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# The Role of AiiA, a Quorum-Quenching Enzyme from *Bacillus thuringiensis*, on the Rhizosphere Competence

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**Bacteria sense their population density and coordinate the expression of target genes, including virulence factors in Gram-negative bacteria, by the *N*-acylhomoserine lactones (AHLs)-dependent quorum sensing (QS) mechanism. In contrast, several soil bacteria are able to interfere with QS by enzymatic degradation of AHLs, referred to as quorum quenching. A potent AHL-degrading enzyme, AiiA, from *Bacillus thuringiensis* has been reported to effectively attenuate the virulence of bacteria by quorum quenching. However, little is known about the role of AiiA in *B. thuringiensis* itself. In the present study, an *aiiA*-defective mutant was generated to investigate the role of AiiA in rhizosphere competence in the root system of pepper. The *aiiA* mutant showed no detectable AHL-degrading activity and was less effective for suppression of soft-rot symptom caused by *Erwinia carotovora* on the potato slice. On the pepper root, the survival rate of the *aiiA* mutant significantly decreased over time compared with that of wild type. Interestingly, viable cell count analysis revealed that the bacterial number and composition of *E. carotovora* were not different between treatments of wild type and the *aiiA* mutant. These results provide evidence that AiiA can play an important role in rhizosphere competentce of *B. thuringiensis* and bacterial quorum quenching to Gram-negative bacteria without changing bacterial number or composition.**

**Keywords:** Quorum sensing, quorum quenching, *N*-acylhomoserine lactone, AHL-lactonase, *Bacillus thuringiensis*

*Bacillus thuringiensis* belongs to a group of spore-forming Gram-positive bacterium. The bacterial species can easily be isolated from soil and plant surfaces as well as dead insects. For a long time, *B. thuringiensis* has been regarded as an insect pathogen alone by producing toxin. During sporulation, various *B. thuringiensis* subspecies produce different insecticidal crystal proteins encoded by *cry* genes [11]. In addition, these different insecticidal toxins have a narrow range of insect hosts. However, toxicity to mammalian species has not yet been reported. Therefore, *B. thuringiensis* has been widely used as a biological control agent against insect pests in several crop systems [11]. Recent study shows that many *B. thuringiensis* subspecies produce AiiA homolog enzymes to degrade *N*-acylhomoserine lactones (AHLs), signal molecules of quorum sensing in Gram-negative bacteria [1, 3, 5]. Quorum sensing is known as a bacterial regulatory mechanism to control the expression of various biological activities such as virulence factors of plant pathogenic bacteria by a cell density-dependent manner [4]. The AHL-degrading enzyme, AHL-lactonase (AiiA) first identified in *Bacillus* species, has been reported to inactivate bacterial virulence by quorum sensing through hydrolysis of the lactone ring of AHLs [1]. The introduction of the *aiiA* gene in bacterial pathogens or plants disrupts bacterial quorum sensing, consequently inhibiting the production of pathogenesis factors and protecting plants against infection of plant pathogens like *Erwinia carotovora,* which is a casual pathogen on the crucifer plant species and of which pathogenesis controlled by the AHL-based quorum-sensing system [1, 2]. However, little has been studied on the role of AiiA in the physiology of bacteria or under *in situ* condition [13]. Besides AiiA from Gram-positive *Bacillus* spp., many Gram-negative bacteria species including *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Arthrobacter* sp., *Rhodococcus* sp., *Variovorax paradoxus*, and *Acinetobacter* sp. were also reported to produce AHL-degrading enzymes [7, 9]. Collectively, these results indicate that quorum quenching is a common mechanism during interactions of plant-associated bacteria groups. However, the role of quorum quenching on the interactions cannot be intensively elucidated. To investigate the function of quorum-quenching enzyme from plant-associated bacteria such as *Bacillus* spp. with plant,

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we constructed an *aiiA*-defective mutant of *B. thuringiensis* and characterized it under *in vitro* condition. In addition, the root competency of *B. thuringiensis* wild type and its *aiiA* mutant was assessed by examining the population dynamics on pepper roots. The two strains were cultivated in tryptic soy agar (TSA) plates at 30°C. *Chromobacterium violaceum* CV026 was used as a reporter strain for the bioassay and cultivated in LB medium. For the bioassay plate, an overnight culture of *C. violaceum* CV026 was added to LB soft agar at an $OD_{600}$ of 0.1, and then 5 ml of the mixture was overlaid on the surface of an LB agar plate [6]. If necessary, antibiotics were added at the following concentrations: 100 μg/ml rifampicin for wild-type *B. thuringiensis* (generated a spontaneous mutant before conducting experiment), 10 μg/ml tetracycline for *aiiA*-defective *B. thuringiensis*, 100 μg/ml ampicillin for *E. carotovora*; and 5 μg/ml vancomycin for G(−) bacteria. Cyclohexamide at 100 μg/ml was added for inhibition of fungal growth.

To construct the *aiiA* mutant strain, *B. thuringiensis* subsp. *israelensis* 4Q7, whose *aiiA* homolog shares 95% identity with *aiiA* of *Bacillus* 240B1 [1, 5], without natural plasmid was obtained from the Bacillus Genetic Stock Center. To test the role of *B. thuringiensis* AiiA to degrade quorum-sensing signals, wild type and *aiiA* mutant strains were assessed for their quorum quenching capacity using two assay systems: (1) attenuation of *E. carotovora* pathogenesis, which is governed by quorum sensing on the potato slices and (2) direct capacity to degrade the quorum sensing signal (AHL) on the LB plate. Potato slices were placed on Petri dishes with wet paper to maintain moisture. The actively growing *E. carotovora* and *B. thuringiensis* strains were harvested and resuspended in saline solution. The bacterial suspensions were adjusted to $2.5\times10^4$ colony-forming unit (CFU)/ml for *E. carotovora* and $3.0\times10^5$ CFU/ml for *B. thuringiensis*, and equal volumes were then mixed. Ten μl of the mixture was loaded onto potato slices after mild tip punching and incubated for 18 h at 30°C. Watery rotten lesions around inoculation sites were examined as evidence of activation of virulence.

For the bioassay of AHL-degrading activity, *B. thuringiensis* was cultured overnight in LB medium at 30°C, and harvested and resuspended in 100 mM Tris-HCl (pH 7.0). Then, 50 μl of the cell suspension ($OD_{600}$=2.0) and an equal volume of 40 μM *N*-hexanoylhomoserine lactone (HHL) were mixed, and the mixture was 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 appropriate concentrations and loaded into the hole of *C. violaceum* CV026-overlaid plates. The residual amount of HHL was calculated using a relationship equation based on the color zone size and known amounts of HHL.

Single colonies were transferred to an LB agar plate containing each antibiotic and incubated for 2 days. For experimental use, fully grown bacteria were scraped off plates and resuspened into sterilized distilled water (SDW). The bacterial suspensions were adjusted to $10^9$ CFU/ml based on optical density. Five ml of each bacterial suspension was drench-applied to pepper seedlings grown in the potting media. The pots were placed in the greenhouse with natural light at 22–25°C and 70–80% relative humidity. To evaluate the effect of AiiA on the challenge of the pathogen *E. carotovora*, gfp-tagged *E. carotovora* subsp. *carotovora* SCC1 was cultivated in LB agar containing ampicillin [10]. As described above, *E. carotovora* was collected and adjusted to $10^9$ CFU/ml. Five ml of *E. carotovora* suspension was drench-applied to pepper plants at 10 days after the treatment of *B. thuringiensis* strains. Every 10 days after the treatment with each strain, a root system was taken and washed in a 100-ml flask. The dilution plating method was used to isolate colonies on TSA, with appropriate antibiotics for each strain and cyclohexamide to inhibit fungal growth. *E. carotovora* from the root system was isolated on LB with ampicillin and GFP expression confirmed by fluorescence. This experiment was designed as a randomized complete block (RCB) with 3 replications and one plant per replication. The experiment was conducted three times. The effects of treatment in three trials were similar, therefore, representative data from one trial are presented in the results. Data were subjected to analysis of variance using JMP software version 4 (SAS Institute Inc., Cary, NC, U.S.A.). The different capacity of the introduced bacterial strains was considered significant according to the magnitude of the F value ($P$=0.05). When a significant F test was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD).

**Construction and Identification of *B. thuringiensis* AiiA-Deficient-Mutant**

Previously, we reported that many *B. thuringiensis* subspecies have AHL-degrading activity against various AHLs [5]. These AHL-degrading activities result from the AiiA homolog encoded by *aiiA* homolog genes. To study the role of AiiA in *B. thuringiensis*, we disrupted the *aiiA* gene in *B. thuringiensis* subsp. *israelensis* 4Q7 by double-crossover recombination. The *aiiA*-deleted fragments were amplified by PCR with the following primer pairs; up-AiiAF (5'-cgAA-GCTTatgacagtaaa-aaagc) and up-AiiAR (5'-cgTCTAGAgaaaaagcaccatttc), down-AiiAF (5'-ctGCGGCCGCagagaagaat-attt) and down-AiiAR (5'-cgACGCGTcaaagaaaataattgg), which carried the HindIII and XbaI, NotI and MluI restriction sites, respectively. Each amplified fragments were digested with restriction enzymes, and ligated upstream and downstream of the tetracycline resistance gene in the temperature-sensitive suicide vector pUCTV2 ($Ap^r$, $Tc^r$, and $Ori^{ts}$) [12]. The resulting mutant plasmid was introduced into *B. thuringiensis* subsp. *israelensis* 4Q7 by electroporation, and tetracycline-resistant colonies were selected. Purified transformant colonies

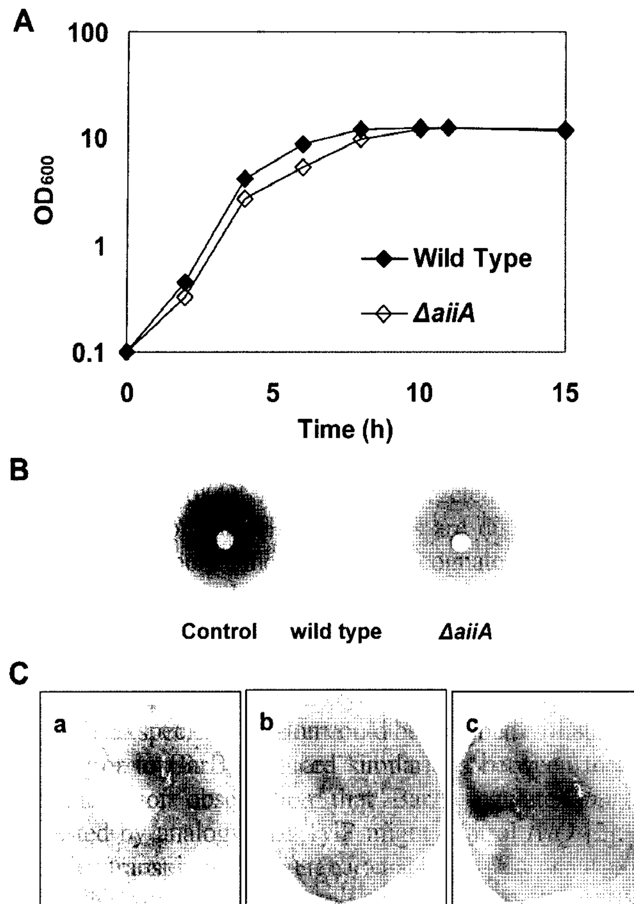


**Fig. 1.** Characterization of *B. thuringiensis* and its AiiA-defective mutant (*ΔaiiA*).

**A.** Growth curve of wild type and *ΔaiiA*, (*aiiA*-defective mutant.) **B.** AHL-degrading activity by wild type and *ΔaiiA*. **C.** Soft-rot symptoms caused by *E. carotovora* SCC1 of which the final cell number inoculated on the potato slices was $4\times10^5$ CFU. a: *E. carotovora* SCC1 inoculation on a potato slice pretreated with water. b: Mix treatment with the wild type *B. thuringiensis* suspension cultures at $6\times10^6$ CFU/ml. c: Mix treatment with the *ΔaiiA* suspension cultures at $7.5\times10^6$ CFU/ml.

were then incubated overnight at 45°C (the nonpermissive temperature for plasmid replication) on LB broth containing 10 μg/ml tetracycline to permit selection of colonies that had integrated the plasmid into the chromosome at the cloned locus *via* homologous recombination. The specific mutation of the *aiiA* gene was confirmed by PCR analysis (data not shown), and the selected *aiiA*-deletion mutant (*ΔaiiA*) did not shown any AiiA activity when using *C. violaceum* CV206 as a reporter strain. Although the growth of the *aiiA* mutant was slower compared with that of the wild type, both strains reached the same final cell densities (Fig. 1). The AHL-degrading activity of wild type was growth dependent and reached the highest at early stationary phase (Fig. 1). In contrast, however, we did not detect AHL-degrading enzyme activity in the *aiiA* mutant (Fig. 1). To confirm the role of AiiA in virulence attenuation of *E. carotovora* subsp. *carotovora* SCC1, a potato slice assay was employed with wild type and the *aiiA* mutant. The result showed that the macerated area was decreased on the potato slice samples that were pretreated with wild type, whereas the treatment of *aiiA*-defective *B. thuringiensis* could not prevent maceration caused by *E. carotovora* (Fig. 1). These results verified that AiiA of *B. thuringiensis* is essential for attenuating the virulence of *E. carotovora.*

### Effect of *B. thuringiensis* and its AiiA defective Mutant on Bacterial Number on Pepper Roots

To determine the effect of AiiA on the competency of *B. thuringiensis* in the rhizosphere of pepper, we evaluated the population dynamics of the introduced *B. thuringiensis* wild type and *aiiA* mutant at different time points (0, 10, 20, and 30 days after inoculation) (Table 1). At 0 day after application, the number of total bacteria ranged from $3.2\times10^8$ to $4.3\times10^8$ CFU/g fresh root, which was sustained at $10^8$ CFU/g fresh root for 10–30 days after application. In contrast, the number of wild type *B. thuringiensis* was 89-and 267-fold higher than its *aiiA*-defective mutant at days 10 and 20, respectively. At 30 days of treatment, the total number of bacteria on the root of pepper did not differ statistically, comparing wild type *B. thuringiensis* or its *aiiA*

**Table 1.** Population dynamics of *B. thuringiensis* and its *aiiA*-defective mutant on the pepper roots.

| Treatments | Population density (CFU/g fresh root) | | | |
|---|---|---|---|---|
| | Total bacteria | G(−) bacteria | *B. thuringiensis* | *E. carotovora* |
| Day 0 | | | | |
| Water | $4.3\times10^8$a | $1.0\times10^8$a | - | |
| Wild type | $3.2\times10^8$a | $4.0\times10^7$a | $4.6\times10^7$a | |
| *ΔaiiA* | $3.6\times10^8$a | $6.8\times10^7$a | $1.3\times10^7$a | |
| Day 10 | | | | |
| Water | $1.5\times10^8$a | $8.3\times10^7$a | - | $2.0\times10^8$a |
| Wild type | $3.0\times10^8$a | $1.2\times10^8$a | $2.6\times10^7$a | $2.7\times10^8$a |
| *ΔaiiA* | $1.5\times10^8$a | $8.8\times10^7$a | $2.9\times10^5$b | $2.2\times10^8$a |
| Day 20 | | | | |
| Water | $2.1\times10^8$a | $1.0\times10^8$a | - | $4.4\times10^7$a |
| Wild type | $2.4\times10^8$a | $8.6\times10^7$a | $1.5\times10^7$a | $3.3\times10^7$a |
| *ΔaiiA* | $2.4\times10^8$a | $1.4\times10^8$a | $5.6\times10^4$b | $4.1\times10^7$a |
| Day 30 | | | | |
| Water | $3.5\times10^8$a | $1.9\times10^8$a | - | $9.1\times10^7$a |
| Wild type | $1.7\times10^8$a | $8.6\times10^7$a | $1.5\times10^7$a | $6.4\times10^7$a |
| *ΔaiiA* | $1.9\times10^8$a | $9.4\times10^7$a | $1.3\times10^4$b | $5.3\times10^7$a |

Numbers represent the mean of three replications per treatment. Bacterial numbers were counted 0, 10, 20, and 30 days after inoculation of *B. thuringiensis* wild type and *ΔaiiA* on the roots. To select targeted bacterial population, antibiotics were added at the following concentrations: 100 μg/ml rifampicin for wild type *B. thuringiensis* (a spontaneous mutant), 10 μg/ml tetracycline for *aiiA*-defective *B. thuringiensis,* 100 μg/ml ampicillin for *E. carotovora*; and 5 μg/ml vancomycin for G(−) bacteria. Cyclohexamide at 100 μg/ml was added for inhibition of fungal growth.

Different letters indicate significant differences using Fisher's protected LSD test at $P$=0.05.

mutant treatment with water treatment. However, the viability of *aiiA* mutant decreased significantly, showing $1.3\times10^4$ of bacterial population density, which is 1,153-fold lower than that of its wild type. The populations of *E. carotovora* were not significantly different between treatments through inoculation times but were lower at day 10 and 20 compared with day 0. Intriguingly, root application of the *aiiA* mutant in pepper failed to protect the plant from root rot caused by *E. carotovora,* whereas wild type protected plant root (data not shown). However we did not obtain consistent results. These results indicate that AiiA of *B. thuringiensis* might play an important role in rhizosphere competence when *B. thuringiensis* survives on the plant root system.

Since the first finding of an AHL-degrading enzyme from *Bacillus* sp. 240B1 by Dong *et al.* [1], AiiA has been studied to interfere with the quorum sensing signal molecule, in order to inhibit the expression of target genes that encode virulence proteins of plant and animal pathogenic bacteria [1, 8]. However, the nature of AiiA in the *B. thuringiensis* itself has not yet been fully understood. Our present results showed that AiiA from *B. thuringiensis* plays a critical role in the rhizosphere competence when *B. thuringiensis* survives on the plant root system, which is an ecological niche for *B. thuringiensis. B. thuringiensis* can mostly be isolated from soil and plant tissues such as root or leaf. Our data also showed that AiiA protein can protect the viability of *B. thuringiensis* itself, suggesting that AiiA can be used as an important enzyme for cell metabolism or survival machinery during bacterial growth.

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