Insecticidal *Bacillus thuringiensis* Silences *Erwinia carotovora* Virulence by a New Form of Microbial Antagonism, Signal Interference

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It is commonly known that bacteria may produce antibiotics to interfere with the normal biological functions of their competitors in order to gain competitive advantages. Here we report that *Bacillus thuringiensis* suppressed the quorum-sensing-dependent virulence of plant pathogen *Erwinia carotovora* through a new form of microbial antagonism, signal interference. *E. carotovora* produces and responds to acyl-homoserine lactone (AHL) quorum-sensing signals to regulate antibiotic production and expression of virulence genes, whereas *B. thuringiensis* strains possess AHL-lactonase, which is a potent AHL-degrading enzyme. *B. thuringiensis* did not seem to interfere with the normal growth of *E. carotovora*; rather, it abolished the accumulation of AHL signal when they were cocultured. In planta, *B. thuringiensis* significantly decreased the incidence of *E. carotovora* infection and symptom development of potato soft rot caused by the pathogen. The biocontrol efficiency is correlated with the ability of bacterial strains to produce AHL-lactonase. While all the seven AHL-lactonase-producing *B. thuringiensis* strains provided significant protection against *E. carotovora* infection, *Bacillus fusiformis* and *Escherichia coli* strains that do not process AHL-degradation enzyme showed little effect in biocontrol. Mutation of *aiiA*, the gene encoding AHL-lactonase in *B. thuringiensis*, resulted in a substantial decrease in biocontrol efficacy. These results suggest that signal interference mechanisms existing in natural ecosystems could be explored as a new version of antagonism for prevention of bacterial infections.

It has now been well established that single-celled bacterial cells talk frequently to one another through secretion, uptake, or recognition of small signal molecules (4, 5, 13, 17). In many cases, such a cell-cell communication is population density dependent, a mechanism known as quorum sensing (18). Quorum-sensing bacteria normally produce a basal level of quorum-sensing signals at low population density and respond to increased concentrations of signals as they proliferate. Different bacterial species may produce and respond to different quorum-sensing signals, but they use quorum-sensing mechanisms in a similar manner: to synchronize target gene expression and coordinate cellular activities. N-Acyl homoserine lactones (AHLs), which are present in the quorum-sensing systems of many gram-negative bacteria, are one family of the most characterized quorum-sensing signals. AHLs regulate diverse microbial biological functions, including antibiotic production, virulence factor expression, and biofilm formation (8, 9, 21, 28, 30, 35).

Because quorum sensing controls a range of activities implicated in pathogen-host interaction and microbe-microbe competition, such as expression of virulence genes (21, 28, 30) and production of antibiotics (2, 3, 20), it is thought that such a mechanism of gene regulation could presumably provide quorum-sensing bacteria with a competitive advantage in their natural environment (32). Because microbe-microbe interactions are common in the natural ecosystem, it is not surprising that microorganisms could also develop different versions of signal interference mechanisms to counteract the quorum-sensing signaling of their competitors (6, 32, 38). Among the several characterized quorum-sensing signal interference mechanisms (6, 38), also known as quorum quenching (12, 38), there are two groups of AHL-degrading enzymes produced by several soil bacterial species. AHL-lactonase, which was first identified in a *Bacillus* species, inactivates AHLs by hydrolyzing the lactone ring of the signals (10, 11, 24). AHL-acylases from *Ralstonia* and *Variovorax paradoxus* degrade signals by breaking the amide linkage of AHLs (23, 25). These AHL-degrading enzymes, when expressed either in transgenic plants or in bacterial pathogens, blocked bacterial quorum sensing and disintegrated bacterial population density-dependent infection (12, 25, 38). However, much less is clear whether these soil bacteria that produce AHL signal interference enzymes could effectively counteract the quorum-sensing-dependent bacterial pathogens, and whether such a signal interference mechanism could be used as a new form of antagonism in biocontrol.

The soil bacterium *Bacillus thuringiensis* is the most widely used biocontrol agent for insect control. Recently, it was shown that many *B. thuringiensis* isolates produce and display strong AHL-lactonase activity (10, 24). It is of significant interest to investigate whether *B. thuringiensis* could also be used as a biocontrol reagent to control infectious bacterial diseases. Plant bacterial pathogen *Erwinia carotovora* was selected as the target organism for this purpose. The virulence of this pathogen is correlated with its ability to produce and secrete plant cell wall-degrading enzymes, including pectate lyase, pectin lyase, and polygalacturonase (21, 30, 35). We had shown previously that expression of AHL-lactonase in transformed *E. carotovora* significantly reduced the production and release of these pectolytic enzymes (11). In this study, we tested the effect of *B. thuringiensis* on the growth and quorum sensing of *E. carotovora* and assessed the effect of *B. thuringiensis* on control of the potato soft rot disease caused by *E. carotovora*. We
further determined the role of AHL-lactonase of \textit{B. thuringiensis} in biocontrol by generation of an AHL-lactonase-null mutant.

**MATERIALS AND METHODS**

### Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. COT1, which was originally reported as a \textit{Bacillus} isolate showing a high level of 16S ribosomal DNA (rDNA) homology to \textit{B. thuringiensis} (10), was confirmed to be a \textit{B. thuringiensis} strain based on its ability to produce parasporal crystal proteins (data not shown). The other six subspecies of \textit{B. thuringiensis} strains were described previously (10). Erwinia \textit{coli} strains were grown at 37°C in Luria-Bertani (LB) medium. The other bacterial strains were grown at 28°C in LB medium. The antibiotics ampicillin and tetracycline were added at concentrations of 100 and 10 µg/ml, respectively. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Promega) was included in medium at 50 µg/ml for detection of β-galactosidase enzyme activity.

#### AHL bioassay.

To determine the level of \(N\)-acetoxy-\(N\)-homoserine lactone (OHHL), the AHL produced by \textit{E. carotovora} SCG1, the cell supernatant of bacterial culture at different time points, as indicated in Fig. 1A, was loaded onto an AHL bioassay plate (minimal agar medium supplemented with X-Gal) and quantified as described previously (11, 39). The synthetic OHHL was used as the positive control. \textit{Agrobacterium tumefaciens} strain 749, containing a lacZ fusion with the \textit{tra} gene of pTiC58, was used as an indicator strain for AHL activity (29).

#### In vitro pathogenicity assay.

\textit{Potatoes} (\textit{Solanum tuberosum} L. cv. Binjet) were obtained from local stores. After being washed with tap water and dried on a paper towel, potato tubers were surface sterilized with 70% ethanol and then sliced evenly about 5 mm in height. For pretreatment, potato slices were dipped into a suspension of \textit{B. thuringiensis} or other bacterial strains at a concentration of 5 \times 10^6 CFU/ml for about 20 s. Sterilized water was used as a control. The treated slices were then dried in a laminar flow cabinet for about 20 min to reduce surface moisture before inoculation with 2.5 µl of an \textit{E. carotovora} SCG1 bacterial suspension of different concentrations. For mix treatment, an equal volume of each test organism was mixed with the \textit{E. carotovora} SCG1 bacterial suspension as stated. The cut surface of the potato slice was inoculated with the mixture (2.5 µl). All potato slices were placed in covered petri dishes and incubated at 28°C. The maceration area (in square millimeters) was measured at the time specified. Each treatment was repeated 4 to 12 times (12 times for COT1), and each repeat was used to inoculate 1 to 3 sites per slice. For the colonization experiment, each treatment was repeated four times; each slice was inoculated at the center of slice. To test the effect of \textit{B. thuringiensis} and the \(a\_\text{iiA}^\text{::Tc}\) (the gene encoding AHL-lactonase) mutant on \textit{E. carotovora} infection, six sub-species of \textit{B. thuringiensis} and the mutant B23\(\Delta\text{ai}^\text{::Tc}\) were used separately to treat potato slices before inoculation with \textit{E. carotovora} SCG1.

#### In vitro competition between \textit{B. thuringiensis} and \textit{E. carotovora}.

Competition experiments were conducted by coinoculation of \textit{B. thuringiensis} and \textit{E. carotovora} in LB medium. \textit{E. carotovora} was inoculated to a final concentration of about 10^5 CFU/ml, and the others were inoculated at 10^6 CFU/ml. The mixture was incubated at 28°C. At different time points, the bacteria samples were taken for bioassay of AHL and spread on plates for colony counting after proper dilutions. \textit{B. thuringiensis} and \textit{E. carotovora} colonies were easily distinguishable based on their unique colony morphologies. The experiment was repeated four times.

#### Construction of an AHL-lactonase mutant.

To determine the role of AHL-lactonase in suppression of \textit{Erwinia} virulence, the gene replacement approach was used to generate the \(a\_\text{iiA}^\text{::Tc}\) mutant of \textit{B. thuringiensis} subspecies \textit{israelensis} B23 (BGSC 4Q7). The fragments about 300 bp from both the 5' and 3' ends of \(a\_\text{iiA}^\text{::Tc}\) were separately ligated upstream and downstream of the tetracycline resistance gene in the gene replacement vector \textsc{pUCTV}2 (36) to generate \textsc{pUCTV}2\(\Delta\text{ai}^\text{::Tc}\) (Table 1). This construct was transferred into B23 by electroporation with \textit{Electro Cell Manipulator} 600 (1.5 kV, 240 µF, 2-mm cuvette, BTX, San Diego, Calif.), and the transformants were incubated at 42°C to get rid of the plasmid. After consecutive culture for 3 days (recultured at 12-h intervals), tetracycline-resistant colonies were picked up. The correct mutation was confirmed by PCR analysis and by the AHL-lactonase-null phenotype.

### RESULTS

\textit{B. thuringiensis} blocked \textit{E. carotovora} AHL signal accumulation but did not affect its growth.

To test the effect of \textit{B. thuringiensis} on AHL accumulation and growth of \textit{E. carotovora} SCG1, SCG1 was cocultured with \textit{B. thuringiensis} strains COT1 and B1, \textit{E. coli DH}5\(\alpha\), and \textit{B. fusiformis}, respectively. Figure 1A shows that the AHL produced by strain SCG1 was detectable 2 h after inoculation, and a rapid increase was observed between 2 to 6 h after incubation, parallel to the exponential proliferation stage of the bacterial cells (Fig. 1B). However, no AHL was detected in the culture supernatant of SCG1 cocultured with either COT1 or B1, which produce AHL-lactonase. However, coculture of SCG1 with either \textit{E. coli DH}5\(\alpha\) or \textit{B. fusiformis} cells, which do not produce AHL-degrading enzyme (10), had much less effect on AHL accumu-
development, the inoculated potato slices were incubated at causing severe tissue maceration symptoms (Fig. 2A). To produce AHL degradation enzymes, failed to prevent SCG1 from mix treatment with AHL-lactonase. In contrast, both pretreatment and symptoms (Fig. 2). The biocontrol efficiency appeared to decrease with SCG1 with COT1 (mix treatment) also attenuated soft rot population density of the inoculum, the larger the maceration area was developed on potato slices. However, when potato slices were pretreated with COT1 suspension (pretreatment) before inoculation with SCG1, the maceration symptom was significantly alleviated (Fig. 2A, left, and 3A). Coinoculation of SCG1 with COT1 (mix treatment) also attenuated soft rot symptoms. The extent of maceration was positively correlated to pretreatment, the maceration symptom was significantly alleviated before inoculation with SCG1, and the number of macerated spots and area of maceration were determined. Fewer maceration incidents were found on the potato slices pretreated with six B. thuringiensis strains than the control slices (Fig. 4A), and the maceration area per site on the pretreated slices was also significantly smaller than that on the control slices (Fig. 4B). Strain B18, which displayed lower AHL inactivation activity (10), showed less protection against Erwinia infection than other B. thuringiensis strains (Fig. 4).

**Effect of B. thuringiensis on colonization of E. carotovora in planta.** To facilitate investigation of colonization of E. carotovora strain SCG1 on potato slices, strain SCG1-GFP was obtained by transformation of a green fluorescence protein (GFP) gene carried by expression vector pGEM7 into strain SCG1 (Table 1). There was no difference in virulence between strain SCG1-GFP and wild-type SCG1. To investigate the effect of B. thuringiensis bacteria on the survival and growth of SCG1 on

**FIG. 1.** Effect of B. thuringiensis on AHL accumulation and growth of E. carotovora. (A) AHL accumulation during bacterial growth. E. carotovora SCG1 was inoculated alone (■) or coinoculated, respectively, with B. thuringiensis strain COT1 (*) or B1 (●). E. coli DH5α (△), or B. fusiformis (□) in LB medium. (B) Time course of bacterial growth. SCG1 (■), COT1 (●), and B1 (▲) were incubated and grown separately in LB medium. (C) Cell numbers of SCG1 (■) and COT1 (●) when coinoculated. (D) Cell numbers of SCG1 (■) and B1 (▲) when coinoculated. The experiment was repeated four times. The mean data are presented.
plants, potato slices were pretreated with a bacterial suspension of COT1 and then inoculated with SCG1-GFP. Changes in bacterial cell numbers and development of soft rot symptom on potato tissue were monitored daily for 4 days. There were no significant changes in cell numbers of SCG1-GFP on the COT1-treated slices and control slices (water treated) during the first 2 days of incubation, and then a slight decrease was noticed at the third and fourth days in both cases (data not shown). However, the bacterial distributions on the slices pretreated with COT1 or treated with water were quite different. On the first day after incubation, the control slices displayed soft rot symptoms, and most of the E. carotovora SCG1 bacteria were observed at the edge of the rotten area. However, on the COT1-pretreated slices, SCG1 cells were confined around the inoculated site, indicating the aggressive SCG1 lost its virulence (Fig. 5A and B). These results confirm that B. thuringiensis bacteria suppressed the virulence of E. carotovora via interference of quorum-sensing signaling among pathogenic cells rather than killing the pathogen.

The AHL-lactonase-null mutant of B. thuringiensis is less effective in silencing the virulence of E. carotovora. To further confirm the role of AHL-lactonase in silencing the virulence of E. carotovora, we disrupted the aiiA gene, which encodes AHL-lactonase, in B. thuringiensis strain B23 by double-crossover recombination using the tetracycline resistance gene as the marker. The mutation was confirmed by PCR with aiiA-specific primers and by AHL bioassay. No AHL-degrading enzyme activity was detected in the AHL-lactonase-null mutant B23Δai (Fig. 5C). The virulence assay showed that all SCG1-inoculated potato slices were macerated, whereas B23Δai pretreatment failed to prevent SCG1 infection, although the extent of maceration was restricted (Table 2). The positive control, in which potato slices were pretreated with wild-type B23, did not show the symptoms of SCG1 infection (Table 2). The data indicate that AHL-lactonase is essential for silencing the virulence of E. carotovora. However, other mechanisms of B. thuringiensis might also play a role in biocontrol, because B23Δai pretreatment reduced the area of maceration in comparison with that of the control (pretreated with water).
**DISCUSSION**

*B. thuringiensis* has been used extensively as a microbial insecticide in the last few decades because of its ability to produce selective insecticidal crystal proteins that are usually environmentally safe (15, 22). *B. thuringiensis* strains showed biocidal activity against several families of pest insects, such as lepidopteran, dipteran, and colepteran at larval stages, as well as mites, nematodes, flatworms, and protozoa (16, 22). However, most insectcidal *B. thuringiensis* strains have not been exploited for disease control—probably because they normally do not produce effective antibiotics against bacterial and fungal pathogens. In this study, we showed that gram-positive *B. thuringiensis* bacteria interrupted quorum-sensing signaling of gram-negative *E. carotovora* when they live as commensals (Fig. 1A and 5C), and such signal interference resulted in drastic attenuation of *E. carotovora* virulence (Table 2 and Fig. 2 to 5). All seven randomly selected *B. thuringiensis* bacterial isolates displayed biocontrol activity against the potato soft rot disease caused by *E. carotovora*, either when they were used to pretreat potato slices before inoculation of the pathogen or when they were coinoculated with the pathogen on plant tissues. These results show for the first time that the biocontrol spectrum of *B. thuringiensis* could be further expanded to include at least the soft rot disease caused by *E. carotovora*.

Our data showed that the *B. thuringiensis* strains tested did not produce an antibiotic-like substance to interfere with the proliferation of *E. carotovora* (Fig. 1C and D). The efficacy of *B. thuringiensis* at preventing *E. carotovora* infection depends upon its ability to produce AHL-lactonase, a potent enzyme that inactivates AHL quorum-sensing signals by hydrolyzing the homoserine lactone ring (12, 36). *B. thuringiensis* isolate B18, which displayed the poorest AHL-lactonase activity among the *B. thuringiensis* strains tested (10), also showed the least effect in biocontrol (Fig. 4A and B). *B. fusiformis*, which...
does not process an AHL-lactonase (10), showed little effect in biocontrol (Fig. 2A). Moreover, null mutation of the aiiA gene encoding AHL-lactonase substantially decreased the biocontrol potency of *B. thuringiensis* (Fig. 5 and Table 2). The results suggest that signal interference might represent a novel form of microbial antagonism that could be explored for the control and prevention of AHL quorum-sensing signal-mediated bacterial diseases.

AHL-lactonase appears to be widely conserved. *Bacillus cereus* and *Bacillus mycoides*, species closely related to *B. thuringiensis*, also produce AHL-lactonases (10). These *Bacillus* enzymes are highly conserved, sharing more than 90% homology at the peptide level. A recent report showed that *Bacillus* sp. strain A24, showing AHL-lactonase activity, provided significant preventive and curative biocontrol against the potato soft rot caused by *E. carotovora* and crown gall of tomato incited by *A. tumefaciens* (26). AHL-lactonase has also been identified in gram-negative bacterial species, such as *A. tumefaciens* (7, 27, 37). Although levels of homology between *Bacillus* AHL-lactonase and the AHL-lactonases from gram-negative bacterial species are low (usually about 30 to 35%), they share a highly conserved motif, HXD(H–D)H–D, which is essential for enzyme activity (10). Except for *A. tumefaciens*, in which the AHL-lactonase encoded by aiiA plays a vital function in quorum-sensing signal turnover in response to changes in growth (37), the role of AHL-lactonase in other organisms remains unclear. However, because AHL signals (in particular, the short chain members) diffuse conveniently into bacterial cells (34), any microorganism that processes a potent AHL degradation enzyme could have a significant impact on the AHL-dependent quorum-sensing bacteria if they live as commensals. Because microbe-microbe interactions are ubiquitous and AHL signals are involved in regulation of a range of biological functions important for survival, such as antibiotic production (2, 3, 20), swarming and swimming motility (14), and biofilm formation (1, 8), it is likely that AHL-lactonase could play a significant role in obtaining competitive advantages for its producer over competitors in natural ecosystem. This notion is strengthened by the finding that the presence of AHL-lactonase-producing *B. thuringiensis* effectively stopped the otherwise rapid spread of *E. carotovora* cells in plant tissues (Fig. 3 and 5A and B).

Antibiotic production has been the major mechanism of microbial antagonisms that are commonly exploited in biocontrol of bacterial and fungal diseases (31). These antibiotics function by either killing or stopping bacterial growth. In recent years, other versions of microbial antagonisms, which do not directly kill pathogens, have also been investigated. One interesting example is that *Lactobacillus fermentum* RC-14, a probiotic bacterial isolate, inhibited acute *Staphylococcus aureus* infection (19). The probiotic bacteria did not appear to affect pathogen growth; rather, the pathogen secretes cell surface extracellular matrix-binding proteins and biosurfactant that somehow prevented pathogen adherence to surgical implants and inhibited *S. aureus* infection. More recently, Molina et al. (26) reported that the recombinant *Pseudomonas fluorescens* strain overexpressing AHL-lactonase attenuated the virulence of *E. carotovora* on potatoes. These findings, as well as the data presented in this study, illustrate the promising potential to explore the microbial antagonistic mechanisms other than antibiotic production, such as signal interference, for the control and prevention of infectious diseases.

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**REFERENCES**


**TABLE 2.** *E. carotovora* virulence assay on potato slices pretreated with wild-type *B. thuringiensis* and its mutant lacking AHL-lactonase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of sites with maceration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Maceration area (mm&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>5.0 × 10&lt;sup&gt;5&lt;/sup&gt; CFU</td>
<td>2.5 × 10&lt;sup&gt;5&lt;/sup&gt; CFU</td>
</tr>
<tr>
<td>Water</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>B23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B23aai</td>
<td>100</td>
<td>100</td>
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<sup>a</sup>The pretreated potato slices were inoculated with *E. carotovora* SCG1 at 5.0 × 10<sup>5</sup>, 2.5 × 10<sup>5</sup>, and 5.0 × 10<sup>4</sup> CFU. The percentage of inoculation sites showing maceration symptoms was determined 3 days after inoculation.

<sup>b</sup>Maceration areas were measured 1 to 3 days after inoculation with an inoculum of 5.0 × 10<sup>5</sup> CFU. Data are the means of six replicates.


