Biological Control 52 (2010) 17–23

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

# Reducing *Pectobacterium* virulence by expression of an *N*-acyl homoserine lactonase gene $P_{lpp}$-*aiiA* in *Lysobacter enzymogenes* strain OH11

Guo-Liang Qian [1], Jia-Qin Fan [1], De-Feng Chen [1], Yue-Jing Kang, Bing Han, Bai-Shi Hu *, Feng-Quan Liu *

*Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, Key Laboratory of Monitoring and Management of Crop Diseases and Pest Insects, Ministry of Agriculture, Nanjing 210095, China*

ARTICLE INFO

*Article history:*
Received 8 October 2008
Accepted 19 May 2009
Available online 29 May 2009

*Keywords:*
*Lysobacter enzymogenes*
Acyl homoserine lactone
AiiA
*Pectobacterium carotovorum*

ABSTRACT

An *N*-acyl homoserine lactonase gene *aiiA*, transcribed by a strong and constitutive *Escherichia coli* promoter $P_{lpp}$ (Accession No. EU723847), was transformed into *Lysobacter enzymogenes* strain OH11, creating strain OH11A. The *N*-acyl-homoserine lactone (AHL)-degradation assay showed that transformant OH11A acquired the ability to degrade AHL molecules produced by *Agrobacterium tumefaciens*, *Pectobacterium carotovorum, Pseudomonas syringae* pv. *tomato* strain DC3000 and *Acidovorax avenae* subsp. *citrulli*. Pathogenicity tests showed that while the parental strain OH11 did not reduce *P. carotovorum* infection, the transformant OH11A caused a strong reduction of *Pectobacterium* virulence on Chinese cabbage and cactus, whereas strain OH11A did not seem to interfere with the normal growth of this pathogen in cabbages. In antimicrobial activity assays, strain OH11A and OH11 showed similar antimicrobial activity against *Phytophthora capsici* and *Sclerotinia sclerotiorum*. This work provided a new strategy for developing genetically engineered multi-functional *L. enzymogenes* strains that possessed the ability to biologically control fungal pathogens and reduce bacterial pathogenicity.

© 2009 Published by Elsevier Inc.

## 1. Introduction

The genus *Lysobacter* is characterized with a high G + C content and gliding motility. Members of this genus are typically found in soil and water (Christensen and Cook, 1978; Folman et al., 2003). *Lysobacter enzymogenes* (Christensen and Cook, 1978) has been shown to produce extracellular lytic enzymes, and is described as a potential biological control agent for fungal plant diseases (Zhang and Yuen, 1999; Yuen and Zhang, 2001; Folman et al., 2003).

Recently, four different *Lysobacter enzymogenes* strains were characterized as biological control agents for fungal or oomycetous plant diseases (Kobayashi and Barrad, 1996; Folman et al., 2003; Sullivan et al., 2003; Jiang et al., 2005). Experiment evidences showed that strain 3.1T8 and strain N4-7 are effective in biocontrol of *Pythium* root and crown rot caused by *P. aphanidermatum* and summer patch disease of turfgrass caused by *Magnaporthe poae*, respectively (Kobayashi and Barrad, 1996; Folman et al., 2003). Strain C3 (previously identified as *Stenotrophomonas maltophilia*) was field-effective in reducing plant diseases such as Bipolaris leaf spot (caused by *Bipolaris sorokiniana*) and brown patch (caused by *Rhizoctonia solani*) in turfgrass (Giesler and Yuen, 1998; Zhang and Yuen, 1999; Yuen and Zhang, 2001). Strain OH11, isolated from cayenne-root soil, was reported for the first time as a biological control agent in China (Qian et al., 2009). It displayed *in vitro* activity against fungal and oomycetous plant pathogens, including *Phytophthora capsici*, *Pythium ultimum*, *R. solani*, *Sclerotinia sclerotiorum* and *Fusarum solani* (Jiang et al., 2005). A chitinase gene and an α-lytic protease gene were cloned from strain OH11 and expressed in *Escherichia coli*. The resultant strains displayed strong *in vitro* activity against *R. solani* (Zhu et al., 2007, 2008). However, at the time of this writing, no reports on controlling bacterial plant diseases by *L. enzymogenes* were documented.

Quorum sensing (QS) is a type of decision-making process used by decentralized groups to coordinate behavior. It has been well established that many species of bacteria use QS to coordinate their gene expression according to the local density of their population (Fuqua and Greenberg, 1998; Fuqua et al., 2001; Zheng et al., 2006). Many gram-negative bacteria use autoinducer *N*-acyl homoserine lactones (AHLs) to regulate diverse microbial biological functions, including biofilm formation in *Pseudomonas fluorescens* and *P. aeruginosa* (Allison et al., 1998; Davies et al., 1998), antibiotic production in *P. aureofaciens* and *Pectobacterium carotovorum* (Costa and Loper, 1997; Pirhonen et al., 1993), and virulence factor expression in *P. carotovorum* and *P. aeruginosa* (Jones et al., 1993).

AiiA, an enzyme that inactivates the AHL quorum-sensing signal, was used for controlling bacterial plant diseases (Dong et al., 2001; Zhu et al., 2006). The first disease control application of AiiA protein was introduction of the *aiiA* gene cloned from *Bacillus* sp.

* Corresponding authors. Fax: +86 25 84395325.
*E-mail addresses:* hbs@njau.edu.cn (B.-S. Hu), fqliu20011@sina.com (F.-Q. Liu).
[1] The first three authors contributed equally to this research.

1049-9644/$ - see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.biocontrol.2009.05.007

into transgenic tobacco and potato plants (Dong et al., 2001). Expression of the *aiiA* gene and production of AHL-lactonase by genetically modified plants and biocontrol agents paralyzed the QS system of phytopathogenic bacteria, resulting in attenuation of the bacterial pathogens' virulence on hosts and increasing of the plants' resistance (Dong et al., 2000, 2001).

Bacterial soft rot of Chinese cabbages caused by *P. carotovorum* is a serious plant disease in China, and responsible for considerable losses in the crop of this plant (Bainton et al., 1992; Thomson et al., 1999). Several research works proved that the QS system in *P. carotovorum* regulates extroenzyme (described as the main determinants of virulence in *P. carotovorum*) production and is crucial to its virulence on hosts (Bainton et al., 1992; Barras et al., 1994; Thomson et al., 1999). Therefore, the main objective of this work was to control this serious plant disease using an engineered *L. enzymogenes* strain with AHL-lactonase activity, ultimately providing a novel pathosystem between *L. enzymogenes* and *P. carotovorum*. In the present study, we reported that: (i) an engineered *L. enzymogenes* strain (strain OH11A) with AHL-lactonase activity was constructed by expression of the *aiiA* gene under a strong and constitutive *E. coli* promoter $P_{lpp}$ in strain OH11; (ii) the ability of strain OH11A to degrade AHL molecules was measured; (iii) the control efficiency of strain OH11A for soft rot disease of Chinese cabbages was demonstrated.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study were described in Table 1. *Lysobacter enzymogenes*, *Agrobacterium tumefaciens*, *Pectobacterium carotovorum* strain 12, *Pseudomonas syringae* pv. *tomato* strain DC3000 and *Acidovorax avenae* subsp. citrulli strain NJL10 were routinely grown on Luria-Bertani (LB) agar at 28 °C. *Escherichia coli* JM109, DH5α and SM10λpir were grown on LB agar at 37 °C. *Phytophthora capsici* and *Sclerotinia sclerotiorum* were grown on potato dextrose agar (PDA) plates at 25 °C. When required, antibiotics were used at the following concentrations: tetracycline (Te), 12.5 μg/mL for *E. coli*/pME6863, 2 μg/mL for *A. tumefaciens* R10 and 1 μg/mL for *A. tumefaciens* JZA1; ampicillin (Amp), 100 μg/mL; gentamicin (Gm), 15 μg/mL for *E. coli*/pBBR1-MCS5, 100 μg/mL for *A. tumefaciens* JZA1 and 200 μg/mL for *L. enzymogenes* transformants; spectinomycin (Spc), 100 μg/mL chloramphenicol (Cm), 100 μg/mL.

### 2.2. DNA isolation and manipulation

Plasmid DNA was prepared from strains of *E. coli* DH5α by an alkaline phenol extraction method (Sambrook et al., 1989). Chromosomal DNA of *L. enzymogenes* strain OH11 was prepared using a TaKaRa MiniBest Bacteria Genomic DNA Extraction Kit (code: D4810A). Restriction enzymes and DNA modification enzymes were purchased from TaKaRa Biotechnology Co. Ltd. (DaLian, China), and were used according to the manufacturers' protocol.

### 2.3. Construction of plasmid pBRA13 and transformation

The promoter $P_{lpp}$ and the *aiiA* gene were spliced by overlap extension (Ho et al., 1989). Briefly, Primer P1 (5′-GGGCTGCAGCGATAACCAGAAGCAATAAAAAATCAAATCGGATTTC-3′, PstI site (underlined)) and Primer P2 (5′-GGACGAAATAAAGCTTCTTTACTGTCATTATTAATACCCTCTAGATTGAGTTAATCTCC-3′) were used to clone promoter $P_{lpp}$ from *E. coli* strain JM109. Primer P3 (5′-GGAGATTAACTCAATCTAGAGGGTATTAATAATGACAGTAAAGAAGCTTTATTTCGTCC-3′) and Primer P4 (5′-GGGGAATTCCTATATATATTCAGGGAACACTTTACATCCCCTTTCCTGCTCTATATCATG-3′, EcoRI site (underlined)) were used to clone the *aiiA* gene from plasmid pME6863. The two PCR fragments of the promoter $P_{lpp}$ and the *aiiA* gene were spliced by overlap extension with primers P1 and P4. The spliced fragment termed $P_{lpp}$-*aiiA* was purified and digested with EcoRI/PstI, then cloned into the corresponding sites of broad-host-vector pBBR1-MCS5. The resulting construct, termed pBBRA, was mated into strain OH11 using *E. coli* SM10λpir. The transformant was designated strain OH11A. In addition, strain OH11P was obtained by

**Table 1**
Bacterial, fungal strains and plasmids used in the study.

| Strains or plasmids | Relevant characteristic[a] | Reference |
|---|---|---|
| *Escherichia coli* | | |
| JM109 | *recA, supE, hsR,* Δ(*lac-pro*) | TaKaRa |
| DH5a | *supE44lacU169*(Δ*lacZ*ΔM15)*hsdR17 recA lendA1gyrA96 thi-1 relA11* | TaKaRa |
| SM10λpir | *thi thr tonA lacY supE recA*::RP4-2-Te::Mu Km$^r$ ; host for p-requiring plasmids; cogjugal donor | Miller and Mekalanos (1998) |
| *Lysobacter enzymogenes* | | |
| OH11 | Wild-type, Sm$^r$, Cm$^s$, Km$^r$, Gm$^s$ | Lab collection |
| OH11A | Derivative of OH11 harboring a plasmid pBBRA | This work |
| OH11P | Derivative of OH11 harboring a plasmid pBBR1-MCS5 | This work |
| OH11-3 | A *hmgA*-deletion mutant of strain OH11 | Lab collection |
| *Agrobacterium tumefaciens* | | |
| JZA1 (pJZ372) (pJZ384) (pJZ410) | AHL biosensor strain | Zhu et al., 2003 |
| R10 (pCF218) | TraR overexpressed in *A. tumefaciens* | Fuqua and Winans, 1994 |
| *Pectobacterium carotovorum* strain 12 | Soft rot pathogen of Chinese cabbages, AHL producer; Cm$^r$, Km$^s$ | "Song et al., 2002" |
| *Acidovorax avenae* subsp. *citrulli* NJL10 | The pathogen of bacterial fruit blotch of watermelons, AHL producer; Gm$^s$, Km$^s$ | Lab collection |
| *Pseudomonas syringae* pv. *tomato* strain DC3000 | The pathogen of *Arbidopsis thaliana*, AHL producer | Lab collection |
| *Oomycetes* | | |
| *Phytophthora capsici* | The pathogen of Phytophthora blight of pepper | Lab collection |
| Fungi | | |
| *Sclerotinia sclerotiorum* | The pathogen of rape sclerotinia stem rot | Lab collection |
| *Plasmids* | | |
| pBBR1-MCS5 | Broad-host-range vector with a $P_{lac}$ promoter | Kovach et al., 1995 |
| pBBRA | $P_{lpp}$–*aiiA* in pBBR1-MCS5 | This work |
| pME6863 | pME6000 carrying the *aiiA* gene from *Bacillus* sp. A24 under the $P_{lac}$ promoter | Molina et al., 2003 |

[a] Sm$^r$, Km$^r$, Cm$^r$, Gm$^r$ = streptomycin-, kanamycin-, chloramphenicol-, and gentamicin-resistant, respectively.

mating broad-host-vector pBBR1-MCS5 into strain OH11 using *E. coli* SM10λpir.

### 2.4. RNA extraction and reverse transcription (RT)-PCR assay for the *aiiA* gene transcript

Primers aiiA-F: (5′-ATGACAGTAAAGAAGCTTTAT-3′) and aiiA-R: (5′-CTATATATATTCAGGGAACAC-3′) were designed to amplify a 753-bp fragment for evaluating transcriptional expression of the *aiiA* gene in *L. enzymogenes* strain OH11A.

Total RNAs of *L. enzymogenes* strains were isolated using an RNA extraction kit (TaKaRa) according to the manufacturer's instructions and subsequently treated with RNase-free DNase (TaKaRa). Fifty nanograms of RNA were used as a template for RT-PCRs, which were performed using a SuperScript one-step RT-PCR kit (Invitrogen). The initial cDNA synthesis was conducted at 42 °C for 90 min and was followed by a predenaturation step of 94 °C for 5 min, a 94 °C melting step for 30 s, a 55 °C annealing step for 30 s, and extension at 72 °C for 45 s. This was repeated for 35 cycles before a final extension of 10 min at 72 °C.

### 2.5. AHL-degradation bioassay

An AHL preparation of each *A. tumefaciens* R10 (pCF218), *P. carotovorum*, *P. syringae* pv. *tomato* strain DC3000 and *A. avenae* subsp. *citrulli* was conducted following a procedure described previously (Zheng et al., 2006). In brief, each strain was cultivated in 100 mL LB at 28 °C and stopped when its $OD_{600}$ reached 0.4, then 200 mL product was collected by centrifugation at 12,000*g* for 5 min. The supernatant containing AHLs was extracted and concentrated 200-fold by ethyl acetate. After that, 20 μL of the culture ($10^7$ colony forming units (CFU)/mL) of strain OH11A was mixed with 180 μL of LB–AHL medium (LB broth containing 10% (w/v) of each concentrated AHLs). The mixtures were incubated at 28 °C for 9 h. The following steps of AHL-degradation bioassay were performed according to the "Bioassay of AI activity" as described previously (Zhu et al., 2003). Briefly, 20 μL of the supernatant from the mixtures of strain OH11A and each concentrated AHLs was added into AT medium (Fuqua and Winans, 1994) and approximately $10^7$ CFU/mL AHL-bioassay strain JZA1 together with 5-bromo-4-chloro-3-indolyl β-galactoside (X-gal) (100 μg/ml) (Zhu et al., 2003) were added. These cultures were incubated with aeration for 12 h and assayed for β-galactosidase specific activity (Miller, 1972) (AHL-like molecules were capable of induction of the bioassay reporter strains' β-galactosidase to hydrolyze X-gal, resulted in a blue color). Here, the supernatant of strain OH11 was used to detect AHL production from *L. enzymogenes* itself.

### 2.6. In vitro P. carotovorum pathogenicity test and bacteria recovery

The *in vitro P. carotovorum* pathogenicity test was performed as described previously (Zhu et al., 2006), with some modification. In brief, fresh Chinese cabbages were used for *in vitro P. carotovorum* pathogenicity tests. The surfaces of cabbages were washed with sterilized double distilled water ($ddH_2O$), sterilized with 70% ethanol. After that, the Chinese cabbages were sliced into 10 × 4 cm pieces. The *L. enzymogenes* and *P. carotovorum* were grown in LB broth for 48 h, then adjusted to a similar concentration ($\approx 1 \times 10^7$ CFU/mL). Before inoculation, the surfaces of Chinese cabbages were once penetrated downward 2 mm deep, then, 50 μL of the culture of each *L. enzymogens* strain (OH11, OH11P or OH11A) was mixed with equal volume of the culture of *P. carotovorum*, and the mixture was incubated in the incubator at 28 °C for 4 h (Zhu et al., 2006). In the following step, 5 μL of the mixtures was inoculated on the generated wounds of Chinese cabbages. Here, the LB broth was used as the negative control. All inoculated Chinese cabbage slices were placed in the box possessing sterilized humid filter paper, and incubated at 28 °C for 12 h. After incubation, the maceration areas of Chinese cabbages were used for assessing disease severity. For this, the lengths of the long axis and the short axis were averaged, the radius was determined, and the formula: area = $\pi \times (\text{radius})^2$ was used (Hu et al., 2005). In addition, the bacterial populations of each *L. enzymogenes* strain and *P. carotovorum* were recovered from rotted tissues by the dilution-plate method (Molina et al., 2003), with some modification. Briefly, about 0.2 g rotted tissues of Chinese cabbages were put into a 2 mL plastic tube, triturated by a sterilized chopstick, and suspended by 0.5 mL sterilized $ddH_2O$. After that, the prepared samples were diluted in series using sterilized $ddH_2O$. In the following steps, 100 μL of each diluted sample was spread on LB agar supplemented with Cm and Km, respectively, and incubated at 28 °C for 2 days for the growth of the *P. carotovorum* (Cm-resistant and Km-sensitive) and *L. enzymogenes* strains (Cm-sensitive and Km-resistant). Finally, the cell numbers (CFU/g) of *P. carotovorum* and *L. enzymogenes* in the rotted tissues of Chinese cabbages were determined by calculating the number of colonies on the corresponding plate together with its dilution ratio. In this study, three independent *in vitro* pathogenicity tests were conducted, and each treatment involved three replicates. Furthermore, the data from multiple experiments was analyzed separately by using Microsoft Excel software.

### 2.7. In vivo P. carotovorum pathogenicity test

The *in vivo P. carotovorum* pathogenicity test was carried out as described previously (Zhu et al., 2006), with some modification. In brief, pieces of cactus stems were purchased from Nanjing Institute of Vegetables. The cut surface of each stem was washed with 70% ethanol. The stems were put in the sun for 1 h until the ethanol was completely volatilized. Then each stem was grown in a flowerpot piled with dry soil. The pathogenicity test was performed after the stems started their radiation. In brief, the *L. enzymogens*, *P. carotovorum* strains were grown in LB broth for 48 h, then adjusted into the similar concentration ($\approx 1 \times 10^7$ CFU/mL). After that, 100 μL of the *P. carotovorum* culture was mixed with equal volume of the *L. enzymogenes* culture. The mixtures were incubated at 28 °C for 4 h (Zhu et al., 2006). Before inoculation, each stem was washed with 70% ethanol and penetrated downwards 5 mm inside after ethanol volatilization. Then 15 μL of the mixture was inoculated into the cut of the stem. Pictures were taken from the maceration areas after 14-day cultivation. In this study, three independent *in vivo* pathogenicity tests were conducted, and each treatment involved three replicates. The data analysis was performed by using Microsoft Excel software.

### 2.8. Antimicrobial activity assays

According to the method described by Kobayashi et al., 2005, the *in vitro* antimicrobial activities of each *L. enzymogenes* strain (OH11, OH11P or OH11A) were determined by co-culturing bacteria strains with the oomycete *P. capsici* and fungus *S. sclerotiorum* on agar plates. The *L. enzymogenes* strain (OH11, OH11P or OH11A) was grown in LB broth for 20 h, then adjusted to a concentrations ($\approx 1 \times 10^7$ CFU/mL) using spectrophotometry. Then five microliters of the culture of each *L. enzymogens* strain was spot inoculated on the periphery of PDA plates (four bacterial spots per plate), and a mock inoculated plate was prepared as a control. The center of each plate was inoculated with a mycelial plug containing either *P. capsici* or *S. sclerotiorum* (three plates per pathogen). After the plates were incubated for 2–5 days at 25 °C, the radii of growth inhibition zones around bacterial colonies were measured. The experimental data was analyzed by using Microsoft

Excel software. Three independent antimicrobial activity assays were repeated.

## 3. Results

### 3.1. RT-PCR analysis

In order to determine whether the *aiiA* gene could be expressed under the strong promoter $P_{lpp}$, the RT-PCR analyses were conducted by primers aiiA-F and aiiA-R to amplify the 753-bp fragment. As shown in Fig. 1, total RNA was successfully extracted from *L. enzymogenes* strains (Fig. 1A), and amplified RT-PCR products of expected sizes (753-bp) were obtained when RNA was extracted from strain OH11A (Fig. 1B), indicating that the *aiiA* gene was successfully expressed in strain OH11A. In contrast, no RT-PCR product was observed when the same primers were used with the RNA extracted from strain OH11 or OH11P.

### 3.2. Transformant OH11A degrades AHLs

To determine whether the transformant OH11A could degrade the AHLs, an AHL-degradation bioassay was performed. According to the intensity of blue color in each well, it was concluded that strain OH11 and OH11P demonstrated no ability to degrade AHLs (Fig. 2A), and the transformant OH11A showed a stronger degradation of AHLs than strain OH11 (Fig. 2A). We also performed liquid AHL bioassays to compare the AHL activity in the supernatant of the mixtures of AHLA (AHLs, produced by *A. tumefaciens* R10) and *L. enzymogens* strains. Similarly, few AHL molecules were detected in the supernatant of the mixtures of AHLA and strain OH11A (β-galactosidase activity (Miller unite): 128.7 ± 25.5) (Fig. 2B). Meanwhile, AHL activity in the supernatant of the mixture of AHLA and strain OH11 (β-galactosidase activity (Miller unite): 1565.1 ± 52.1) or OH11P (β-galactosidase activity (Miller unite): 1521.1 ± 28.8) was as strong as the case of the mixture of AHLA and LB medium (β-galactosidase activity (Miller unite): 1609.1 ± 15.7). To determine whether strain OH11A could degrade AHL-like molecules produced by different plant-pathogenic bacteria, the AHL-producing bacteria, *P. carotovorum*, *A. avenae* subsp. *citrulli* and *P. syringae* pv. *tomato* strain DC3000 were chosen for further AHL-degradation assay. As shown in Table 2, strain OH11A showed a stronger ability to degrade AHLs produced by *P. carotovorum*, *A. avenae* subsp. *citrulli* and *P. syringae* pv. *tomato* strain DC3000 than strain OH11 or OH11P. Moreover, strain OH11 was not detected to produce AHLs by the AHL-bioassay strain JZA1 (Fig. 2).

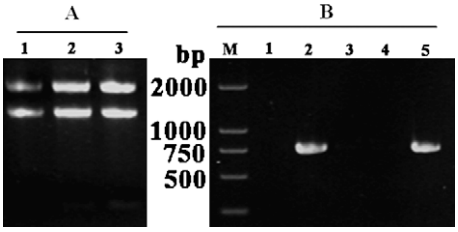


**Fig. 1.** RNA extraction and Reverse-transcription (RT)-PCR for the *aiiA* gene transcription. (A) RNA extraction. (1) *Lysobacter enzymogenes* strain OH11; (2) *L. enzymogenes* strain OH11P; (3) *L. enzymogenes* strain OH11A; (B) RT-PCR for the *aiiA* the gene transcription in strain OH11A. (1) $ddH_2O$, a negative control; (2) pME6863, a positive control; (3) strain OH11; (4) strain OH11P; 5, strain OH11A.

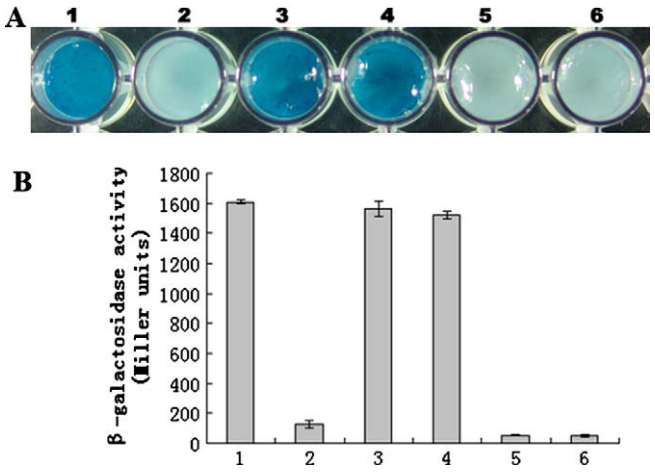


**Fig. 2.** AHL-degradation activity of the transformant OH11A. (A and B) AHL like activity in the supernatant. (1) AHLA; (2) AT medium; (3) strain OH11 + AHLA; (4) strain OH11P + AHLA; (5) strain OH11A + AHLA; (6) strain OH11. AHLA, the AHLs produced by *Agrobacterium tumefaciens* R10 (pCF218), which produces 3-O-$C_6$-HSL and 3-O-$C_8$-HSL, was used as a positive control.

### 3.3. Transformant OH11A reduced P. carotovorum infection on Chinese cabbages

The *in vitro* pathogenicity test showed that OH11A attenuated the virulence of *P. carotovorum* on Chinese cabbage. As shown in Fig. 3, the sections of Chinese cabbage, which were inoculated with *P. carotovorum* turned macerated, and rotten (maceration area: 1.2 ± 0.02 $cm^2$). The sections inoculated with *P. carotovorum* and strain OH11 or OH11P were as rotten as that inoculated with *P. carotovorum* (maceration area co-inoculation both *P. carotovorum* with OH11 or OH11P was 1.15 ± 0.06 and 1.16 ± 0.04 $cm^2$, respectively), showing that strain OH11 and OH11P could not reduce the soft rot disease of Chinese cabbages. Little maceration (maceration area: 0.05 ± 0.02 $cm^2$) of sections co-inoculated both *P. carotovorum* and transformant OH11A were observed, showing

**Table 2**
AHL-degradation activity of transformant OH11A for phytopathogenic bacteria (three replication, with S.D.).

| Experimentation | Treatment | β-galactosidase specific activity (Miller unite) |
|---|---|---|
| AHLE[2]-degradation assay | AHLA[1] (Positive control) | 1384.3 ± 47.1 |
| | AT medium (Negative control) | 21.1 ± 0.1 |
| | AHLE | 1279.6 ± 38.0a[6] |
| | AHLE + strain OH11 | 1299.1 ± 59.3a |
| | AHLE + strain OH11P[5] | 1212.2 ± 66.4a |
| | AHLE + strain OH11A | 279.6 ± 66.0b |
| AHLC[3]-degradation assay | AHLA (Positive control) | 1296.6 ± 51.6 |
| | AT medium (Negative control) | 32.5 ± 1.3 |
| | AHLC | 1075.2 ± 38.9a |
| | AHLC + strain OH11 | 979.7 ± 68.9a |
| | AHLC + strain OH11P | 1042.1 ± 46.9a |
| | AHLC + strain OH11A | 217.7 ± 36.2b |
| AHLP[4]-degradation assay | AHLA (Positive control) | 1347 ± 36.4 |
| | AT medium (Negative control) | 29.5 ± 2.3 |
| | AHLP | 1434.4 ± 38.2a |
| | AHLP + strain OH11 | 1418.4 ± 63.1a |
| | AHLP + strain OH11P | 1385.7 ± 46.8a |
| | AHLP + strain OH11A | 256.7 ± 42.1b |

[1] AHLA, the AHLs produced by *Agrobacterium tumefaciens* R10 (Fuqua and Winans, 1994), was used a positive control.
[2] AHLE, the AHLs produced by *Pectobacterium carotovorum* strain 12.
[3] AHLC, the AHLs produced by *Acidovorax avenae* subsp. *citrulli* NJL10.
[4] AHLP, the AHLs produced by *Pseudomonas syringae.* pv. *tomato* strain DC3000.
[5] Strain OH11P, derived from strain OH11 carrying the plasmid pBBR1-MCS5.
[6] Fisher's least significant difference (LSD) test among the strain OH11, strain OH11P and strain OH11A within a column ($p = 0.05$).

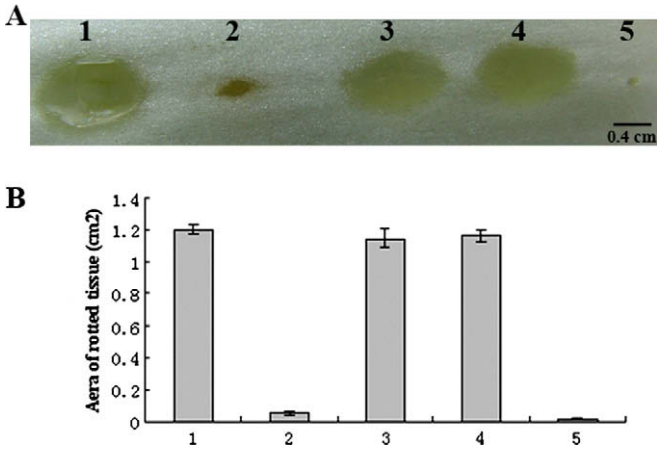


**Fig. 3.** Biocontrol of bacterial soft rot of Chinese cabbages caused by *Pectobacterium carotovorum*. (A) Visual observation of soft-rot symptoms on Chinese cabbages; (B) Data showed that the maceration areas on Chinese cabbages. (1) *P. carotovorum*; (2) *P. carotovorum* + strain OH11A; (3) *P. carotovorum* + strain OH11; (4) *P. carotovorum* + strain OH11P; (5) LB broth, a negative control.

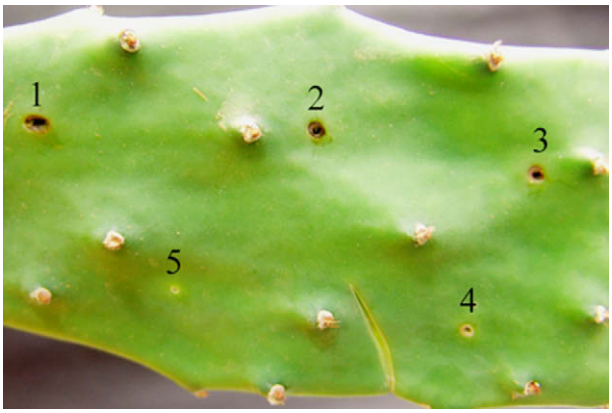


**Fig. 5.** Effects of strain OH11A on symptom's color and area caused by *Pectobacterium carotovorum* on the epidermis of cactus. (1) *P. carotovorum*; (2) *P. carotovorum* + OH11; (3) *P. carotovorum* + OH11P; (4) *P. carotovorum* + OH11A; (5) LB, a negative control.

that strain OH11A reduced the *P. carotovorum* virulence to Chinese cabbages. However, pathogen (*E. catorovora*) growth inhibition by strain OH11A was not observed in the Chinese cabbage slices (Fig. 4).

### 3.4. Strain OH11A attenuated P. carotovorum virulence on cactus

To assess whether strain OH11A was able to control *Pectobacterium* infection on live plants, the cactus, a plant that is susceptible to infection by *P. carotovorum* and bears good tolerance to other bacterial pathogenicity, was chosen as a subject of a study on the reduction of strain OH11A to *P. carotovorum* virulence. As shown in Fig. 5, the sections were inoculated with *P. carotovorum* turned macerated and brown. Similarly, the sections inoculated with *P. carotovorum* and OH11 or OH11P were as macerated and brown as that inoculated with *P. carotovorum* alone, indicating that wild type strain OH11 was unable to control the soft rot disease of cactus. In contrast, no maceration of the sections inoculated with *P. carotovorum* and OH11A were observed, showing that strain OH11A reduced the *P. carotovorum* virulence on cacti.

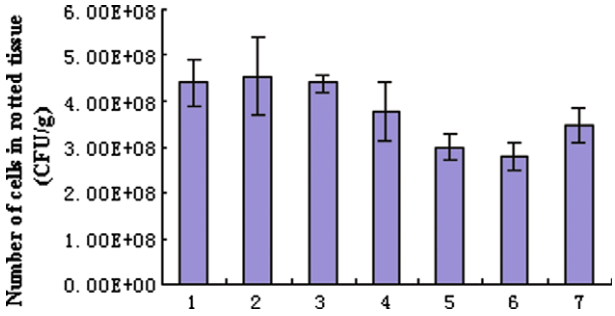


**Fig. 4.** Effect of AHL-degrading strain OH11A on cell numbers of *P. carotovorum* in Chinese cabbages. The Chinese cabbage slices were inoculated with *P. carotovorum* alone or co-inoculated with strain OH11, strain OH11P or strain OH11A. Cell numbers of the pathogen inoculated alone (1) co-inoculated with strain OH11 (2) with strain OH11P (3) or with strain OH11A (4) were determined. Meanwhile, the numbers of the strain OH11 (5) strain OH11P (6) or strain OH11A (7) was determined in the presence of the pathogen. Values represent the mean of three trials with three treatment replications per trial. Bars indicate standard deviation of the mean.

### 3.5. Antimicrobial activity assays

The *L. enzymogenes* strains (strain OH11, OH11P and OH11A) were selected to test antimicrobial activity against plant pathogens *P. capsici* and *S. sclerotiorum*. The antimicrobial activity was determined based on inhibition zones on PDA plates. It was observed that strain OH11 displayed a strong antimicrobial activity against *P. capsici* (inhibition zones (radius): 13.5 ± 2.6 mm) and *S. sclerotiorum* (inhibition zones (radius): 15.1 ± 1.6 mm). Compared to strain OH11, both strains OH11P and OH11A exhibited similar antimicrobial activity against *P. capsici* (inhibition zones (radius): 13.1 ± 2.2 and 13.3 ± 1.9 mm displayed by strains OH11P and OH11A, respectively) and *S. sclerotiorum* (inhibition zones (radius): 14.9 ± 1.4 and 15.3 ± 1.8 mm displayed by strain OH11P and OH11A, respectively).

## 4. Discussion

We reported for the first time that construction of a genetically-engineered *L. enzymogenes* strain with AHL-degrading capacity to biological control of bacterial soft rot of Chinese cabbages *in vitro* and cactus *in vivo*. In this work, the *aiiA* gene was successfully expressed under the promoter $P_{lpp}$ in strain OH11A. Compared to well studied *E. coli* promoters $P_{lac}$, $P_{tac}$ and T7 (the activity of these promoters should be induced by isopropyl-beta-D-thiogalactopyranoside (IPTG)), promoter $P_{lpp}$ (*lpp* is the gene encoding the major lipoprotein of *E. coli*) was described as a strong and constitutive promoter (Inouye and Inouye, 1985). Therefore, expression of the *aiiA* gene under promoter $P_{lpp}$ was unnecessary for IPTG induction, which facilitated the further large-scale fermentation for strain OH11A. To further confirm whether promoter $P_{lpp}$ could be applied for complementation of functional genes in strain OH11, a *hmgA*-deletion mutant (termed strain OH11-3), which could produce dark brown pigment in LB medium (wild type strain OH11 had not such a trait), was complemented by expression of the *hmgA* gene (Accession No. EU717786) under the promoter $P_{lpp}$ in strain OH11-3 (Fig. 6 in Supplementary file, these results revealed that the promoter $P_{lpp}$ could be used not only for expression of heterogenous genes, but also for the complementation of functional genes in strain OH11.

It has been well established that AiiA proteins in plants or bacteria can efficiently degrade AHLs and consequently enhance the resistance to *P. carotovorum* infection on hosts (Dong et al., 2001; Lee et al., 2002). However, here we have demonstrated for the first

time that an engineered *L. enzymogenes* strain OH11A with AHL-lactonase activity can be used for control of bacterial plant diseases. Two biocontrol agents (*Bacillus thuringiensis* and *P. fluorescens* P3/pME6863) are well studied on the effects of AHL interference on *P. carotovorum* virulence (Molina et al., 2003; Dong et al., 2004; Zhu et al., 2006). Therefore, here this pathogen was selected as a model to evaluate the effects of strain OH11A on the pathogen's virulence and growth. Our results showed that strain OH11A, with AHL-lactonase, effectively quenched the AHL signalling by *P. carotovorum* and stopped the spread of soft rot symptoms in Chinese cabbages tissues, whereas strain OH11A did not seem to interfere with the normal growth of this pathogen. These results were in accordance with the reports on *B. thuringiensis* and *P. fluorescens* P3/pME6863 described by Dong et al. (2004) and Molina et al. (2003), respectively.

In an antimicrobial activity assay, strain OH11 and OH11A showed similar antimicrobial activity against plant pathogen *P. capsici* and *S. sclerotiorum*. This result indicates that introduction of the *aiiA* gene expands the biocontrol spectrum against bacterial plant pathogens, but does not affect the antimicrobial activity of strain OH11 against fungal plant pathogens. This work reported the great potential of using AHL lactonase to construct bifunctional *L. enzymogenes* recombinant strains that possessed biocontrol activity against fungal plant pathogens and reduced bacterial pathogenicity. Work to integrate $P_{lpp}$-*aiiA* gene into strain OH11 chromosome will be performed in order to obtain a much more stable biocontrol agent for control of bacterial plant diseases.

## Acknowledgments

We thank Dr. Jun Zhu (Nanjing Agricultural University) for providing the AHL-bioassay strain JZA1 and *A. tumefaciens* R10. We also thank Dr. Yu Chen (Nanjing Agricultural University) and Puja Chebrolu (University of Georgia) for critical revisions of this manuscript. This work was supported by the National High Technology Research and Development Program of China (No. 2006AA10A211).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocontrol.2009.05.007.

## References

Allison, D.G., Ruiz, B., SanJose, C., Jaspe, A., Gilert, P., 1998. Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. FEMS Microbiology Letters 167, 179–184.

Bainton, N.J., Stead, P., Chhabra, S.R., Bycroft, B.W., Salmond, G.P.C., Stewart, G.S.A.B., Williams, P., 1992. *N*-(3-Oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochemical Journal 288, 997–1004.

Barras, F., van Gijsegem, F., Chatterjee, A.K., 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annual Review Phytopathology 32, 201–234.

Christensen, P., Cook, FD., 1978. *Lysobacter*, a new genus of nonfruiting, gliding bacteria with high base ratio. International Journal of Systematic and Evolutionary Microbiology 28, 367–393.

Costa, J.M., Loper, J.E., 1997. EcbI and EcbR: homologs of LuxI and LuxR affecting antibiotic and exoenzyme production by *Erwinia carotovora* subsp. *Betavasculorum*. Canadian Journal of Microbiology 43, 1164–1171.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., Greenberg, E.P., 1998. The involvement of cell-to-cell signals in the development of a bacteria biofilm. Science 280, 295–298.

Dong, Y.H., Wang, L.H., Xu, J.L., Zhang, H.B., Zhang, X.F., Zhang, L.H., 2001. Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature 411, 813–817.

Dong, Y.H., Xu, J.L., Li, X.Z., Zhang, L.H., 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proceedings of the National Academy of Sciences 7, 3526–3531.

Dong, Y.H., Zhang, X.F., Xu, J.L., Zhang, L.H., 2004. Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. Applied and Environmental Microbiology 70 (2), 954–960.

Folman, L.B., Postma, J., Van-Veen, J.A., 2003. Characterisation of *Lysobacter enzymogenes* (Christensen and Cook 1978) strain 3.1T8, a powerful antagonist of fungal diseases of cucumber. Microbiology Research 158, 1–9.

Fuqua, C., Greenberg, E.P., 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. Current Opinion in Microbiology 1, 183–189.

Fuqua, C., Parsek, M.R., Greenberg, E.P., 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annual Review of Genetics 35, 439–468.

Fuqua, W.C., Winans, S.C., 1994. A LuxR–LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. Journal of Bacteriology 176, 2796–2806.

Giesler, L.J., Yuen, G.Y., 1998. Evaluation of *Stenotrophomonas maltophilia* strain C3 for biocontrol of brown patch disease. Crop Protection 17, 509–513.

Ho, S.N., Hunt, H.D., Horton, R.M., Horton, J., Pullen, K., Pease, L.R., 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77, 51–59.

Hu, X.J., Roberts, D.P., Jiang, M.L., Zhang, Y.B., 2005. Decreased incidence of disease caused by *Sclerotinia sclerotiorum* and improved plant vigor of oilseed rape with *Bacillus subtilis* Tu-100. Applied Microbiology and Biotechnology 68, 802–807.

Inouye, S., Inouye, M., 1985. Up-promoter mutations in the *lpp* gene of *Escherichia coli*. Nucleic Acids Research 13, 3101–3110.

Jiang, Y.H., Hu, B.S., Liu, F.Q., 2005. Selection and identification of antagonistic bacteria against soil-borne plant pathogens. Chinese Journal of Biological Control 21, 260–264.

Jones, S., Yu, B., Bainton, N.J., Birdsall, M., Bycroft, B.W., Chhabra, S.R., Cox, A.J., Golby, P., Reeves, P.J., Stephens, S., Winson, M.K., Salmond, G.P.C., Stewart, G.S.A.B., Williams, P., 1993. The Lux autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. The EMBO Journal 12, 2477–2482.

Kobayashi, D.Y., Barrad, N.E.L., 1996. Selection of bacterial antagonists using enrichment cultures for the control of summer patch disease of Kentucky bluegrass. Current Microbiology 32, 106–110.

Kobayashi, D.Y., Reedy, R.M., Palumbo, J.D., Zhou, J.M., Yuen, G.Y., 2005. A *clp* gene homologue belonging to the Crp gene family globally regulates lytic enzyme production, antimicrobial activity, and biological control activity expressed by *Lysobacter enzymogenes* strain C3. Applied and Environmental Microbiology 71, 261–269.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop 2nd, R.M., Peterson, K.M., 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175–176.

Lee, S.J., Park, S.Y., Lee, J.J., Yum, D.Y., Koo, B.T., Lee, J.K., 2002. Genes encoding the *N*-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. Applied and Environmental Microbiology 68, 3919–3924.

Miller, J.H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Miller, V.L., Mekalanos, J.J., 1998. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. Journal of Bacteriology 170, 2575–2583.

Molina, L., Constantinescu, F., Michel, L., Reimmann, C., Duffy, B., Défago, G., 2003. Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. FEMS Microbiology Ecology 45, 71–81.

Pirhonen, M., Flego, D., Heikinheimo, R., Palva, E.A., 1993. Small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. The EMBO Journal 12, 2467–2476.

Qian, G.L., Hu, B.S., Jiang, Y.H., Liu, F.Q., 2009. Identification and Characterization of *Lysobacter enzymogenes* as a biological control agent against some fungal pathogens. Agricultural Sciences in China 8 (1), 68–75.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Song, C.F., Pan, X.M., Lu, P., Wang, J.S., 2002. Multiplication and extension of two motility mutants of *Erwinia carotovora* subsp. *carotovora* in chinese cabbages. Acta Phytopathologica Sinica 32 (2), 165–169.

Sullivan, R.F., Holtman, M.A., Zylstra, G.J., White Jr., J.F., Kobayashi, D.Y., 2003. Taxonomic positioning of two biological control agents for plant diseases as *Lysobacter enzymogenes* based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. Journal of Applied Microbiology 94, 1079–1086.

Thomson, N.R., Thomas, J.D., Salmond, G.P.C., 1999. Virulence determinants in the bacterial phytopathogen *Erwinia*. Methods in Microbiology 29, 347–426.

Yuen, G.Y., Zhang, Z., 2001. Control of brown patch using the bacterium *Stenotrophomonas maltophilia* C3 and culture fluid. International Turfgrass Society Research Journal 9, 742–747.

Zhang, Z., Yuen, G.Y., 1999. Biological control of *Bipolaris sorokiniana* on tall fescue by *Stenotrophomonas maltophilia* C3. Phytopathology 89, 817–822.

Zheng, H.M., Zhong, Z.T., Lai, X., Chen, W.X., Li, S.P., Zhu, J., 2006. A LuxR/LuxI-type quorum sensing system in a plant bacterium, *Mesorhizobium*

*tianshanense*, controls symbiotic nodulation. Journal of Bacteriology 188, 1943–1949.

Zhu, C.G., Yu, Z.N., Sun, M., 2006. Restraining *Erwinia* virulence by expression of *N*-acyl homoserine lactonase gene *pro3A-aiiA* in *Bacillus thuringiensis* subsp. *leesis*. Biotechnology and Bioengineering 95, 526–532.

Zhu, H., Wang, Y.X., Hu, B.S., Liu, F.Q., 2007. Cloning and expression of α-lytic protease gene from *Lysobacter enzynogenes* stain OH11. Chinese Journal of Biological Control 23, 358–362.

Zhu, H., Wang, Y.X., Hu, B.S., Liu, F.Q., 2008. Cloning and expression of a chitinase gene from *Lysobacter enzymognes* strain OH11. Journal of Nanjing Agricultural University 31, 47–50.

Zhu, J., Chai, Y., Zhong, Z., Li, S., Winans, S.C., 2003. *Agrobacterium* bioassay strain for ultrasensitive detection of *N*-acylhomoserine lactone-type quorum-sensing molecules: detection of autoinducers in *Mesorhizobium huakuii*. Applied and Environmental Microbiology 69, 6949–6953.