J. Microbiol. Biotechnol. (2010), **20**(2), 281–286
doi: 10.4014/jmb.0908.08005
First published online 25 December 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

A Genetically Engineered *Pseudomonas fluorescens* Strain Possesses Dual Activity Against Phytopathogenic Fungi and Insects

Lu, Wenwei[1,2], Weiqiong Zhang[1], Yan Bai[1], Yingying Fu[1], Jun Chen[1], Xiaolu Geng[1], Yujing Wang[1], and Ming Xiao[1]*

[1]*College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, China*
[2]*Department of Food Science, Shanghai Business School, Shanghai 200233, China*

Received: August 9, 2009 / Revised: September 20, 2009 / Accepted: October 1, 2009

A *Pseudomonas fluorescens* strain was isolated and found to show antagonistic activity against phytopathogenic fungi and to possess a gene responsible for production of antibiotic 2,4-diacetylphloroglucinol. For the extension of biocontrol range, a gene for an *Androctonus australis* Hector insect toxin 1 (AaHIT1), one of the most known toxic insect-selective peptides, was designed and synthesized according to the preferred codon usage of *Pseudomonas fluorescens*, cloned, and transformed into the strain by pSUP106 vector, a broad-host-range plasmid. Bioassays indicated that the engineered strain was able to produce AaHIT1 with insecticidal activity, and at the same time retain the activity against plant pathogen. The experiments for nonplanted soil and rhizosphere colonization showed that, similar to the population of the wild-type strain, that of the engineered strain remained relatively constant in the first 10 days, and the subsequent 50 days, suggesting that AaHIT1 expression in the bacterial cell does not substantially impair its long-term colonization. It is first reported that a *Pseudomonas fluorescens* strain expressing an active scorpion neurotoxin has dual activity against phytopathogenic fungi and insects, making at attractive for agronomic applications.

Keywords: *Pseudomonas fluorescens*, AaHIT1, biocontrol, scorpion neurotoxin

Crop damage caused by phytopathogens and insect pests is a severe problem in the world agriculture. Chemical controls, such as fungicides and insecticides, are applied to crop protection. However, some chemical controls are expensive, impractical, and harmful to the environment. Biocontrol of the phytopathogens and insect pests provide an attractive alternative to chemical controls. Some strains of *Pseudomonas fluorescens* have been candidates for biofungicide [2, 6, 10, 12, 13, 29]. *Pseudomonas fluorescens* strains can suppress phytopathogens mainly because of their producing the antibiotics, such as 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1-carboxyclic acid (PCA) [8]. In addition, they possess the potential for plant growth promotion, pollutant degradation, and induction of systemic resistance in plants against phytopathogens [17, 24, 27]. However, *Pseudomonas fluorescens* has not been found to control insect pests in many countries and regions.

The *Androctonus australis* Hector insect toxin 1 (AaHIT1) is one of the most known toxic insect-selective peptides, composed of 70 amino acids cross-linked by four disulfide bridges. The structure of AaHIT1 in solution has been solved by ^{1}H NMR [5]. Its strict selectivity for insects has been documented by toxicity, electrophysiological, and ligand binding assays [30]. Scorpion toxins active on insects can be classified into two groups on the basis of the effects observed following injection into the tested insects: excitatory contractive toxins to induce an immediate fast paralysis, and flaccid depressant toxins to cause a slow progressive flaccid paralysis [31]. The AaHIT1 belongs to excitatory contractive toxins and has been applied to improve insecticidal activity of some baculoviruses and fungi [4, 26]. It has been found that scorpion neurotoxins are actively expressed in some eukaryotic and prokaryotic cells [11, 18, 23]. However, expression of active scorpion neurotoxins in *Pseudomonas fluorescens* has not been reported. In this report, a genetically engineered *Pseudomonas fluorescens* strain was constructed by transformation of AaHIT1, which was able to produce AaHIT1 with insecticidal activity, and at the same time retain the activity against the plant pathogen.

*Corresponding author
Phone: +86-(0)-21-64321022; Fax: +86-(0)-21-65642468;
E-mail: xiaom88@shnu.edu.cn

Materials and Methods

Isolation and Culture of Bacteria, and Assays of Fungicidal Activity

Strains of *Pseudomonas fluorescens* were isolated from the rapeseed (*Brassica napus* L.) rhizosphere in the Pudong areas of Shanghai. A suspension of rapeseed rhizosphere soil was obtained by shaking 10 g of root plus lightly adhering soil in 90 ml of 0.85% NaCl for 10 min at 180 rpm in a rotary shaker. One hundred μl of the suspension was spread onto King's medium B (KB) [14] and incubated at 28°C for 48 h. Single colonies that fluoresced under UV light (366 nm) were selected and further cultured to establish pure cultures. Some colonies with antagonistic activity were further picked and stored. Other colonies without antagonistic activity were abandoned.

Quantitative assays of fungicidal activity were performed as described previously [10]. The liquid culture was centrifuged to pellet the bacteria when it reached a level of turbidity of 0.5 at 600 nm in Luria–Bertani (LB) broth. The resulting supernatant was filter-sterilized through a 0.22-μm Whatman filter (Cat. 6900-2502), stored in phosphate saline buffer. For the assay, the wells, with a diameter of 5 mm, were then cut in the potato dextrose agar (PDA) on which phytopathogens (from Shanghai Pesticide Research Institute) were maintained. Then, 20 μl of the P13 strain's culture supernatant, or other sample, was added to the wells and allowed to diffuse into the agar. The zones of suppression around the well were measured after a 4-day incubation period. Five replicates were done for each treatment.

DNA Manipulations

Total genomic DNA was extracted from strain P13 by standard methods [1]. Partial fragments of the 16S rRNA gene of the strain were amplified with the following primer pair: forward primer 5'-AGAGT TTGATCCTGGCTCAG-3', reverse primer 5'-ACGGCTACCTTGTTA CGACTT-3' [28]. The *phlD* gene of the strain, related to production of antibiotic 2,4-diacetylphloroglucinol, was amplified with the following primer pair: forward primer 5'-GAGGACGTCGAAGACCACCA-3', reverse primer 5'-ACCGCAGCATCGTGTATGAG-3' [29]. The PCR products were purified from gel slices by using a TaKaRa agarose gel DNA purification kit Ver.2.0 (TaKaRa Inc., Dalian, China), and sequenced. The partial 16S rRNA gene sequence was deposited in the GenBank database with accession number EF487999, subjected to a BLAST search against the NCBI nr database. The *phlD* gene sequence was deposited in GenBank (FJ457627).

A DNA sequence encoding AaHIT1 was designed using *Pseudomonas fluorescens* preferred codon usage (Fig. 1A). Eight primers were used for synthesis of the AaHIT1 gene by PCR (Fig. 1B). The first PCR reaction was carried out in 100-μl reactions containing 1.0 U of *Taq* polymerase, 200 μm of dNTPs, and 2.0 μm of each primer, in the following conditions: 50°C for 1 min and extension of 20 min at 72°C. The PCR product was obtained. The second PCR reaction was carried out in 50-μl reactions containing 0.5 U of *Taq* polymerase, 100 μm of dNTPs, 1.0 μm of primer 1, 1.0 μm of primer 8, and about 80 ng of the PCR product (as template) of the first PCR reaction, in the following conditions: 94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; and a final extension of 5 min at 72°C. The PCR product was analyzed on agarose gel.

AaHIT1 Expression and Purification

An initial codon and a terminal codon were respectively engineered to the 5' and 3' terminal ends of the synthesized AaHIT1 sequence. The Shine–Dalgarno sequence was not used to avoid overexpression, which was found to affect the antagonism of the P13 cells against phytopathogenic fungi. The recombinant AaHIT1 sequence was cloned into the pMD18-T vector. After the orientation and sequence encoding AaHIT1 were confirmed by sequencing, the *Hin*dIII–*BamH* I fragment containing AaHIT1 sequences was subcloned into the pSUP106 vector, a broad-host-range plasmid often used for expression of heterologous genes in Rhizobacteria [16, 22], and then electroporated directly into P13 as described previously [7]. Transformants were selected and tranferred to LB broth [8]. The transformed P13 cells were subjected to ultrasonication at 300 W after reaching a level of turbidity of 0.5 at 600 nm in LB broth. The sonicated material was centrifuged at 10,000 ×*g* at 4°C. The resulting supernatants from pSUP106 and AaHIT1/pSUP106 transformed cells were designated as Sample 1 and

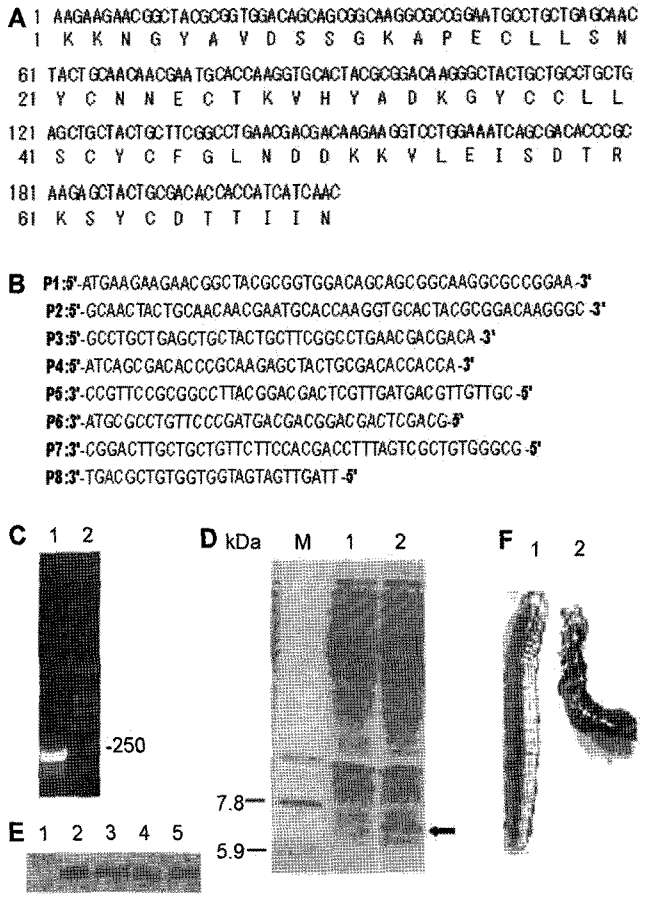

Fig. 1. Gene synthesis, expression, and paralysis assay for AaHIT1.
A. A DNA sequence encoding AaHIT1 was designed using *Pseudomonas fluorescens* preferred codon usage. **B.** Eight primers were used for synthesis of the AaHIT1 gene by PCR. **C.** Analysis of PCR product on agarose gel. Lane 1, PCR product; lane 2, DNA ladder. **D.** Tris-Tricine–SDS–PAGE (16.5%) analysis of AaHIT1 expressed in strain P13. Lane 1 is for Sample 1, lane 2 for Sample 2. **E.** Western blot analysis. Sample 1 (lane 1), Sample 2 (lane 2), Sample 3 (lane 3), and total protein extracts from the tissues (with removal of tracheal epithelium) of 30 larvae killed by feeding the media containing Sample 3 (lane 4) or AP13 (lane 5) were separated on Tris-Tricine–SDS–PAGE (16.5), transferred onto nitrocellulose, and reacted with an antiserum to *A. australis* venom (MicroPharm, U.K.). **F.** AaHIT1 paralysis assays. The fourth-instar *Mythimna seperata* Walker larvae were injected with 30 μl of Sample 3 (lane 2) and Sample 4 (lane 1).

Sample 2, respectively, stored in phosphate saline buffer (NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l, KH_2PO_4 0.24 g/l, pH 7.4), and subjected to SDS–PAGE analysis. Western blot analysis was performed using an antiserum to *A. australis* venom (MicroPharm, U.K.). Cell lysates were separated on Tris-Tricine–SDS–PAGE (16.5), transferred onto nitrocellulose, and reacted with an antiserum to *A. australis* venom.

Purification of AaHIT1 was performed as described previously [9]. In brief, Sample 2 was subjected to an anion-exchange chromatography (Q Sepharose fast flow resin; Amersham-Pharmacia Biotech, Sweden). AaHIT1-containing fractions were collected, concentrated, and then passed through a Superdex-75 column, resulting in Sample 3. The liquid culture of P13 cells transformed by AaHIT1/pSUP106 was designated as AP13 when it reached a level of turbidity of 0.5 at 600 nm in LB and after AaHIT1 expression in the cells was confirmed.

Assays of Insecticidal Activity

One mg of Sample 1, Sample 2, and AP13, and 50 μg of Sample 3 were mixed in 1 g of the growth media, respectively, on which second-instar larvae (*Mythimna seperata* Walker, from Shanghai Pesticide Research Institute) were fed for 24 h, and then transferred to regular growth media. Testing larvae were raised in separate tubes at 25°C on a 14:10 h light:dark cycle. The final death rate, the developmental stage, and its weight of surviving larvae were determined 120 h after commencement of the experiment. The time for half insect death was designed as LT_{50}. Average weight was calculated from 30 testing larvae. The values ± SE are based on five replicates.

Monitoring of Bacteria in Soil

For monitoring of bacteria in nonplanted soil, the bacterial inoculum was added to the pots (20 cm dia.) containing pasteurized sandy soil at a density of 1×10^7. For bacteria introduced to planted soil, the pots (20 cm dia.) contained pasteurized sandy soil in which the seeds of rapeseed (*Brassica napus* L.) soaked for 30 min in bacterial suspension (10^8 CFU/ml) were sown. The pots were maintained in a greenhouse at 16 to 20°C with natural light–dark cycles. Samples were taken at the indicated time points. When monitoring bacteria in planted soil, the first samples were taken from the rhizosphere after the appearance of the first true leaf. The wild-type strains were enumerated by plating on King's medium B and detecting the *phlD* gene; the engineered strains were enumerated by plating on King's medium B and detecting the AaHIT1 gene.

RESULTS AND DISCUSSION

Isolation of Bacterial Strain with Activity Against Plant Pathogen

A bacterial strain P13 was isolated from the rapeseed (*Brassica napus* L.) rhizosphere in the Pudong areas of Shanghai by plating on King's medium B [14]. Antagonism test showed that the strain has antagonistic activity against some phytopathogenic fungi (Table 1). Furthermore, the *phlD* gene related to production of antibiotic 2,4-diacetylphloroglucinol was detected in the genome of the strain. The 2,4-diacetylphloroglucinol is very important for agriculture because of its activity against phytopathogenic fungi, and is produced by many strains of *Pseudomonas fluorescens* with biocontrol activity against fungal plant pathogens. An operon that includes four genes, *phlA*, *phlB*, *phlC*, and *phlD*, is required for synthesis of 2,4-diacetylphloroglucinol in these strains [2, 8]. Deletion of the *phlD* gene in the genome of strain P13 suggested that the strain has the ability for synthesis of 2,4-diacetylphloroglucinol, which might be a major cause for its antagonistic activity against some phytopathogenic fungi.

Table 1. Antagonism of P13 strain against phytopathogenic fungi.

Phytopathogenic fungi	Antagonistic activity[a]
Sclerotinia sclerotiorum	+++
Fusarium graminearum	+
Rhizoctonia solani	++
Bipolaris maydis	++
Botrytis cinerea	+++
Colletotrichum orbiculare	++
Colletotrichum gloeosporioides	+++

[a]Inhibition of phytopathogenic fungi, defined as hyphal growth less abundant and growth retarded on the area of the plate to the side where the phytopathogenic fungi had been grown. +++, Very strong inhibition, zone of inhibition >20.0 mm; ++, strong with inhibition, zone of inhibition >10.0 mm; +, growth with obvious definitely retarded, zone of inhibition near colony.

Moreover, strain P13 was found to possess the morphological and biochemical properties that are characteristic criteria for *Pseudomonas fluorescens* (results not shown). The partial 16S rDNA sequence of the strain was obtained and deposited in the GenBank database (EF487999), nearly identical to that of some strains of *Pseudomonas fluorescens*. Therefore, strain P13 was temporarily classified as *Pseudomonas fluorescens* (CCTCC AB209102).

Construction of Genetically Engineered Strain P13 to Produce AaHIT1 Toxin

However, the P13 is not effective against plant pests. Although a novel gene cluster encoding an insect toxin and expression of an insect toxin have been found in plant-associated strains of *Pseudomonas fluorescens* [20, 21], *Pseudomonas fluorescens* strains have not been effectively used for agronomic practices in pest biocontrol. The AaHIT1 is one of the most known toxic insect-selective peptides [30]. For the extension of biocontrol range for the strain, a DNA sequence encoding AaHIT1 was designed according to the preferred codon usage of *Pseudomonas fluorescens*, and synthesized by PCR (Fig. 1A, 1B, and 1C), and then subcloned into pSUP106 vector and electroporated directly into P13 for expression. Finally, Sample 1 (the supernatants from pSUP106 transformed cells) and Sample 2 (the supernatants from AaHIT1/pSUP106 transformed cells) were obtained. SDS–PAGE showed an extra protein band with an approximate molecular mass of 5.9–7.8 kDa only in Sample 2 (Fig. 1D, lane 2). Western blot analysis using an antiserum to *A. australis* venom showed that

Sample 2 contains the AaHIT1 (Fig. 1E, lane 2). Samples 3 and 4 were obtained by purification of Samples 2 and 1, respectively. The AaHIT1 in Sample 3 was verified (Fig. 1E, lane 3). Samples 3 and 4 were respectively injected into *Mythimna seperata* Walker larvae, which are important plant pests. All the larvae injected with Sample 3 died with the same symptoms: contraction paralysis, which did not occur in larvae injected with Sample 4 (Fig. 1F). The contraction paralysis is employed as a quick, specific, and sensitive bioassay for the detection and monitoring of the purification of the AaHIT1 toxin [30]. Therefore, the engineered P13 cells are able to produce AaHIT1 toxin with biological activity.

To stabilize the antagonism of strain P13 against phytopathogenic fungi, we did not used the Shine–Dalgarno (SD) sequence to avoid overexpression during the introduction of the AaHIT1 gene into its cells. At the beginning of the experiments, we used different SD sequences to express AaHIT1 on the basis of Komarova's suggestion that an extended SD sequence adversely affects translation in *Escherichia coli*. A stronger SD domain usually contains a shorter sequence [15]. Although the AaHIT1 expression level increased in strain P13, its antagonistic activity was significantly reduced, below detection limits (data not shown). AaHIT1 overexpression might inhibit the cells to produce antibiotics, or interfere with the effect of antibiotics, therefore affecting the antagonism of the P13 cells against phytopathogenic fungi. Our aim was to establish a *Pseudomonas fluorescens* strain that possesses the dual activity against phytopathogenic fungi and insects. Ultimately, we did not use any SD sequence. Results showed that the engineered strain was able to produce AaHIT1 with insecticidal activity, and at the same time retained the activity against plant pathogen (Table 2 and Fig. 2). In fact, SD interaction was found to be not essential for correct initiation of prokaryotic translation, and its impairment was paralleled by a decrease in their protein synthesis [3].

Evaluation of the Dual Activity Against Plant Pathogen and Pest

For a quantitative biological assay of activity against plant pest, a toxicity test was performed (Table 2). Compared with Sample 1, Sample 2, Sample 3, and AP13 killed 30%, 56%, and 48% of the testing larvae, respectively. There was also a significant difference in total weight between the control group (Sample 1) and the test groups (Samples 2 and 3, AP13). The average weight of the larvae fed on the Sample 2, Sample 3, or AP13-containing media was approximate 13%, 6%, or 8% of that of the control group. Moreover, the expressed toxin was detected by anti-AaHIT1 antibody in the tissues (with removal of tracheal epithelium) of larvae killed by feeding with the media containing the neutoxin (Fig. 1E, lanes 4 and 5).

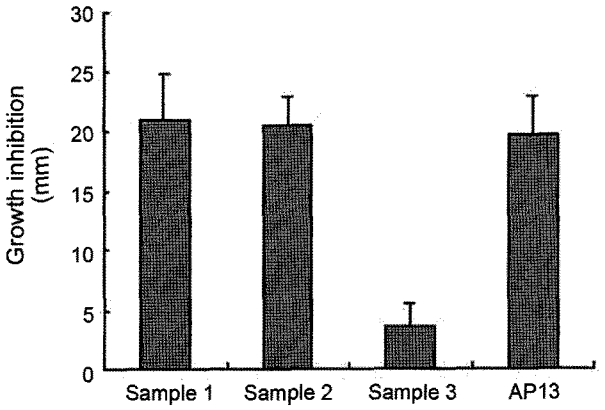

Fig. 2. Antagonism of the different P13 samples and AP13 against phytopathogenic fungi.
The antagonism was examined as described in Materials and Methods.

For investigation of whether the engineered strain still retained the activity against plant pathogen, antagonism testing of the different P13 samples and AP13 against phytopathogenic fungi was performed [10]. Among the different P13 samples, Sample 1, without insecticidal activity, the crude supernatant from pSUP106 transformed cells, showed the highest growth inhibition of *Sclerotinia sclerotiorum*. Sample 2, the crude supernatant from AaHIT1/pSUP106 transformed cells, and AP13, still retained high activity against plant pathogen. Sample 3, the supernatant containing purified AaHIT1, showed the lowest antagonism (Fig. 2). Taken together, the AaHIT1 expressed in the cells of strain P13 did not influence substantially its fungicidal activity (*e.g.*, Sample 2 and AP13). A series of purification

Table 2. Toxicity test on *Mythimna seperata*.

Samples	Average weight at zero h	Average weight at 120 h	New weight increase	Mortality (%)	LT_{50} (h)[a]
Sample 1[b]	3.65	138.20	134.55	0	
Sample 2	3.64	18.53	14.89	30±2.6	192±10
Sample 3	3.64	8.56	4.92	56±4.3	110±5
AP13	3.64	11.87	8.23	48±3.1	121±6

[a]The time for half insect death was designed as LT_{50}. Average weight was calculated from 30 testing larvae. The values ± SE are based on five replicates.
[b]Samples 1 and 2 are the supernatants from pSUP106 and AaHIT1/pSUP106 transformed cells, respectively.
Sample 3 was obtained by purification of Sample 2.
AP13 refers to the liquid culture of P13 cells transformed by AaHIT1/pSUP106.

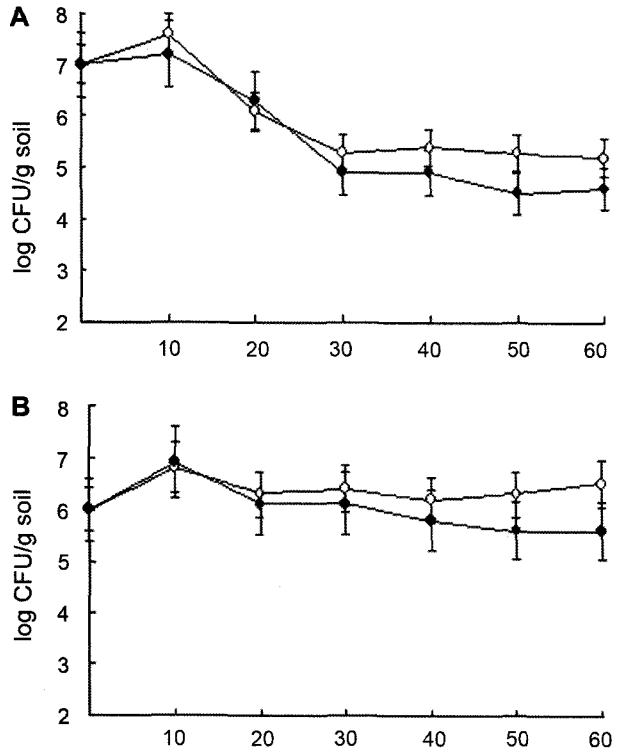

Fig. 3. Monitoring of the wild-type (open symbols) and engineered (closed symbols) strains in soil (**A**) and in rhizosphere (**B**) under greenhouse conditions.
Five replicates were done for each treatment; error bars indicate the standard error from the mean. Two-way analysis of variance (ANOVA) was used to determine statistical significance ($p<0.05$).

processes for obtaining AaHIT1 might result in loss of some materials responsible for the antagonistic activity of P13 (e.g., Sample 3). Although Sample 3 contains purified AaHIT1, some materials responsible for antagonistic activity might be present in low amount. Therefore, Sample 3 still has antifungal activity although it is very low. It is noted that Sample 2 and AP13 have insecticial effect, and at the same time possess high antagonistic activity against phytopathogenic fungi.

Monitoring of Engineered and Wild-Type Strains in Soil
For characterization of the bacterial colonization, the survival of the wild-type and engineered strains was examined in nonplanted soil and in rhizosphere of rapeseed (*Brassica napus* L.) under greenhouse conditions. Nonplanted soil and rhizosphere colonization were evaluated at the indicated times. It was found that the population of the wild-type strain remained relatively constant with time. The population of the engineered strain also remained relatively constant in the first 10 days and the subsequent 50 days (Fig. 3A and 3B). It was indicated that AaHIT1 expression in the bacterial cell does not substantially impair its long-term colonization.

To our knowledge, this is the first report that a *Pseudomonas fluorescens* strain expressing an active scorpion neurotoxin has dual activity against plant pathogen and pest. *Pseudomonas* inoculant products have been considered as environmentally friendly alternatives to chemical agents [25]. The engineered *Pseudomonas fluorescens* strain is attractive for agronomic applications.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (30670445, 30870492), Shanghai Leading Academic Discipline Project (S30406), Leading Academic Discipline Project of Shanghai Normal University (DZL709, DCL704), Shanghai Municipal Science and Technology Commission (07DZ12038, 08JC1416900), and Shanghai Municipal Education Commission (08ZZ66).

References

1. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1995. *Short Protocols in Molecular Biology*. John Wiley & Sons, Inc., New York, NY.
2. Bangera, M. G. and L. S. Thomashow. 1999. Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. *J. Bacteriol.* **181:** 3155–3163.
3. Calogero, R. A., C. L. Pon, M. A. Canonaco, and C. O. Gualerzi. 1988. Selection of the mRNA translation initiation region by *Escherichia coli* ribosomes. *Proc. Natl. Acad. Sci. U.S.A.* **85:** 6427–6431.
4. Cory, J. S., M. L. Hirst, T. Williams, R. S. Halls, and D. Gouson. 1994. Field trial of a genetically improved baculovirus insecticide. *Nature* **370:** 138–140.
5. Darbon, H., C. Weber, and W. Braun. 1991.Two dimensional ^{1}H nuclear magnetic resonance study of AaH IT, an anti-insect toxin from *Androctonus australis* Hector. Sequential resonance assignments and folding of the polypeptide chain. *Biochemistry* **30:** 1836–1845.
6. Delany, I., M. M. Sheehan, A. Fenton, S. Bardin, S. Aarons, and F. O'Gara. 2000. Regulation of production of the antifungal metabolite 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* F113: Genetic analysis of *phlF* as a transcriptional repressor. *Microbiology* **146:** 537–546.
7. Farinha, M. A. and A. H. Kropinski, 1990. High efficiency electroporation of *Pseudomonas aeruginosa* using frozen cell suspensions. *FEMS Microbiol. Lett.* **70:** 221–226.
8. Fenton, A. M., P. M. Stephens, J. Crowley, M. O'Callaghan, and F. O'Gara. 1992. Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Appl. Environ. Microbiol.* **58:** 3873–3878.
9. Hao, C., C. Xu, W. Wang, B. Chai, and A. Liang. 2005. Expression of an insect excitatory toxin, *Bm*K IT, from the

scorpion, *Buthus martensii* Karsch, and its biological activity. *Biotechnol. Lett.* **27:** 1929–1934.

10. Jayaraj, J., T. Parthasarathi, and N. V. Radhakrishnan. 2007. Characterization of a *Pseudomonas fluorescens* strain from tomato rhizosphere and its use for integrated management of tomato damping-off. *Biocontrol* **52:** 683–702.
11. Jiang, G., Y. Xu, X. Zhu, Y. Su, and Y. Zhu. 2001. Prokaryotically expressed *Buthus martensii* Karsch insect depressant toxin has insecticidal effects. *Toxicon* **39:** 469–476.
12. Kamilova, F., G. Lamers, and B. Lugtenberg. 2008. Biocontrol strain *Pseudomonas fluorescens* WCS365 inhibits germination of *Fusarium oxysporum* spores in tomato root exudate as well as subsequent formation of new spores. *Environ. Microbiol.* **10:** 2455–2461.
13. Keel, C., U. Schnider, M. Maurhofer, C. Voisard, J. Laville, U. Burger, P. Wirthner, and D. Haas. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Molec. Plant Microbe Interact.* **5:** 4–13.
14. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* **44:** 301–307.
15. Komarova, A. V., L. S. Tchufistova, and E. V. Supina. 2002. Protein S1 counteracts the inhibitory effect of the extended Shine–Dalgarno sequence on translation. *RNA* **8:** 1137–1147.
16. Kurbanova, I. V., V. A. Velikov, and M. I. Chumakov. 2001. Expression of VirB2 protein-containing structures on *Agrobacterium* in mating cultures. *Antonie Van Leeuwenhoek* **79:** 291–295.
17. Leeman, M., J. A. Van Pelt, F. M. Den Ouden, M. Heinsbroek, P. A. H. M. Bakker, and B. Schippers. 1995. Induction of systemic resistance against *Fusarium* wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. *Phytopathology* **85:** 1021–1027.
18. Martin-Eauclaire, M. F., M. Søgaard, C. Ramos, S. Cestèle, P. E. Bougis, and B. Svensson. 1994. Production of active, insect-specific scorpion neurotoxin in yeast. *Eur. J. Biochem.* **223:** 637–645.
19. Melançon, P., D. Leclerc, N. Destroismaisons, and L. Brakier-Gingras. 1990. The anti-Shine–Dalgarno region in *Escherichia coli* 16S ribosomal RNA is not essential for the correct selection of translational starts. *Biochemistry* **29:** 3402–3407.
20. Péchy-Tarr, M., D. J. Bruck, M. Maurhofer, E. Fischer, C. Vogne, M. D. Henkels, *et al.* 2008. Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. *Environ. Microbiol.* **10:** 2368–2386.
21. Peng, R., A. Xiong, X. Li, H. Fuan, and Q. Yao. 2003. A delta-endotoxin encoded in *Pseudomonas fluorescens* displays a high degree of insecticidal activity. *Appl. Microbiol. Biotechnol.* **63:** 300–306.
22. Priefer, U. B., R. Simon, and A. Pühler. 1985. Extension of the host range of *Escherichia coli* vectors by incorporation of replication and mobilization functions. *J. Bacteriol.* **163:** 324–330.
23. Rhouma, R. B. H., M. Cerutti-Duonor, K. Benkhadir, F. Goudey-Perriere, M. E. Ayeb, M. Lopez-Ferber, and H. Karoui. 2005. Insecticidal effects of *Buthus occitanus tunetanus* BotIT6 toxin expressed in *Eschericchia coli* and baculovirus/insect cells. *J. Insect Physiol.* **51:** 1376–1383.
24. Seong, L. H., J. M. Lee, and S. D. Kim. 2002. A plant growth-promoting *Pseudomonas fluorescens* GL20: Mechanism for disease suppression, outer membrane receptors for ferric siderophore, and genetic improvement for increased biocontrol efficacy. *J. Microbiol. Biotechnol.* **12:** 249–257.
25. Walsh, U. F., J. P. Morrissey, and F. O'Gara. 2001. *Pseudomonas* for biocontrol of phytopathogens: From functional genomics to commercial exploitation. *Curr. Opin. Biotechnol.* **12:** 289–295.
26. Wang, C. and R. J. St. Legal. 2007. A scorpion neurotoxin increases the potency of a fungal insecticide. *Nat. Biotechnol.* **25:** 1455–1456.
27. Wang, Y., M. Xiao, X. Geng, J. Liu, and J. Chen. 2007. Horizontal transfer of genetic determinants for degradation of phenol between the bacteria living in plant and its rhizosphere. *Appl. Microbiol. Biotechnol.* **77:** 733–739.
28. Weissburg, W. G., S. M. Barns, D. A. Pelletier, and D. L. Lane. 1991. 16S Ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173:** 697–703.
29. Zha, W., S. B. Rubin-Pitel, and H. Zhao. 2006. Characterization of the substrate specificity of PhlD, a type III polyketide synthase from *Pseudomonas fluorescens*. *J. Biol. Chem.* **281:** 32036–32047.
30. Zlotkin, E., Y. Fishman, and M. Elazar. 2000. AaIT: From neurotoxin to insecticide. *Biochimie* **82:** 869–881.
31. Zlotkin, E., D. Kadouri, D. Gordon, M. Pelhate, M. F. Martin, and H. Rochat. 1985. An excitatory and depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch. Biochem. Biophys.* **240:** 870–887.