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Biological Control Activity of Two Isolates of *Pseudomonas fluorescens* against Rice Sheath Blight

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Two isolates of mucous bacteria, mc75 and pc78, were isolated from fungal culture plate as culture contaminants with an interesting swarming motility. Both isolates were identified as *Pseudomonas fluorescens* based on microscopy, biochemical analysis, Biolog test and DNA sequence analysis of the 16S rRNA gene. Both strains have the exactly the same 16S rRNA gene sequences, and yet their biological control activity were not identical each other. *In vitro* analysis of antagonistic activity of two isolates against several plant pathogenic fungi indicated that both produced diffusible and volatile antifungal compounds of unknown identities. Treatment of the bacterial culture of *P. fluorescens* pc78 and its culture filtrate exhibited a strong biological control activity against rice sheath blight *in vivo* among six plant diseases tested. More effective disease control activity was obtained from treatment of bacterial culture than that of culture filtrate. Therefore, in addition to antifungal compound and siderophore production, other traits such as biofilm formation and swarming motility on plant surface may contribute to the biological control activity of *P. fluorescens* pc78 and mc75.

Keywords : Biological control, *Pseudomonas fluorescens*, Rice sheath blight, Swarming

Fluorescent pseudomonads have received much attention as potential biological control agent to suppress mainly soilborne plant pathogens. A number of bacterial traits contribute to the control of soilborne plant disease, including bacterial colonization of plant surface, nutrient competition, siderophore production and antimicrobial metabolite production (Haas and Keel, 2003; Handelsman and Parke, 1989; O'Sullivan and O'Gara, 1992). Because of the traits useful for biological control, fluorescent pseudomonads have been applied to seeds and rhizosphere. However, it has been recognized that there are problems to commercialize the fluorescent pseudomonads as a biocontrol agent for soilborne disease. It is because of inconsistent disease control activity in different soils and poor survival of the bacteria in soil and on seed (O'Sullivan and O'Gara, 1992).

Nevertheless, there have been consistent efforts to use fluorescent pseudomonads as a biocontrol agent. This is mainly because fungal pathogens frequently acquire resistance against fungicides, although fungicides application effectively controls many plant diseases (Ferrin and Rhode, 1992; Yourman and Jeffers, 1999). Furthermore, for environmental reasons, there is growing public concern over pesticide use. Therefore, new approaches focus on increasing and optimizing the biological control activity by combining several desirable traits of potential biocontrol organisms (Cook, 1993). The improvement of the biocontrol mechanisms of potential biological control agents by genetic means is also an important way to enhance the biological control activity (Dowling and O'Gara, 1994).

In this study, we isolated and identified two fluorescent pseudomonads isolates from frequent fungal culture contamination. These isolates possessed desirable bacterial traits as a potential biocontrol agent. Those include the production of diffusible antifungal compounds, volatile antifungal metabolites, siderophore and exopolysaccharides to form biofilm on plant surface. The isolates also showed swarm motility, which is probably due to exopolysaccharide production. Since the multiple traits of the isolates may be desirable for biological control, we investigated the biological control activity of the isolates *in vivo* against six plant diseases including rice sheath blight.

Materials and Methods

Microorganisms, media and culture conditions. *Pseudomonas* species cultures were routinely grown at 28 °C on mannitol-glutamate medium (MG) (Keane et al., 1970) supplemented with yeast extract (0.25 g/liter) (MGY) or in MGY broth. When necessary for bacterial growth in rich medium, *P. fluorescens* strains were cultured on YpSs medium (2% glucose, 0.2% yeast extract, 0.5% polypep-

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tone, 0.05% $MgSO_4$, 0.1% KH_2PO_4) under the same conditions. Fungal plant pathogens such as *Magnaporthe grisea, Corticium sasaki, Botrytis cinerea, Fusarium oxysporum, Pythium ultimum, Phytophthora capsici* and *Phytophthora infestans* were routinely grown on potato dextrose agar (PDA) as previously described (Kim et al., 2004). Two obligate parasitic fungal pathogens *Puccinia recondita* and *Erysiphe graminis* f. sp. *hordei* were maintained by periodical transfer on plants as previously described on wheat and barley plants, respectively (Kim et al., 2004).

Microscopy, biochemical test and Biolog analysis. Gram staining, microscopic observation of bacterial cells and biochemical test were performed by the standard procedure (Gerhardt et al., 1981). Biochemical test in this study includes lipid hydrolysis, gelatin hydrolysis, starch hydrolysis, lecithinase, catalase, oxidase, fermentation in triple sugar iron agar, polyhydroxybutyrate formation, and nitrate reduction. Swarming motility of bacterial cells was investigated on different culture media such as MGY, PDA, nutrient agar (NA), Luria-Bertani medium (LB), and tryptic soy agar (TSA) at different temperature. Bacterial identification based on carbohydrate utilization was conducted using GN2 MicroLog plate (Biolog Inc, Hayward, CA, USA) by following the provided standard procedure.

DNA sequencing of 16S ribosomal RNA gene. Bacterial 16S ribosomal RNA (rRNA) gene was amplified with the two bacterial universal primers, 8F (5'-AGAGTTTGATCCTGGCTCAG-3' *E. coli* position 8-27) and 1492R (5'-GGTTACCTTGTTACGACTT-3' *E. coli* position 1510-1492) (Edwards et al., 1989) using genomic DNA obtained from *P. fluorescens* isolates. The PCR amplification was carried out under the following conditions: 94 °C for 3 min initial denaturation; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min 30 sec, and final extension at 72 °C for 5 min. The reaction mixture (final volume, 50 μl) contained 1 μl DNA (approximately 40-50 ng), 1X reaction buffer with 2 mM $MgCl_2$ and 2.5 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA). The amplified products were cloned into the pGEM-Teasy vector (Promega,). The nucleotide sequencing was performed commercially at the DNA sequencing facility of GenoTech Corp. (Daejeon, Korea). DNA sequences of 16S rRNA gene were analyzed by using software from the Blastn program provided by the National Center for Biotechnology Information.

Analysis of *in vitro* antifungal activity. Antifungal activity of the *P. fluorescens* isolates mc75 and pc78 was tested on PDA against *M. grisea, P. ultimum* and *F. oxysporum*. Antagonistic bacteria and a nonantagonistic bacterium *P. putida* 06909 (Lee and Cooksey, 2000) were streaked at a distance of 5 cm from the agar discs (5 mm in diameter) of a target fungus. Fungal growth inhibition was determined 5-7 days after incubation at 25 °C. In order to determine if the *P. fluorescens* isolates produce any volatile compounds inhibiting fungal growth, the following experiment was conducted. An agar strip (1 cm width) of PDA was removed from the center of the PDA plate and the medium was air-dried in the sterile condition to remove any remained moisture completely. This is to exclude the possible diffusion of any non-volatile compounds from bacterial side to fungal mycelia. The tested bacteria, *P. fluorescens* isolates and *P. putida* 06909 were streaked in the center of one side of PDA and an agar disc of a target fungus was inoculated in the center of the other side of the PDA plate. The culture plates were sealed with plastic wrap and incubated for 5-7 days at 25 °C.

***In vivo* biological control activity.** Bacterial cell culture and the culture filtrate were tested *in vivo* for biological control activity. The cells from the isolates mc75 and pc78 grown at 28 °C for 24 hrs in 100 ml of YpSs broth with shaking of 150 rpm were directly used for evaluation of *in vivo* biological control activity. On the other hand, the *in vivo* antifungal activity of bacterial metabolites was investigated using bacterial culture filtrate. The *P. fluorescens* mc75 and pc78 were grown at 28 °C for 48 hrs in 100 ml of YpSs broth with shaking of 150 rpm and the bacterial cells were completely removed by the repeated centrifugation at 8,000 rpm. Plants used for *in vivo* biological control activity were grown to 4 weeks in a controlled glasshouse at 25 (± 5)°C and the activity was investigated against six plant diseases. The six plant diseases include rice blast (RCB, *M. grisea*), rice sheath blight (RSB, *C. sasaki*), tomato gray mold (TGM, *B. cinerea*), tomato late blight (TLB, *P. infestans*), wheat leaf rust (WLR, *P. recondita*), barley powdery mildew (BPM, *E. graminis* f. sp. *hordei*). The plant seedlings were sprayed to run-off on plant leaves with bacterial culture, bacterial culture filtrate and chemical fungicides, respectively. Control plants were sprayed with fresh YpSs broth. The treated plants were inoculated with respective fungal pathogens. The fungal pathogen inoculation, incubation and disease rating were conducted as previously described (Kim et al., 2004). Chemical fungicides were treated with two concentrations to compare biological control activity of the tested bacteria with chemical fungicides; Balsticidin-S for RCB, 1 and 50 μg/ml; Validamycin for RSB, 5 and 50 μg/ml; Fludioxonil for TGM, 5 and 50 μg/ml; Dimethomorph for TLB, 2 and 10 μg/ml; Flusilazole for WLR, 2 and 10 μg/ml; Flusilazole for BPM, 0.5 and 10 μg/ml. The disease control value was calculated using the following formula: Disease control

value (%) = ((A-B)/A) x 100, where A is the disease severity caused by control treatment and B is the disease severity after various treatments. The treatments were arranged in a completely randomized design, with three replicates per treatment.

Results and Discussion

Isolation and identification of antagonistic bacteria. During periodical sub-culturing of plant pathogenic fungi, *M. grisea* and *P. ultimum*, we fortuitously isolated two fungal hyphae contaminants showing mucous colony morphology as antifungal bacteria. Both isolates designated mc75 and pc78, respectively produced large amount of exopolysaccharides on sugar-containing media and fluorescent siderophore on King's B medium. Since both isolates showed consistent antifungal activity with unique cultural characteristics, we continued to investigate their biological control activities. Both were rod shaped aerobic Gram-negative bacteria. Biochemical analysis exhibited the typical characteristics of fluorescent pseudomonads such as no fermentation with any supplied sugars. Most of the tested characteristics of two isolates were exactly consistent with those of pseudomonads described in Bergey's manual of systematic bacteriology (Table 1).

Biolog analysis using GN2 MicroLog plate showed that both isolates were highly similar to *P. fluorescens* with 0.56-0.58 similarity index. Analysis of DNA sequences of 16S rRNA gene revealed that the two isolates were identical with each other (Fig. 1) and the closest species in GenBank database was *P. putida* or *P. fluorescens* with 99% identity. *P. fluorescens* and *P. putida* could be distinguished based on gelatin hydrolysis. While *P. putida* does not hydrolyze gelatin, *P. fluorescens* is positive for gelatin hydrolysis. Therefore, we identified mc75 and pc78 as *P. fluorescens*. In addition, we concluded that two isolates are not identical because their biological control activity were different each other, despite the identities in biochemical characteristics and the 16S rRNA sequences.

Table 1. Microscopy and biochemical characteristics of *P. fluorescens* isolates mc75 and pc78

Characteristics	Isolate mc75	Isolate pc78	Bergey's description
Gram stain	–	–	–
Swarming on PDA, MGY	+	+	nd
Swarming on LB, TSA, NA	+	+	nd
Lipid hydrolysis	+	+	+/–
Gelatin hydrolysis	+	+	+
Starch hydrolysis	–	–	–
Lecithinase	+	+	+/–
Fermentation on triple sugar[a]	–	–	–
Gas production	–	–	–
H_2S production from ferrous sulfate	–	–	–
Polyhydroxybutyrate production	–	–	–
Catalase	+	+	+
Oxidase	+	+	+
Nitrate reduction	+	+	+/–
Fluorescent pigment production	+	+	+

[a]The triple sugar comprise glucose, sucrose and lactose. 'nd' indicates not described characteristics.

Cultural characteristics of two isolates. *P. fluorescens* isolates mc75 and pc78 showed an interesting cultural characteristic with mass production of exopolysaccharides to form a biofilm on sugar containing media. When they were grown in a medium with reduced agar content, they showed the typical swarm motility (Fig. 2). The swarming in bacteria is complex pattern of bacterial cells organized as radiation tendrils. The swarming motility was reported in biocontrol bacterium *P. fluorescens* CHA0 (Kay et al., 2005) and during biofilm formation in *P. aeruginosa* (Caiazza et al., 2005). Biofilm formation and swarming motility of *P. fluorescens* mc75 and pc78 were more apparent when cultured at 25 °C rather than 30 °C (data not shown).

Biofilm formation and swarming may be desirable traits for biocontrol bacterium because those traits could enhance bacterial attachment and persistence on plant surface (Handelsman and Stabb, 1996). In fact, spraying YpSs broth on rice leaves did result in the moisturization of plant surface, while bacterial culture of *P. fluorescens* pc78 formed thin moisturizing film on rice leaves when sprayed (data not shown). This observation suggested that certain characteristics of the isolate pc78 were probably involved in the surfactant properties of the bacteria and plant surface colonization. At this stage, it is not clear if exopolysaccharides production and swarming motility are directly responsible for good attachment and biofilm formation on plant surface. Since *P. fluorescens* pc78 produces lipase and probably biosurfactant, those can also contribute to the biofilm formation to attach the plant surface. Mutants lacking lipase production or exopolysaccharides production could be generated and tested for the attribute to the bacterial interesting phenotype. It is interesting that a phenotypic variant of *P. fluorescens* F113 with increased motility showed enhanced colonization of plant root (Martinez-Granero et al., 2006). Although the origin of our strains is unknown, their characteristics might be useful for the application in biological control of plant diseases.

Antifungal activity on culture media. Antifungal activity of *P. fluorescens* mc75 and pc78 was investigated *in vitro*

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
GGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCT
GCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGA
GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
CACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACA
GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTGGTG
GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG
ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAG
TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
AAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGG
GAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
GGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT
GGAGCTAATCCCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA
AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTC
GGGAGGACGGTTACCACGGTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACC

Fig. 1. The nucleotide sequence of 1,498 bp of 16S rRNA gene from *P. fluorescens* pc78. The underlined are sequences from primers used for amplification of 16S rRNA gene.

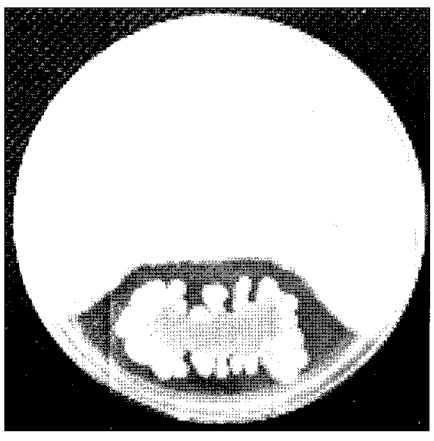

Fig. 2. Swarming motility of *P. fluorescens* pc78 and its antifungal activity against *P. ultimum* on PDA with reduced (1%) agar content.

against three-plant pathogenic fungi *M. grisea, P. capsici* and *F. oxysporum.* Both strains exhibited a strong fungal growth inhibition on PDA against *M. grisea* and *P. capsici* (Fig. 3) but a weak inhibition against *F. oxysporum* (data not shown). *In vitro* activity analysis indicated that both strains produced diffusible antifungal compounds and volatile antifungal compounds as shown in Fig. 3. One of the biocontrol strains *P. putida* 06909 did not produce antifungal compound, neither diffusible nor volatile as previously described (Yang et al., 1994).

Fluorescent pseudomonads with biological control activity produce diffusible or volatile antifungal compounds *in situ* in rhizosphere (Haas and Keel, 2003; O'Sullivan and O'Gara, 1992). One of the major volatile compounds produced from biocontrol bacteria is hydrogen cyanide that is involved not only in biocontrol activity but also in suppression of weed seedling growth (Kremer and Souissi, 2001). Since our isolate pc78 inhibited seed germination of red pepper significantly (data not shown), this strain probably produced the volatile cyanide. The *in vitro* antifungal activity by pc78 and mc75 could be the result of volatile metabolites production in addition to the diffusible antibiotics.

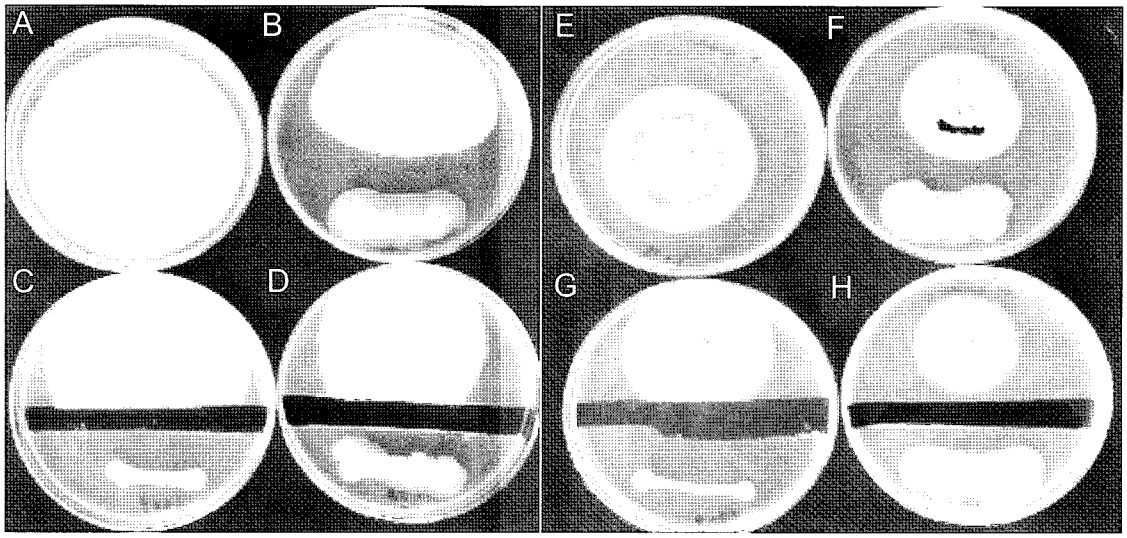

Fig. 3. *In vitro* antifungal activity of *P. fluorescens* pc78 against *P. capsici* (A-D) and *M. grisea* (E-F) on PDA. (A) and (E), fungal inoculation alone. (B) and (F), antifungal activity of *P. fluorescens* pc78. (C) and (G) no production of any volatile antifungal compounds by *P. putida* 06909. (D) and (H) volatile antifungal compound production by *P. fluorescens* pc78.

Biological control activity of *P. fluorescens*. Biological control activity of *P. fluorescens* mc75 and pc78 were evaluated *in vivo* by investigating the disease control value of the bacterial cultures and culture filtrates against six plant diseases. Both bacterial culture and culture filtrate of the strain pc78 exhibited the strongest disease control activity against rice sheath blight caused by *C. sasaki* (Table 2). The disease control value of the strain pc78 was the higher than that of chemical fungicide validamycin. The strain pc78 also showed biological control activity against rice blast and tomato gray mold, but the disease control values were not comparable to the respective chemical fungicide. The disease control activity by mc75 was generally lower than that of pc78. Both strains were not effective to control tomato late blight, wheat leaf rust and barley powdery mildew. Interestingly, treatment of bacterial cultures was more effective to control the most diseases tested than that of culture filtrate (Table 2). This result suggested that application of bacterial cells as well as antifungal compound produced by the cells are important for biological control activity of the strains pc78 and mc75. It is likely that the bacterial cells might colonize plants and contribute to inhibit the fungal pathogen invasion.

It is previously recognized that successful colonization and competitive fitness of treated microorganisms are the essential traits for biocontrol agents (Handelsman and Stabb, 1996; Weller, 1988). To control the rice sheath blight, the successful colonization of bacterial cells and attachment of antifungal compounds on the hydrophobic rice sheath surface would be one of the most important traits. It is likely

Table 2. Disease control value of *P. fluorescens* mc75 and pc78 treatments on six plant diseases

Treatment[a]	Disease control value (%)[b]					
	RCB	RSB	TGM	TLB	WLR	BPM
mc75cu	75	20±14.1	33±18.9	19±8.8	3±4.7	42±11.8
mc75sn	38±17.7	100	47±18.9	6±8.8	20	42±11.8
pc78cu	63	100	67±9.4	0	3±4.7	50
pc78sn	0	100	67±9.4	0	0	0
Cont-low	38	48	87±9.4	31±8.8	89±4.7	84±4.7
Cont-high	100	100	100	84	100	100

[a]mc75 and pc78 indicate *P. fluorescens* isolates. 'cu' and 'sn' represent bacterial cell culture and bacterial culture supernatant, respectively. Cont-low and cont-high represent chemical fungicide for respective disease treated at low concentration and high concentration, respectively.

[b]RCB, rice blast (*Magnaporthe grisea*); RSB, rice sheath blight (*Corticium sasaki*); TGM, tomato gray mold (*Botrytis cinerea*); TLB, tomato late blight (*Phytophthora infestans*); WLR, wheat leaf rust (*Puccinia recondita*); BPM, barley powdery mildew (*Erysiphe graminis* f. sp. *hordei*). Each value represents the mean ± standard deviation of three replicates.

that exopolysaccharides production, biofilm formation and swarming motility contribute to the bacterial colonization and stable maintenance of antifungal compounds on plant surface. Antagonistic bacteria are thought to protect plants more effectively against pathogen attack when they successfully colonize the rhizosphere (Weller et al., 1988). The contribution of the biofilm formation and swarming motility to the essential biocontrol traits such as bacterial colonization should be analyzed for our strains in the future. Since *P. fluorescens* pc78 and mc75 were highly effective to control rice sheath blight caused by *C. sasaki*, it would be interesting to investigate if these bacteria could be applied to control other plant diseases caused by the same pathogen.

Several desirable traits of potential biocontrol organisms isolated in this study may be useful to increase and optimize the biological control activity of other biocontrol agent by genetic engineering (Cook, 1993). In addition, the improvement of the biocontrol mechanisms of our isolates by genetic means could be also possible to enhance the biological control activity.

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