6.5) containing 2 mM $ZnSO_4$ until an OD_{600} of 1.0 was achieved. Then, 40 μl of the cell resuspension mixture and 40 μl of OHHL (final concentration, 20 μM) were mixed and incubated at 37°C for 1 h with gentle shaking, followed by 95°C for 5 min to stop the reaction. Twenty microliters of sample was loaded in the hole of a CV026-overlaid plate. Recombinant *E. coli* BL21(DE3) carrying pETAIM or pETAIK was cultivated for 3 h after IPTG induction, and the cells harvested from 3 ml of culture broth were resuspended in 300 μl of morpholineethanesulfonic acid buffer. Forty-microliter volumes of OHHL (final concentration, 10 μM), HHL (final concentration, 10 μM), and OHL (final concentration, 10 μM) were added to 40-μl volumes of the cell suspensions and mixed. The sample was incubated at 37°C for 1 h, followed by incubation at 95°C for 5 min to stop the reaction. After heating, the original AHLs in the samples were diluted until they reached the appropriate concentrations. Subsequently, 5 μl of OHHL (0.1 μM) and 25 μl of OHL (1 μM) were loaded in the holes of NT1 (pDCI41E33)-overlaid plates and 50 μl of OHHL (5 μM) and 30 μl of HHL (1 μM) were loaded in the holes of CV026-overlaid plates. The plates were incubated at 30°C for 16 h for color development. The residual amounts of AHLs were evaluated according to the decrease in size of purple- and blue-colored areas around the holes in the CV026 and NT1 (pDCI41E33) plates, respectively.

Virulence tests. *Erwinia carotovora* strain IBN98 was cultivated until the OD_{600} was 1.0 and was then diluted with saline solution (0.15 M NaCl). Recombinant *E. coli* carrying pETAIK was harvested 3 h after IPTG induction and resuspended in saline solution to a final OD_{600} of 5. Equal volumes of *Erwinia carotovora* IBN98 (OD_{600} = 0.05) and recombinant *E. coli* (OD_{600} = 5) were mixed. Twenty microliters of the mixture was loaded onto potato slices and incubated at 30°C for 18 h. Watery rotten lesions around inoculation sites were observed as evidence of the activation of virulence.

Nucleotide accession numbers. The nucleotide sequences of *aiiA* homologue genes reported in this paper have been submitted to the GenBank-EMBL database under the following accession numbers (the *B. thuringiensis* subspecies names are shown in parentheses): AF478045 (*aizawai* HD11), AF478046 (*alesti* HD4), AF478047 (*canadensis* HD224), AF478048 (*darmstadiensis* HD146), AF478049 (*galleriae* HD29), AF478050 (*indiana* HD521), AF478051 (*israelensis* HD567), AF478052 (*kyushuensis* HD541), AF478053 (*morrisoni* HD12), AF478054 (*ostriniae* HD501), AF478055 (*pakistani* HD395), AF478056 (*subtoxicus* HD109), AF478057 (*thompsoni* HD542), AF478058 (*toumanoffi* HD201), AF478059 (*kurstaki* HD263), AF478060 (*tolworthi* HD537), and AF478061 (soil-isolated *Bacillus* sp. strain IBN35).

RESULTS

***aiiA* homologous genes in the subspecies of *B. thuringiensis*.** The *aiiA* gene, first cloned from *Bacillus* sp. strain 240B1, consists of 750 nucleotides encoding a polypeptide of 250 amino acids (5). In the incomplete genome database of *Bacillus anthracis* in The Institute for Genomic Research, an *aiiA* homologue was found which has 90% similarity in nucleotide sequence with the *aiiA* gene of *Bacillus* sp. strain 240B1. An *aiiA* homologue was searched for in our company's incomplete genome database of *B. thuringiensis* subsp. *morrisoni* HD12. As a result, a 3.7-kb contig containing an *aiiA* homologue was also found. Three *aiiA* homologue genes were aligned, and the most conserved oligonucleotide pair (AIF and AIR) was selected to investigate the presence of *aiiA* genes in other subspecies of *B. thuringiensis*. PCR amplification of the *aiiA* gene was carried out using the chromosomal DNAs of 16 strains of *B. thuringiensis* and one strain of soil-isolated *Bacillus* sp. strain IBN35 as templates. As a result, PCR products from all the strains of *B. thuringiensis* were obtained and subcloned into the T vector, and the sequences of each of the PCR products were determined. Figure 1 shows the deduced amino acid sequences of *aiiA* homologue genes and their alignments. Comparison of the *Bacillus* sp. strain 240B1 *aiiA* gene with the *B. thuringiensis* *aiiA* homologue genes revealed high homologies of 89 to 95% and 90 to 96% in nucleotide sequence and deduced amino acid sequence, respectively. Reportedly, two small conserved regions, ^{103}SHLHFDH^{109} and ^{165}HTPGHTPGH^{173}, exist in AiiA, glyoxalase II, metallo-lactamase, and arylsulfatase (5). In the sequence alignment shown in Fig. 1, ^{103}SHLHFDH^{109} is completely conserved between AiiA families, while ^{165}HTPGHTPGH^{173} is not completely conserved. In a search using the BLAST program, ^{96}DLLYIISSHLHFDHAGGNG^{114} is closely related to glyoxalase II in *Arabidopsis thaliana* (data not shown). The sequence alignment among the AiiA family might yield information in the study of AiiA specificity for different AHLs and in the engineering of enzymes with even greater activity or more refined specificity.

Figure 2 shows the *aiiA* homologue gene dendrogram from various *Bacillus* species. The *aiiA* gene of *B. thuringiensis* subsp. *toumanoffi* HD201 is most similar to that of *Bacillus* sp. strain 240B1. There are two *Bacillus* groups that have the same amino acid sequences in their AiiA proteins. One group is composed of *B. thuringiensis* subsp. *aizawai* HD11, *indiana* HD521, and *kurstaki* HD263. The other group is composed of *B. thuringiensis* subsp. *alesti* HD4, *darmstadiensis* HD146, *israelensis* HD567, *morrisoni* HD12, and *thompsoni* HD542. Although they have different serotypes against H1 flagella antigens, they have the same amino acid sequence as AiiA. As a result of analysis of a contig containing the *aiiA* gene of *B.*

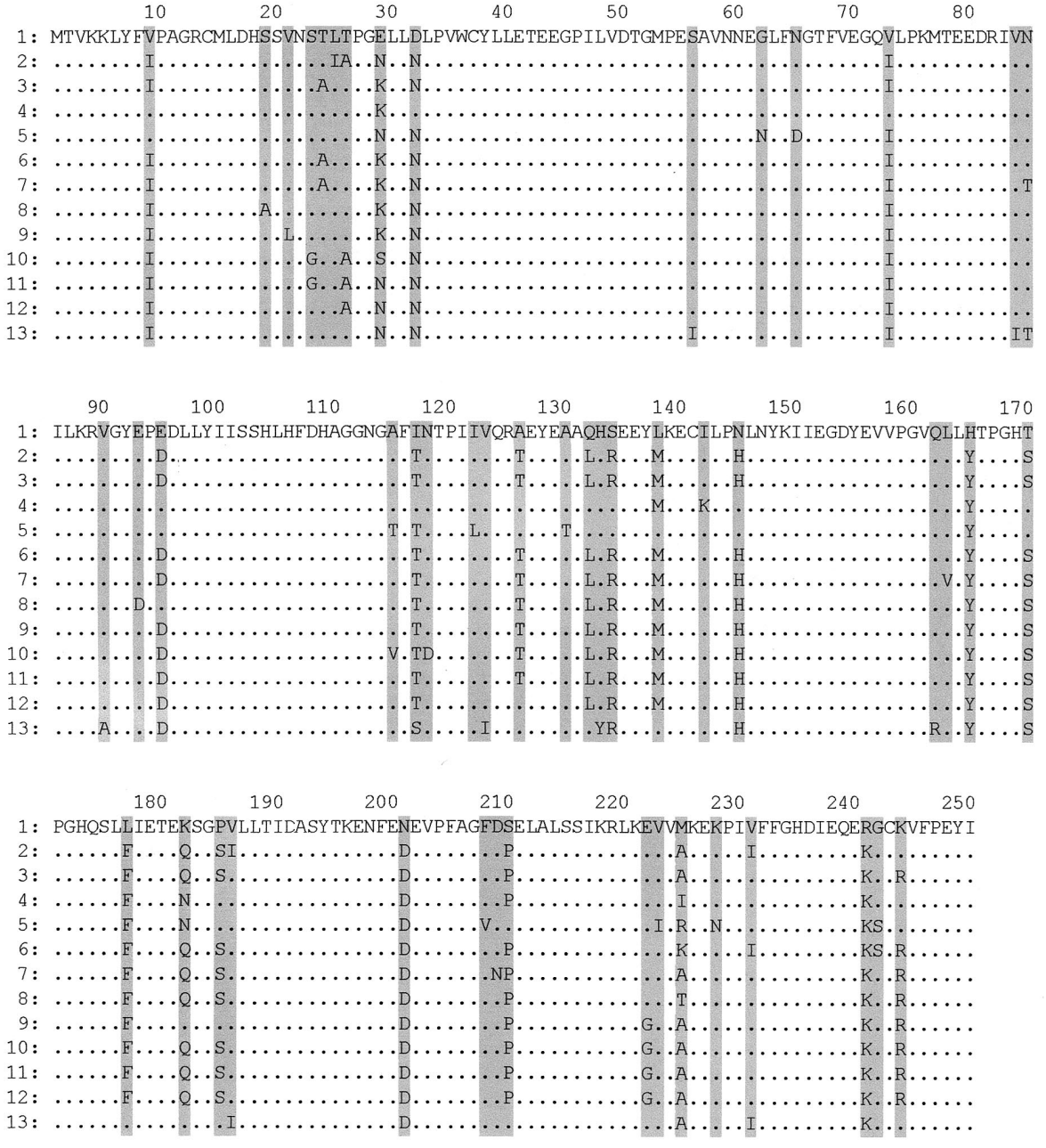


FIG. 1. Sequence alignment of deduced amino acids of putative AHL-degrading enzyme genes from *B. anthracis, Bacillus* sp. isolates, and various subspecies of *B. thuringiensis.* Sequences of the following strains were used (accession numbers in parentheses): 1, *Bacillus* sp. strain 240B1 (AF196486); 2, *B. anthracis* Ames strain (contig number 4847 from unfinished *B. anthracis* genome; The Institute for Genomic Research); 3, *Bacillus.* sp. strain IBN35 isolated from soil (AF478061); 4, *B. thuringiensis* subsp. *toumanoffi* HD201 (AF478058); 5, *B. thuringiensis* subsp. *kyushuensis* HD541 (AF478052); 6, *B. thuringiensis* subsp. *aizawai* HD11 (AF478045), *B. thuringiensis* subsp. *indiana* HD521 (AF478050), and *B. thuringiensis* subsp. *kurstaki* HD263 (AF478059); 7, *B. thuringiensis* subsp. *galleriae* HD29 (AF478049); 8, *B. thuringiensis* subsp. *ostriniae* HD501 (AF478054); 9, *B. thuringiensis* subsp. *subtoxicus* HD109 (AF478056); 10, *B. thuringiensis* subsp. *pakistani* HD395 (AF478055); 11, *B. thuringiensis* subsp. *tolworthi* HD537 (AF478060); 12, *B. thuringiensis* subsp. *canadensis* HD224 (AF478047); 13, *B. thuringiensis* subsp. *alesti* HD4 (AF478046), *B. thuringiensis* subsp. *darmstadiensis* HD146 (AF478048), *B. thuringiensis* subsp. *israelensis* HD567 (AF478051), *B. thuringiensis* subsp. *morrisoni* HD12 (AF478053), and *B. thuringiensis* subsp. *thompsoni* HD542 (AF478057). Numbers 6 and 13 contain multiple conserved sequences. Shaded regions indicate all amino acid residues that were not completely conserved.

thuringiensis subsp. *morrisoni* HD12, a 293-amino acid alginate lyase homologous gene was located upstream of the *aiiA* gene (data not shown). The alginate lyase gene is homologous to that of *B. halodurans.* We could not find the *aiiA* homologue gene around the alginate lyase gene BH0738. However, there is a predicted protein sequence (GenBank accession no. BAB06979) that is significantly related to AiiA within the *B. halodurans* genome.

Degradation of AHL by *B. thuringiensis.* To determine whether *B. thuringiensis* strains with the *aiiA* gene can degrade

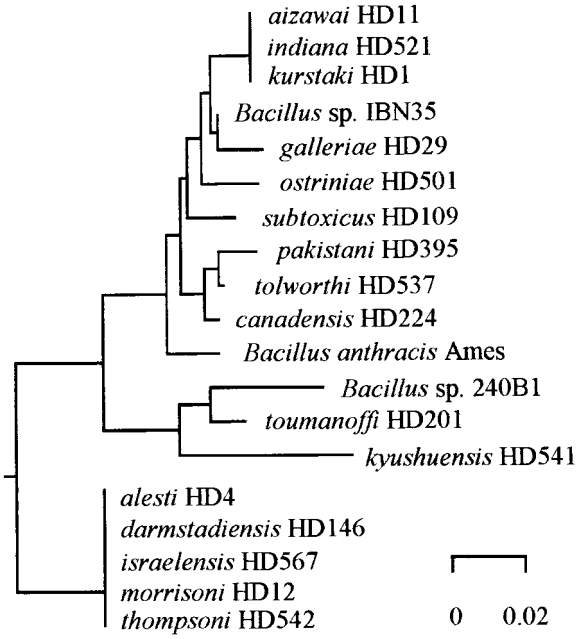
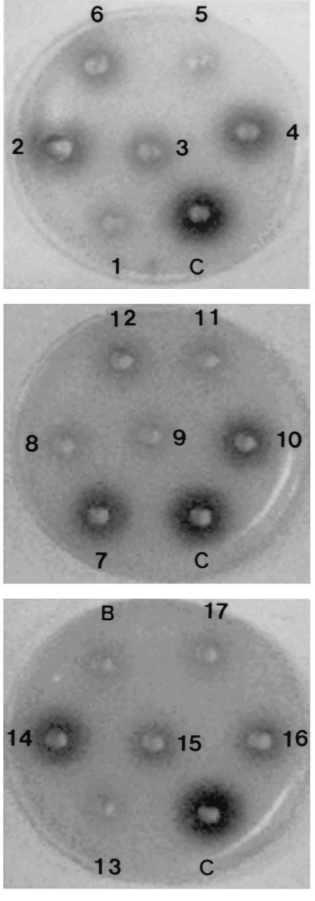


FIG. 2. Phylogenetic dendrogram of AHL-degrading enzymes, constructed using the neighbor-joining method. The bar indicates 2% estimated sequence divergence.

AHL, we designed and performed a whole-cell bioassay. A mixture of *B. thuringiensis* and OHHL was incubated at 37°C for 1 h with gentle shaking, followed by heating at 95°C. Then, the sample was loaded in the holes of *C. violaceum* CV026 plates. After overnight incubation at 30°C, biodegradation of OHHL by *B. thuringiensis* could be observed. Figure 3 shows that some strains of *B. thuringiensis* can strongly degrade OHHL and others can weakly degrade OHHL. In the case of a longer incubation time for biodegradation (>12 h), residual OHHL was not detected in the CV026 plates (data not shown). This result shows that some strains of *B. thuringiensis* effectively degrade AHL, a signal molecule of gram-negative bacteria.

AHL-degrading activity of recombinant AiiA proteins. In order to verify that the AiiA homologues of *B. thuringiensis* have AHL-degrading activity, the *aiiA* genes were expressed in *E. coli* and the activity of the recombinant AiiA proteins was tested. Two *aiiA* genes were amplified from the chromosomes of *B. thuringiensis* subsp. *morrisoni* and subsp. *kyushuensis* and were then overexpressed in *E. coli.* We confirmed the overexpression of two recombinant AiiAs of correct size (about 28 kDa) as major bands in *E. coli* by SDS-PAGE analysis (Fig. 4). The enzyme activity was confirmed by whole-cell bioassay. AHLs with different acyl side chains (OHHL, HHL, and OHL)



No.	Strain	HD code	Relative AiiA activity
1	*aizawai*	HD11	+++
2	*alesti*	HD4	+
3	*canadensis*	HD224	++
4	*darmstadiensis*	HD146	+
5	*galleriae*	HD29	+++
6	*indiana*	HD521	++
7	*israelensis*	HD567	+
8	*kurstaki*	HD1	+++
9	*kyushuensis*	HD541	+++
10	*morrisoni*	HD12	+
11	*ostriniae*	HD501	+++
12	*pakistani*	HD395	++
13	*subtoxicus*	HD109	+++
14	*thompsoni*	HD542	+
15	*tolworthi*	HD537	++
16	*toumanoffi*	HD201	++
17	*Bacillus* sp. IBN35*	-	+++

FIG. 3. Relative AHL-degrading activity of various *B. thuringiensis* subspecies by bioassay. *Chromobacterium violaceum* CV026 was used as a reporter strain, and OHHL (N-[3-oxohexanoyl]-L-homoserine lactone) was used as a substrate of AHL. C, reaction buffer with OHHL; B, reaction buffer only. **Bacillus* sp. strain IBN35 was isolated from soil.

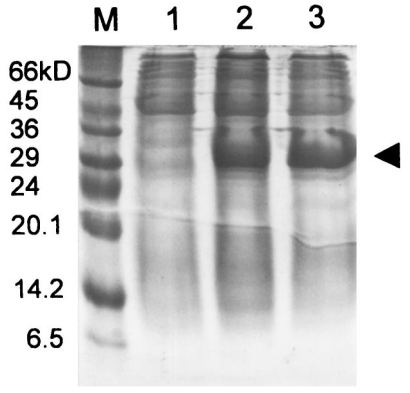


FIG. 4. SDS–15% PAGE analysis of AiiA overexpression in *E. coli*. M, marker; 1, *E. coli* carrying pET22b(+); 2, *E. coli* carrying pETAIK; 3, *E. coli* carrying pETAIM. The arrow indicates the bands of recombinant AiiA proteins.

were used for the substrates of AiiA proteins. Recombinant *E. coli* expressing AiiA could effectively degrade all the substrate, regardless of the *N*-acyl side chain (Fig. 5). This result indicates that the *aiiA* gene in *B. thuringiensis* indeed encodes AHL-degrading enzymes having broad substrate specificity.

Attenuation of plant pathogenicity of *Erwinia carotovora* by *E. coli* producing an AHL-degrading enzyme. The change in plant pathogenicity of *E. carotovora* by recombinant AHL-degrading enzyme-overproducing *E. coli* was tested. Recombinant *E. coli* carrying pETAIK and *E. carotovora* IBN98 were mixed and inoculated onto potato slices. A decrease of the watery rotten lesions in the potato slices treated with the mixture of *E. carotovora* and the recombinant *E. coli* was observed (Fig. 6). This result showed that the potato virulence of *E. carotovora* was attenuated by AHL-degrading enzyme-overproducing *E. coli.* In contrast to the result with recombinant *E. coli,* attenuation was not observed with the wild type of *B. thuringiensis* (data not shown). This might be due to the lower level of expression of the *aiiA* gene in *B. thuringiensis* than in recombinant *E. coli.* These data strongly suggest that AHL-degrading enzyme-overproducing microbes can be used for the control of gram-negative plant-pathogenic bacteria in the production of crops.

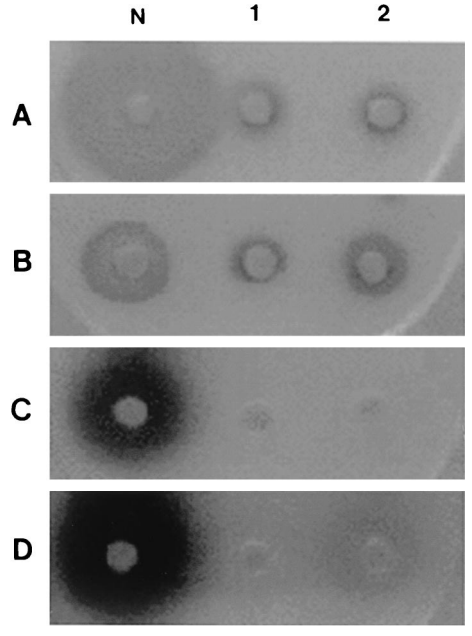


FIG. 5. Bioassay for recombinant AHL-degrading enzyme activity in recombinant *E. coli.* OHL, OHHL, HHL, and OHHL were used as substrates for A, B, C, and D, respectively. The reporter strain for A and B was *A. tumefaciens* NT1 (pDCI41E33); that for C and D was *C. violaceum* CV026. N, *E. coli* carrying pET22b(+); 1, *E. coli* carrying pETAIK; 2, *E. coli* carrying pETAIM.

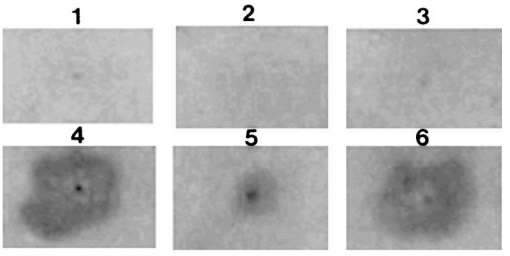


FIG. 6. Attenuation of potato pathogenicity of *Erwinia carotovora* by recombinant AiiA-producing *E. coli.* 1, saline solution; 2, *E. coli* carrying pETAIK; 3, *E. coli* carrying pET22b(+); 4, *E. carotovora*; 5, mixture of *E. carotovora* and *E. coli* carrying pETAIK; 6, mixture of *E. carotovora* and *E. coli* carrying pET22b(+).

DISCUSSION

Gram-negative microorganisms commonly use AHLs to regulate the expression of diverse phenotypic traits, such as bioluminescence (17), antibiotic production (16), and virulence factor synthesis (20). In the plant pathogen *E. carotovora*, virulence-related exoenzyme genes are regulated through an AHL-dependent quorum-sensing system (10). Because many animal and plant pathogens use quorum sensing to regulate virulence, the quorum-sensing system is an attractive target for novel antiinfective therapy (27). One strategy is to disrupt the signal generation process. This could be achieved by the inhibition of synthesis of metabolic precursors of AHL or by the direct inhibition of AHL synthesis (9). Secondly, the active efflux and diffusion system of AHLs could be a target for drug development (21). Thirdly, small AHL-mimetic antagonists such as halogenated furanones could compete with AHL for LuxR homologues (14, 15). Fourthly, an AHL-degrading enzyme or AHL-sequestering antibody might interfere with AHL-mediated cell-to-cell communications. Recently, a novel AHL-degrading enzyme gene (*aiiA*) from *Bacillus* sp. strain 240B1 was cloned (5). Transgenic tobacco and potato expressing the bacterial gene *aiiA* inactivated exogenous AHL and reduced the virulence of gram-negative *E. carotovora* (4).

As stated above, there has been increasing interest in AHL degradation in the control of quorum sensing. To our knowledge, this is the first publication to report the existence of *aiiA* homologue genes in many subspecies of *B. thuringiensis* and to identify *aiiA* homologue genes in *B. anthracis* and *Bacillus* species closely related to *B. cereus* (Fig. 1). Helgason et al. (8) reported the possibility of close relations among food-poisoning-inducing *B. cereus*, anthrax-inducing *B. anthracis*, and insecticidal *B. thuringiensis*, though they have widely different

phenotypes and pathological effects. The sequences of *aiiA* genes in *B. anthracis* and *Bacillus* sp. strain IBN35 belonging to the *B. cereus* group were phylogenetically very close to those in various subspecies of *B. thuringiensis* (Fig. 2). *aiiA* homologue genes might be widespread not only in *B. thuringiensis* but also in other strains in the *B. cereus* group.

Wild-type strains of *B. thuringiensis* show various AHL-degrading activities (Fig. 3). Some strains show strong AHL-degrading activity, and others have weak AHL-degrading activity. When the *aiiA* genes of *B. thuringiensis* subsp. *morrisoni* and *kyushuensis*, showing weak and strong AHL-degrading activity, respectively, were overexpressed in *E. coli* (Fig. 4), both of the recombinant *E. coli* strains efficiently degraded AHLs having various side chains (Fig. 5). This shows that *aiiA* homologue genes in *B. thuringiensis* encode active AHL-degrading enzymes and indicates that the recombinant AiiA proteins could be applied for control of different kinds of AHL-producing gram-negative bacteria.

In the virulence attenuation test, the recombinant *E. coli* could effectively attenuate the virulence of *E. carotovora* in potatoes (Fig. 6). The attenuation of plant pathogenicity shows the possibility of biocontrol of gram-negative bacteria by the use of recombinant AiiA-overproducing microbes or genetically engineered *B. thuringiensis.* The discovery of the existence of *aiiA* genes in many subspecies of *B. thuringiensis,* presently used as an insecticidal agent in agriculture, indicates the possibility of developing a *B. thuringiensis* strain having additional functions, including the regulation of gram-negative pathogenic bacteria.

The reason AHL-degrading enzyme genes are widespread in subspecies of *B. thuringiensis* is not known. It could be supposed that the AHL-degrading activity of AiiA might give insecticidal *B. thuringiensis* the potential to compete with gram-negative bacteria in natural ecosystems, which could explain the frequent occurrence of *B. thuringiensis* on the phylloplane. Further studies should address the identification of the function of AiiA homologue proteins in *B. thuringiensis*.

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