Suppressing *Erwinia carotovora* Pathogenicity by Projecting N-Acyl Homoserine Lactonase onto the Surface of *Pseudomonas putida* Cells

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N-Acyl homoserine lactones (AHLs) serve as the vital quorum-sensing signals that regulate the virulence of the pathogenic bacterium *Erwinia carotovora*. In the present study, an approach to efficiently restrain the pathogenicity of *E. carotovora*-induced soft rot disease is described. *Bacillus thuringiensis*-derived N-acetyl homoserine lactonase (AiiA) was projected onto the surface of *Pseudomonas putida* cells, and inoculation with both strains was challenged. The previously identified N-terminal moiety of the ice nucleation protein, InaQ-N, was applied as the anchoring motif. A surface display cassette with inaQ-N/aiiA was constructed and expressed under the control of a constitutive promoter in *P. putida* AB92019. Surface localization of the fusion protein was confirmed by Western blot analysis, flow cytometry, and immunofluorescence microscopy. The antagonistic activity of *P. putida* MB116 expressing InaQ-N/AiiA toward *E. carotovora* ATCC25270 was evaluated by challenge inoculation in potato slices at different ratios. The results revealed a remarkable suppressing effect on *E. carotovora* infection. The active component was further analyzed using different cell fractions, and the cell surface-projected fusion protein was found to correspond to the suppressing effect.

Keywords: *Pseudomonas putida*, cell surface display, N-acetyl homoserine lactonase, *Erwinia carotovora*, antibacterial activity

*Erwinia carotovora* is a plant-pathogenic bacterium that causes soft rot disease in many crops, resulting in substantial economic losses. This bacterium monitors its own population density via a cell–cell quorum sensing (QS) communication system using signal molecules known as N-acetyl homoserine lactones (AHLs) [19]. Subsequently, the expression of a variety of genes is triggered, including those that encode exoenzymes such as pectinase and cellulose, and carbapenem antibiotics, as well as virulence determinants. The bacterium is then able to invade the host plant to reproduce, ultimately causing plant tissue lesions [4, 15]. AHLs are synthesized in *E. carotovora* cells at a basal level using S-adenosyl methionine and acyl carrier protein as substrates. The AHLs are released from the cells to accumulate to a threshold concentration with increased bacterial density. An AHL then interacts with LuxR to form a LuxR–AHL complex, which activates the expression of a range of infection-associated genes [6, 7, 22]. Therefore, the AHL concentration in the interface between the pathogenic bacteria and the host plant is critical to determining infection occurrence.

Spore-forming *Bacillus* strains produce the intracellular enzyme N-acetyl homoserine lactonase, which inactivates AHL by hydrolyzing its lactone bond [4] and amide linkage [10]. The gene encoding N-acetyl homoserine lactonase, aiiA, was first reported in *Bacillus* sp. 240B1 [5] and was then widely found in many *Bacillus* strains [3, 11]. The expression of aiiA in transformed *E. carotovora* significantly reduces the release of AHLs, decreases extracellular pectolytic enzyme activity, and attenuates plant pathogenicity [5]. Plants expressing the aiiA-encoded protein AiiA also have significantly enhanced resistance to *E. carotovora* infection [4]. Therefore, AiiA-like proteins can be used as biological control agents for preventing soft rot disease.

However, despite the wide distribution of naturally occurring aiiA-harboring bacteria, they do not significantly control soft rot disease. Some researchers have explored recombinant systems expressing AiiA proteins in *Bacillus thuringiensis* [21, 23], *Lysobacter enzymogenes* [16], *Escherichia coli* [1], and *Pichia pastoris* [2]. Although these systems possibly degrade AHLs and effectively control bacterial diseases, they have problems with substrate
slacking or limited diffusion due to cell membrane barriers. AiiAs are expressed intracellularly, whereas the target AHLs are extracellular.

In the present study, an approach to the surface projection of AiiA onto Pseudomonas putida cells is reported. Its purpose is to directly and effectively degrade the activity of AHLs produced and released by E. carotovora cells. *P. putida* is generally regarded as a vigorous and dominant bacterial species with the ability to grow at high densities in a wild environment, enabling the development of large-scale biodegradation processes. To date, the use of AiiA from *P. putida* to degrade AHLs has not been examined. *P. putida* was engineered to project AiiA onto cell surfaces to overcome the mass-transfer limitation and enhance whole-cell biodegradation. Localization of the surface-exposed fusion protein was characterized using Western blot analysis, flow cytometry, and immunofluorescence microscopy. The biological control effect of recombinant *P. putida* cells with surface AiiA on the pathogenicity of *E. carotovora* was evaluated in laboratory trials.

**Materials and Methods**

**Bacterial Strains, Plasmids, and Culture Conditions**
A laboratory stock of *B. thuringiensis* MBM171 was used as the gene source for polymerase chain reaction (PCR) amplification of the N-acyl homoserine lactone gene aiiA (GenBank Accession No. AY198412). *P. putida* AB92019, a wild-type strain with dominant and exuberant vitality in wild environments [14], was used as the host strain for the surface projection of AiiA. *E. carotovora* ATCC25270 was used as the pathogenic bacterium for inducing soft rot in potato slices; *E. coli* DH5α competent cells (TakaRa Bio, Inc.) were used for the construction of recombinant plasmids. *E. coli* JM109 was used for expressing the InaQ-N/AiiA fusion protein.

All strains were cultured in Luria–Bertani (LB) medium. *P. putida*, *E. carotovora*, and *B. thuringiensis* cells were cultured at 30°C. *E. coli* cells were cultured at 37°C. The transformed *P. putida* cells were cultured with 500 µg/ml carbenicillin (Cb). Recombinant *E. coli* cells were cultured in LB containing 100 µg/ml ampicillin (Amp). For screening of recombinant *P. putida* cells, LB was supplemented with a final concentration of 50 µg/ml kanamycin (Kan).

**Cloning of aiiA, and Plasmid Construction and Transformation**
Total bacterial DNA was extracted using a standard procedure [17]. The gene aiiA was amplified by PCR from the *B. thuringiensis* MBM171 genome with primers P<sub>aaiA</sub> (5'-GTCAGATCTATGACAGTAAAGAAGCTTTA-3', BglII site underlined) and P<sub>aaiB</sub> (5'-GTCGAATTCTATATCTAAGGGGAAACA-3', EcoRI site underlined). The PCR-amplified fragment was sequenced before digestion with BglII and EcoRI. The digested fragment was then ligated to the BglII/EcoRI site of a previously constructed plasmid, pMB104 [14], yielding the recombinant plasmid pMB106 (AmpCβ', harboring the inaQ-N/aaiA fusion gene; 6,157 bp). To construct the recombinant plasmid pMB120 (AmpCβ', harboring the aiiA gene; 5,628 bp), primers P<sub>aaiC</sub> (5'-GTCATGCCAGTAAAGAAGCTTTA-3', Nco site underlined) and P<sub>aaiD</sub> were used to amplify the aiiA fragment from the *B. thuringiensis* MBM171 genome by PCR. The aiiA fragment was then digested with NcoI/EcoRI and ligated to the plasmid pMB104. *P. putida* AB92019 and *E. coli* JM109 were transformed using a previously described method [14]. The transformed *P. putida* strains harboring the plasmids pMB106 and pMB120 were designated MB116 and MB120, respectively. Transformed *E. coli* harboring pMB106 was designated as MB117.

**Analytical Assays**
The InaQ-N/AiiA fusion protein expressed in *P. putida* and *E. coli* cells was separated by SDS-PAGE on 12.5% gels. The InaQ-N/AiiA target band was excised and transferred onto a polyvinylidene fluoride membrane. The N-terminal amino acids of the corresponding protein were sequenced using an ABI Procise 491 sequencer. A polyclonal InaQ-N/AiiA antiserum was prepared by excising and purifying the InaQ-N/AiiA band originating from MB117 cells. The antiserum was emulsified with Freund’s complete adjuvant and then subcutaneously injected into the neck region of a New Zealand rabbit. A total of four subsequent booster injections were performed over 5 weeks. The antiserum was collected 10 days after the last injection, and was stored at −80°C until use. Western blot analysis, flow cytometry, and immunofluorescence microscopy were performed using previously described methods [14].

**Suppressing Effect of Antagonistic Inoculation with MB116 and *E. carotovora***
The surfaces of suitable potatoes were swabbed with 70% ethanol, chopped into similar 5-mm-thick slices using sterile knives, and then placed in Petri dishes with a sheet of wetted, sterilized filter paper. For the *P. putida* MB116 vs. *E. carotovora* antagonistic experiments, MB116 and *E. carotovora* cells were adjusted to an optical density of 1 at 600 nm (OD<sub>600</sub>). The cells were mixed at ratios of 1:1, 1:2, and 1:5 (v/v). About 10 µl of a mixed suspension was inoculated into the potato slices, which were then cultured at 28°C for 24 h. Soft rotting was observed, and the areas of lesion spots were measured. For *P. putida* AB92019 vs. *E. carotovora*, the same ratios were used. The same MB116 and *E. carotovora* cells diluted with sterile H<sub>2</sub>O were used as the negative and positive controls for infection, respectively. The average relative lesion area percentage was defined as the percentage of total lesion areas in the total tested slice area.

**Effects of Different MB116 Cell Fractions on Soft Rot**
Cell fractionation was performed as described previously [14]. Exactly 50 ml of *P. putida* MB116 cells (OD<sub>600</sub>=1) was fractionated. The cell envelope and cytosol fractions were separately mixed with *E. carotovora* cells (OD<sub>600</sub>=1) at a ratio of 1:2 (v/v). Each mixture was then inoculated into the potato slices as described above. The areas of lesion spots were measured, and the results were compared with the relative lesion area percentage.

**Suppression of *E. carotovora* Survival by Intact MB116 Cells**
Potato slices cultured for 24 h with a *P. putida* MB116/*E. carotovora* mixed suspension (1:2) were homogenized and centrifuged at 1,000 rpm. The resulting supernatants were diluted with sterile H<sub>2</sub>O and plated on pure and Kan-containing LB agars. The plates were cultured at 28°C for 24 h. The number of growing colony forming
units (CFUs) was determined. The total CFUs of \textit{E. carotovora} was defined as the number of CFUs grown on pure and Kan-containing LB agars.

Data Analysis
All data presented are the averages of at least three assays. Statistical analysis was carried out using SPSS 13.0 statistical software. Statistical significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

Expression of InaQ-N/AiiA in \textit{P. putida} and \textit{E. coli}
In our previous work \cite{14}, a dual-functional surface projection system in both \textit{E. coli} and \textit{P. putida} was developed using a newly identified InaQ-N anchor. To project AiiA onto the surface of \textit{P. putida} AB92019, the same system was used for the expression of the InaQ-N/AiiA fusion protein, which was driven from the \textit{E. coli}–\textit{P. putida} dual active promoter $P_{opr}$ (Fig. 1A). The $aiiA$ gene was amplified from the \textit{B. thuringiensis} BMB171 genome, and its sequence was confirmed to be identical to that of the open reading frame.

The expression of the fusion protein in both \textit{P. putida} MB116 and \textit{E. coli} MB117 was detected by SDS–PAGE (Fig. 1B). InaQ-N/AiiA has a theoretical molecular mass of approximately 47 kDa. A band corresponding to a protein with a predicted molecular mass apparently equal to that of InaQ-N/AiiA was present in the MB117 profile (Fig. 1B, lane 3, indicated by arrow) but was not found in the JM109 profile (the negative control). Therefore, this band was eluted for N-terminal amino acid sequence analysis. The result showed that the first 12 residues were identical to the N-terminal amino acids of InaQ-N, confirming the identity of the protein as InaQ-N/AiiA. It was then purified and used for the preparation of polyclonal antiserum. The observed low level of expression was expected, given that $P_{opr}$ is a constitutive, low-level promoter \cite{14}. $P_{opr}$ is more conducive to transmembrane and surface immobilizations of fusion proteins under weak transcription activity, where cellular transcription and secretion are more coordinated \cite{13, 18}.

Surface Localization of InaQ-N/AiiA
To verify the surface localization of InaQ-N/AiiA on \textit{P. putida} MB116 cells, Western blot analysis (Fig. 1C), flow cytometry of cell fractions (Fig. 2A), and immunofluorescence microscopy (Fig. 2B) were performed. These experiments showed clear signs of the surface immobilization of InaQ-N/AiiA on \textit{P. putida} MB116 cells. In contrast, \textit{P. putida} MB120 cells expressing intracellular AiiA (the negative control) were unable to react with externally added Cy3- or Cy5-labeled antisera. Therefore, these analyses confirmed the surface localization of InaQ-N/AiiA on \textit{P. putida} MB116 cells.

Suppression of \textit{E. carotovora} Infection by \textit{P. putida} MB116
In the antagonistic assays to detect the suppressing activity of \textit{P. putida} MB116 on \textit{E. carotovora} infection, challenge inoculation of potato slices with the two strains at different ratios showed decreased lesion areas (Fig. 3Ad, 3Af, and 3Ah). In contrast, the wild-type strains \textit{E. carotovora} and \textit{P. putida} exhibited a severe infection and a negative lesion, respectively (Fig. 3Aa and 3Ab). Increased MB116 ratios
in mixed suspensions also resulted in decreased lesion areas (Fig. 3Ad, 3Af, and 3Ah). On the other hand, challenge inoculation with *P. putida* AB92019 and *E. carotovora* at the same ratios caused larger lesions (Fig. 3Ac, 3Ae, and 3Ag). This result was similarly obtained from the measured lesion areas of each challenge inoculation (Fig. 3B). The average lesion spot areas from the antagonism of MB116 towards *E. carotovora* were computed as relative percentages. The lesion areas in each challenge inoculation were significantly decreased compared with those of *P. putida* AB92019. This finding indicates that surface-projected AiiA conferred the observed enhanced suppression of soft rot.

The cell lysate and cell envelope fractions of *P. putida* MB116 were also evaluated for suppression of *E. carotovora* in infection using challenge inoculations in potato slices.

### Table 1. Average relative percentages of potato soft lesion areas after challenge inoculation with *E. carotovora* and MB116 cell lysate/cell envelope fraction at different ratios.

<table>
<thead>
<tr>
<th>Inoculation treatment and ratio</th>
<th>Average relative percentage of lesion area (%)</th>
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<tbody>
<tr>
<td><em>E. carotovora</em> + <em>P. putida</em> AB92019 cell lysate fraction (1:2)</td>
<td>98.2</td>
</tr>
<tr>
<td><em>E. carotovora</em> + <em>P. putida</em> AB92019 cell envelope fraction (1:2)</td>
<td>98.7</td>
</tr>
<tr>
<td><em>E. carotovora</em> + H$_2$O (1:2)</td>
<td>98.9</td>
</tr>
<tr>
<td><em>E. carotovora</em> + <em>P. putida</em> MB116 cell lysate fraction (1:2)</td>
<td>61.5</td>
</tr>
<tr>
<td><em>E. carotovora</em> + <em>P. putida</em> MB116 cell envelope fraction (1:2)</td>
<td>64.7</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>0</td>
</tr>
</tbody>
</table>

*Fig. 2.* Flow cytometric analysis (A) and microscopic observation (B) of *P. putida* MB116 and MB120 cells expressing InaQ-N/AiiA and AiiA, respectively. Cells were treated with anti-InaQ-N/AiiA polyclonal antiserum followed by goat anti-mouse Cy5-conjugate antibody for flow cytometric analysis, or with secondary Cy3-conjugated goat anti-mouse IgG for immunofluorescence microscopic examination. (A) For each experiment, 100,000 cells were analyzed. (i) *P. putida* MB116; (ii) *P. putida* MB120. (B) *P. putida* MB120 (expressing intracellular AiiA) was used as the negative control.

*Fig. 3.* Examples of the antagonistic effect of *P. putida* MB116 on potato soft lesions of *E. carotovora* after challenge inoculations at different ratios (v/v) (A), and average relative percentages of potato soft lesion areas (B).

a, *E. carotovora* + H$_2$O (1:1); b, *P. putida* AB92019 + H$_2$O (1:1); c, *E. carotovora* + *P. putida* AB92019 (1:1); d, *E. carotovora* + *P. putida* MB116 (1:1); e, *E. carotovora* + *P. putida* AB92019 (1:2); f, *E. carotovora* + *P. putida* MB116 (1:2); g, *E. carotovora* + *P. putida* AB92019 (1:5); and h, *E. carotovora* + *P. putida* MB116 (1:5).
Table 2. Inhibitory effect of P. putida MB116 on E. carotovora population after challenge inoculation in potato slices.

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>Total CFUs on LB (×10^3)</th>
<th>Total CFUs on Kan-LB* (×10^3)</th>
<th>CFUs of E. carotovora (×10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. carotovora + H2O (1:2)</td>
<td>12.1</td>
<td>0</td>
<td>12.1</td>
</tr>
<tr>
<td>E. carotovora + P. putida AB92019 (1:2)</td>
<td>10.5</td>
<td>4.1</td>
<td>6.4</td>
</tr>
<tr>
<td>E. carotovora + P. putida MB116 (1:2)</td>
<td>4.9</td>
<td>3.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*LB medium supplemented with kanamycin (50 μg/ml).

(1). Compared with the control inoculations with P. putida AB92019 cell fractions and E. carotovora, the challenge inoculation with MB116 cell envelope fractions showed significantly decreased lesion areas. This confirmed the catalytic activity of the surface-exposed fusion enzyme. Notably, the MB116 cell lysates also exhibited comparable effects on pathogenicity (Table 1), signifying that a substantial amount of InaQ-N/AiiA was retained intracellularly.

For the antagonistic assay of intact MB116 cells on the growth of E. carotovora (Table 2), LB media (with and without Kan) were used to culture and enumerate total bacterial CFUs of inoculated potato slices. This procedure was performed because P. putida AB92019 and MB116 cells, but not E. carotovora cells, are resistant to Kan. As expected, the number of E. carotovora CFUs obtained from the challenge inoculation with P. putida MB116 was approximately five times less than that with P. putida AB92019. This result suggests that P. putida MB116 cells with surface-projected InaQ-N/AiiA significantly restrained the population growth of E. carotovora.

A variety of plant-pathogenic bacteria including E. carotovora are capable of using the QS system to carry out various functions, including modulation of population density, cell–cell communication, coordination of community behaviors, and regulation of expression of specific genes. Soft rot disease caused by E. carotovora is a direct consequence of the density of the proliferating community and the unlocking of the expression of infection-related genes using the QS system. As a result, the concentration of environmental AHLs accumulates to a certain threshold. Therefore, decreasing the level of environmental AHLs can possibly prevent the occurrence of soft rot. The expression of the B. thuringiensis AHL-degrading esterase AiiA has been reported. However, intracellularly expressed AiiA apparently does not have a significant controlling effect on soft rot disease [3, 11]. Based on these results, the approach of projecting AiiA onto P. putida cell surfaces was envisaged in the present study. The purpose was to provide a directly catalytic and regenerable AHL-degrading system. To the best of our knowledge, we are the first to describe the immobilization and projection of AiiA in P. putida, a dominant bacterium with exuberant vitality in wild environments.

One of the technical problems in a bacterial cell surface projection system is the promotion of surface-bound target proteins. In Gram-negative bacteria, the ice nucleation protein (INP) from Pseudomonas syringae is generally regarded as one of the most efficient anchor proteins [12, 20]. Target proteins have been surface-immobilized not only by full-length INPs but also by truncated variants [8, 9], which have been proven to be more efficient [13, 14]. Nevertheless, the development of a more efficient INP-mediated surface projection system remains critical. In addition to surface-projected AiiA that endows the suppressing activity against soft rot, about half of the AiiA was retained intracellularly based on the Western blot analysis (Fig. 1C). The possible reasons for this limited transmembrane transport include unmatched fusion protein size (compared with the full-length InaQ), as well as an inconsonant transcription–translation–transport process and promoter activity. The development of more efficient projection systems using an INP anchor is now one of our primary goals.

In conclusion, the present study examined the feasibility of a P. putida whole-cell active system for surface projection of the AHL-degrading enzyme N-acyl homoserine lactonase. The purpose was to suppress the pathogenicity of E. carotovora-induced soft rot disease. The immobilized fusion enzyme InaQ-N/AiiA was confirmed to be surface exposed. This setup enabled the antagonistic activity to be retained not only against the extension of infection, but also against the increase in the pathogen population.

Acknowledgments

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References


