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Biological Control of *Rhizoctonia* damping-off of Cucumber by a Transformed *Pseudomonas putida* Strain Expressing a Chitinase from a Marine Bacterium

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Abstract

To construct an effective biological control agent for plant root diseases, we isolated *Pseudomonas putida* strains from the rhizospheres of cucumber plants and used them as hosts of an expression vector for an antipathogenic gene. The antipathogenic gene encoded a chitinase, and was derived from the marine bacterium *Alteromonas* sp. strain 79401. We screened the genome of *P. putida* PaW8 to obtain a strong promoter for the expression of this gene. The chitinase expression vector pKAC9-p07, containing the chitinase gene and the promoter from PaW8, was introduced into *P. putida* strains that could survive well in the rhizosphere. The transformed *P. putida* strains showed chitinase production and suppressed the damping-off of cucumber seedlings in soils infected with the plant-pathogenic fungus *Rhizoctonia solani*. This study demonstrates a strategy for the construction of a biological control agent for practical use in the rhizosphere.

Discipline: Biotechnology
Additional key words: biological control agent, fluorescent pseudomonads, rhizosphere

Introduction

The biological control of plant diseases using antipathogenic microorganisms is one way to reduce the use of agricultural chemicals, which can have a negative impact on environmental ecosystems and human health. So far, many microorganisms have been screened for antipathogenic activity on agar plates and then tested as biological control agents in agricultural fields[17,18,21,25]. In many cases, however, such microorganisms fail to show a sufficient effect on the outbreak of target diseases in the field[10]. To be useful as biological control agents in the field, it is necessary for such microorganisms to have several crucial characteristics. First, they must be able to survive in the environment where they are introduced. A microorganism that grows well on an agar plate often has poor viability in the soil. Second, such microorganisms should contain effective antipathogenic genes that can suppress the activity of pathogens in the field. Third, they must express the antipathogenic genes at the sites where they are expected to work. Based on these criteria, we developed a new strategy to construct a model recombinant biological control bacterium.

Soil bacteria that colonize the same niches in the rhizosphere as plant root pathogens are likely to be suitable hosts for antipathogenic genes. As the first step, we screened for fluorescent pseudomonads, which are common rhizosphere bacteria that can survive in the rhizosphere for long periods. Many fluorescent pseudomonads are important rhizosphere organisms that can promote plant growth by suppressing phytopathogenic microorganisms, producing plant growth hormones, and/or inducing disease resistance in host plants[6,20]. We chose the plant-pathogenic fungus *Rhizoctonia solani*, which causes damping-off of crop seedlings, as the target to test the model biological control agent. This soil-borne pathogen lives in the rhizospheres of many plants and has a significant effect on agricultural crop production, both in field and greenhouse conditions[23,24]. To achieve high levels of antifungal activity in the model biological control agent, we screened for a strong promoter in *Pseudomonas putida* using a promoter-trap vector, and constructed an expression vector in which antifungal genes

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were placed under the control of this strong promoter. We chose a chitinase gene as the antifungal gene. Chitinases are likely to be useful in the biological control of soil-borne fungal pathogens because they degrade chitin, which is a major component of fungal cell walls[3]. There are many chitinolytic microorganisms in both terrestrial and marine environments[8,13], and they play important roles in converting insoluble chitin to bioavailable growth substrates[4]. The marine bacterium *Alteromonas* sp. strain 79401 shows high chitinolytic enzyme activity and suppresses the conidial germination and hyphal growth of plant pathogenic fungi[11]. We used the *chi9* chitinase gene[19] from this strain of *Alteromonas* and introduced it into rhizosphere-colonizing bacteria.

The aim of this study is to develop an approach for constructing effective biological control agents of pathogens living in the rhizosphere. For this purpose we used a long-surviving rhizosphere organism as the host, and an effective expression system for antipathogenic genes.

Materials and methods

1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. They were grown at 30°C in Luria-Bertani (LB) medium or M9 medium. If necessary, kanamycin (50 μg/mL final concentration) or rifampicin (100 μg/mL final concentration) was added to the medium. For the selection and growth of rifampicin-resistant mutant strains of *P. putida*, cells were grown in 1/3 KMB medium[22] containing 100 μg/mL rifampicin.

2. Isolation of fluorescent pseudomonads from cucumber rhizospheres

Cucumber plants (*Cucumis sativus* L., cv. Shimoshiradzu) were grown in pots with fertilized granulated soils (Kureha Corporation, Tokyo, Japan) for 28 days. The plants were excavated, and after shaking off the loosely adhering soil, the tightly adhering soil was collected as rhizosphere soil samples. The samples were disrupted in a mortar with five volumes of sterilized water, and the resulting soil suspensions were diluted and spread on plates of selective agar medium P-2[12] for the isolation of fluorescent pseudomonads. The plates were incubated at 30°C until fluorescent colonies appeared. A total of 140 fluorescent colonies were picked up under UV light and stored at -80°C. To classify the isolated strains, we performed repetitive sequence-based polymerase chain reactions (rep-PCRs) according to the method of Louws et al[14]. The BOX-A1R primer (5'-CTACGGCAA-

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant properties and remarks	References
Escherichia coli		
DH5α	(used for subcloning)	IFO7030
Pseudomonas putida isolates		
PaW8	a plasmid-cured strain of *P. putida* PaW1[26]	Worsey & Williams[26]
101	isolated from cucumber rhizosphere	This study
104	isolated from cucumber rhizosphere	This study
203	isolated from cucumber rhizosphere	This study
505	isolated from cucumber rhizosphere	This study
PaW8R	spontaneous rifampicin resistant mutant of PaW8	This study
101R	spontaneous rifampicin resistant mutant of 101	This study
104R	spontaneous rifampicin resistant mutant of 104	This study
203R	spontaneous rifampicin resistant mutant of 203	This study
505R	spontaneous rifampicin resistant mutant of 505	This study
101-97	101 harboring pKAC9-p07	This study
203-97	203 harboring pKAC9-p07	This study
PaW8-97	PaW8 harboring pKAC9-p07	This study
Plasmids		
pKT230	Km^r, Sm^r	Bagdasarian *et al.*[1]
pEGFP-V1	*gfp*$^+$, Km^r	Numata *et al.*[19]
pPTEGFP	*gfp*$^+$, Km^r	This study
pPTEGFP-plac	contains the *E. coli lac* promoter *gfp*$^+$, Km^r	This study
pChi9	Km^r, *chi9*	Numata *et al.*[19]
pKAC9-plac	contains the *E. coli lac* promoter Km^r, *chi9*	This study
pKAC9-p07	contains the promoter from *P. putida* PaW8 Km^r, *chi9*	This study

Km^r: kanamycin resistant, Sm^r: streptomycin resistant, *chi9*: a chitinase gene from *Alteromonas* sp. 79401[11], *gfp*$^+$: a green fluorescent protein gene from pEGFP-V1[19].

GGCGACGCTGACG-3') was used with genomic DNA extracted from each strain as a template. All the isolated strains were classified into four groups. We selected the four strains 101, 104, 203, and 505 as representative strains from each group. The representative strains were identified using their 16S ribosomal RNA gene (16S rDNA) sequences. A fragment of the 16S rDNA was amplified from each strain using the 63f primer (5'-CAGGCCTAACACATGCAAGTC-3') and the 1387r primer (5'-GGGCGGWGTGTACAAGGC-3') designed by Marchesi et al[15]. DNA sequencing reactions were performed using an ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystesm, Foster City, CA, USA), and the products were analyzed with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The obtained DNA sequences were used in BLAST searches of the DNA Data Bank of Japan (DDBJ)[9]. Based on sequence homologies, all the representative strains were identified as *P. putida* (data not shown).

3. Assessment of the survival of the representative *P. putida* strains

To assess the survival of the representative strains in the rhizospheres of cucumber seedlings, we selected four spontaneous rifampicin-resistant mutants (101R, 104R, 203R, and 505R) by growing them on 1/3 KMB agar plates containing 100 μg/mL rifampicin at 30°C. To prepare cultures for inoculating seedlings, one rifampicin-resistant mutant of each representative strain and three wild (rifampicin-sensitive) colonies derived from the other three strains were grown separately in 1/3 KMB medium at 30°C. Rifampicin (100 μg/mL) was added to the culture of the rifampicin-resistant mutant. Each culture was adjusted to an optical density at 600 nm of 1.0, and then the cultures were mixed. A mixed culture was prepared for each of the rifampicin-resistant mutant strains (101R, 104R, 203R, and 505R). Germinating cucumber seeds were soaked in the mixtures for 1 h, and then sown in nursery boxes with fertilized granulated soils (Kureha Corporation). At 1 h, 24 h, 72 h, and 168 h after inoculation, some of the seeds were transferred to 50 mL plastic tubes containing sterilized water, and shaken vigorously using a vortex mixer. The resulting suspensions were diluted and spread on 1/3 KMB agar plates containing 100 μg/mL rifampicin and 100 μg/mL cycloheximide. The plates were incubated at 30°C for 24 h. The numbers of rifampicin-resistant colony-forming units (CFU) were determined for each root system.

4. Screening for strong promoters in *P. putida*

To obtain *P. putida* promoters for the efficient expression of an antipathogenic gene in transformed *P. putida* cells, we constructed a promoter-trap vector (pPTEGFP) containing a promoterless green fluorescent protein (GFP)

Fig. 1. Construction of the broad-host-range, promoter-trap vector pPTEGFP

gene as the reporter. The vector was constructed by the ligation of fragments derived from the plasmids pEGFP–V1[19] and pKT230[1] using a TAKARA ligation kit (TAKARA, Shiga, Japan) (Fig. 1). The chromosomal DNA of *P. putida* strain PaW8 was digested with *Sau*3AI and then separated by agarose gel electrophoresis. DNA fragments in the size range 0.1–1.0 kb were recovered from the gel and ligated into pPTEGFP, which had been digested with *Bgl*II and treated with shrimp alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany). The ligated DNAs were introduced into *P. putida* PaW8 cells by electroporation, and transformants were selected on LB agar plates containing 50 μg/mL kanamycin. Promoter-trapped transformants were identified as colonies with bright GFP fluorescence using a Leica MZFL3 microscope with a GFP-plus filter set (Leica Microsystems GmbH, Wetzlar, Germany). A positive control for strong GFP expression was constructed by ligating the *Escherichia coli lac* promoter (the *Eco*47III-*Not*I fragment of pEGFP-V1Plac[19]) into the *Hpa*I-*Not*I sites of pKT-230. The resulting plasmid was named pPTEGFP-plac.

5. Construction of the chitinase gene expression vector

A chitinase gene expression vector was constructed to evaluate the level of chitinase gene expression driven by the trapped promoter from clone no. 7, which was the pPTEGFP-derived transformant with the brightest GFP fluorescence. The vector pPTEGFP-p07 was extracted from clone no. 7

and its GFP gene was replaced with the promoterless chitinase gene (*chi9*) from *Alteromonas* sp. strain 79401. The source of the *chi9* gene was a *Sph*I-*Not*I fragment of the plasmid pChi9[19], and the resulting plasmid was named pKAC9-p07 (Fig. 2). We also sequenced the promoter insert from pPTEGFP-p07. The *P. putida* strains that had been isolated from cucumber rhizospheres were transformed with pKAC9-p07 grown on LB plates containing 0.1% colloidal chitin and 50 μg/mL kanamycin. The plates were incubated at 30°C for six days. We then assessed the production of chitinase by the appearance of visibly cleared zones around the colonies, which indicated the degradation of the colloidal chitin.

6. Assay for chitinase activity of the transformed *P. putida* strains

The cells were grown aerobically at 30°C and 150 rpm in LB medium for 72 h. The cultures were then centrifuged for 3 min at 15,000 rpm and 25°C, and the supernatants were collected. The protein concentrations of the supernatants were determined by the Bradford method[5], using bovine serum gamma globulin as a standard. The chitinase activity in each culture supernatant was determined using the fluorescent substrate 4-methylumbelliferyl-*N*,*N*',*N*''-triacetylchitotrioside (Sigma-Aldrich, St. Louis, Missouri, USA) and the method described by Miyashita et al[16].

7. Bioassay for biological control activity

R. solani strain AG-4 was used to test the abilities of the transformed and untransformed *P. putida* strains to inhibit damping-off. *R. solani* AG-4 was grown in potato dextrose agar (PDA) medium or in potato dextrose broth (PDB) at 25°C. Cucumber seedlings were treated with the bacterial suspensions in the following three ways. 1) For the "soak-and-drop inoculation" method, germinating cucumber seeds were soaked in bacterial suspension (approximately 1×10^8 CFU/mL) for 1 h before seeding in nursery boxes without any pathogen. Three days later, the seedlings were transplanted into nursery boxes containing soil that had been pre-inoculated with 0.05% (vol/vol) *R. solani* AG-4 mycelium. Immediately after and three days after transplantation, a bacterial suspension (1 mL; approximately 1×10^8 CFU/mL) was dropped onto each seedling. 2) For the "soaked inoculation" method, germinating cucumber seeds were soaked in bacterial suspension (approximately 1×10^8 CFU/mL) for 1 h, then seeded in nursery boxes containing soil that had been pre-inoculated with 0.05% (vol/vol) *R. solani* AG-4 mycelium. 3) For the "drop inoculation" method, 3-day-old cucumber seedlings were transplanted into boxes containing soil that had been pre-inoculated as described above, and then 3 mL of bacterial suspension (approximately 1×10^8 CFU/mL) was dropped onto each seedling.

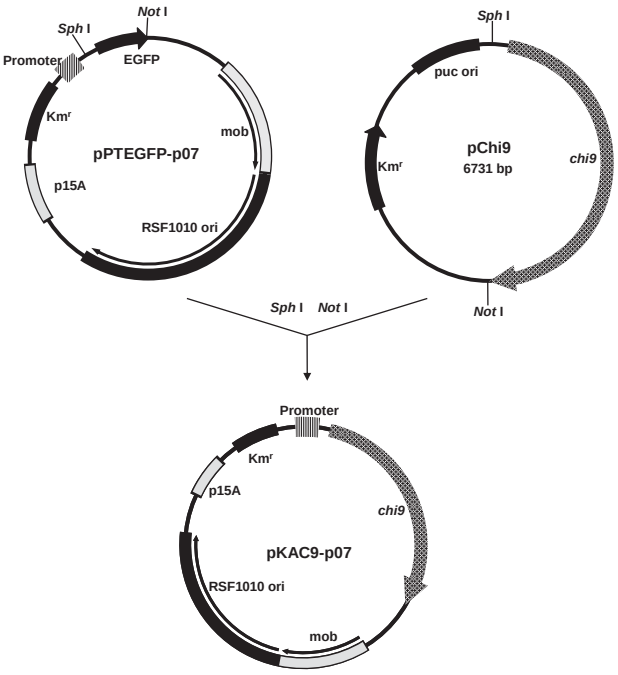

Fig. 2. Construction of the chitinase expression plasmid pKAC9-p07

For each of the three methods described above, we evaluated the disease inhibition ability of each strain one week after transplanting the seedlings into soil contaminated with *R. solani* AG-4. We used a damage index of damping-off as follows: 0 = no browning of the hypocotyls; 1 = some browning of the hypocotyls; 2 = damping-off. Disease incidence (%) was calculated using the following formula: Disease incidence (%) = $100 \times (0 \times n_0 + 1 \times n_1 + 2 \times n_2) / [2 \times (n_0 + n_1 + n_2)]$, where n_{0-2} are the numbers of index-0, -1 and -2 seedlings. We then estimated the disease inhibition activity of each treatment with each *P. putida* strain: Disease inhibition (%) = $100 \times (DI_C - DI_T) / DI_C$, when DI_C was the disease incidence in the control only with pathogen, and DI_T was the disease incidence in the treatment with each *P. putida* strain. The disease incidence in the controls only with pathogen were 34%, 60%, and 60%, respectively, for the "soak-and-drop inoculation," "soak inoculation," and "drop inoculation" methods.

Results

1. Isolation of fluorescent pseudomonads from cucumber rhizospheres and survival tests in the rhizosphere

A total of 140 fluorescent colonies were isolated from the cucumber rhizospheres with the fertilized soils. The isolated strains were classified into four groups by rep-PCR. Representative strains from each group, 101, 104, 203, and

505, were identified as *P. putida* by the homology of their 16S rDNA sequences (data not shown).

We compared the survival rates of these fluorescent *P. putida* strains in the cucumber rhizospheres. To make it easier to detect each strain in the soil, spontaneous rifampicin-resistant mutants (101R, 104R, 203R, and 505R) were selected on 1/3 KMB agar plates containing rifampicin. Each strain was tested for its ability to compete and survive in the rhizosphere by inoculating germinating cucumber seeds with a mixture containing the rifampicin-resistant mutant of that strain and rifampicin-sensitive isolates of the other three *P. putida* strains. Then, the survival of the rifampicin-resistant strain was monitored for up to seven days. The strains 101R and 203R showed the best survival rates during the experimental period. At seven days after inoculation, the levels of 101R and 203R were 7.2×10^5 CFU/root and 9.4×10^5 CFU/root, respectively (Fig. 3). The strains 104R and 505R were less competitive in this experiment. Consequently, we used the parental strains 101 and 203 as hosts for the antipathogenic gene expression vector.

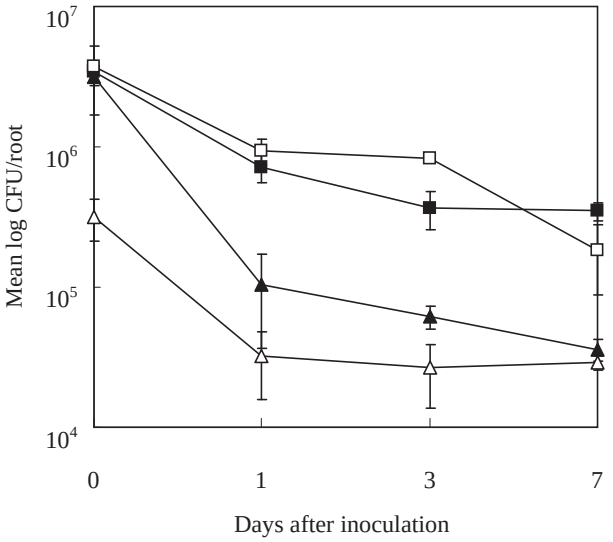

Fig. 3. Survival of *P. putida* strains in the cucumber rhizosphere
—■— : 101R, —△— : 104R, —□— : 203R, —▲— : 505R.

2. Screening for strong promoters in *P. putida* PaW8 and sequence analysis of the GFP-expressing clone

We isolated promoters from *P. putida* PaW8 genomic DNA by screening for GFP-fluorescent colonies after shotgun cloning and transformation using the promoter-trap vector pPTEGFP. Out of approximately 200 clones, ten exhibited GFP fluorescence, and only one (no. 7) fluoresced strongly under the fluorescence microscope (Fig. 4). The vector (pPTEGFP-p07) was isolated from clone no. 7. The insert was sequenced and subjected to a BLAST search of the DDBJ. The DNA insert (226 bp) showed 98% homology with each of the genome sequences from other *P. putida* strains (*P. putida* KT2440: 32180–32403; *P. putida* W619: 48872–49095; *P. putida* GB-1: 44938–45161; *P. putida* F1: 44156–44379). A typical promoter sequence with -35 (TTGCCA) and -10 (GACAAT) consensus sequences was found between bases 136 and 163 in the insert.

3. Construction of transformed *P. putida* strains expressing chitinase

The *P. putida* strains PaW8 (a laboratory strain of *P. putida*), 101, and 203 were transformed with the chitinase gene expression vector pKAC9-p07, and the transformed strains were named PaW8-97, 101-97, and 203-97. Each of these strains formed clear zones around their colonies on colloidal chitin plates, while the parental strains (PaW8, 101, and 203) did not (data not shown). The chitinase activity in supernatants derived from liquid cultures of the transformed strains was significantly higher than that in supernatants derived from cultures of the parental strains (Fig. 5). Furthermore, strains PaW8-97 and 203-97 produced almost twice as much chitinase activity in their culture supernatants as strain 101-97.

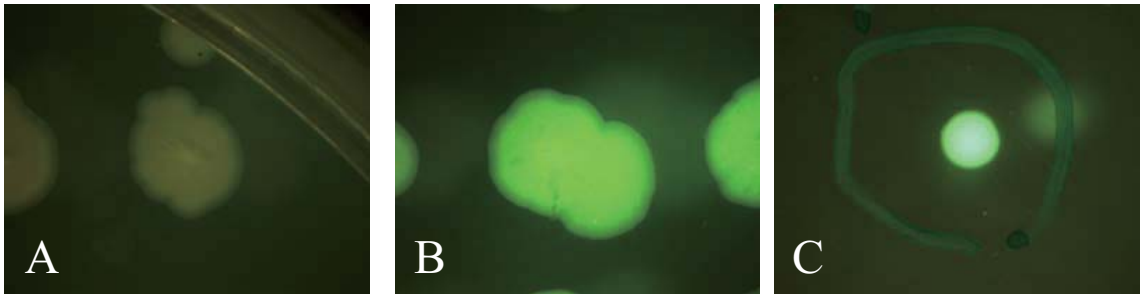

Fig. 4. Expression of GFP fluorescence in wild-type *P. putida* PaW8 (A) and in lines transformed with pPTEGFP-plac (B) and pPTEGFP-p07 (C)
Colonies were photographed using a GFP-Plus fluorescence filter set consisting of an excitation filter (480/40 nm) and a barrier filter (510 nm).

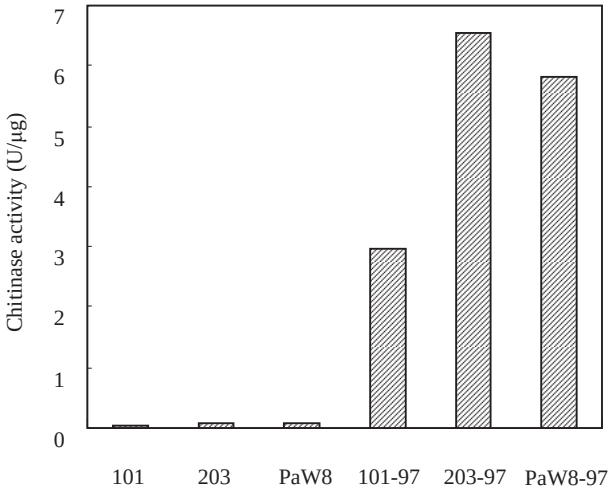

Fig. 5. Chitinolytic enzyme activity in the culture supernatants of *P. putida* strains

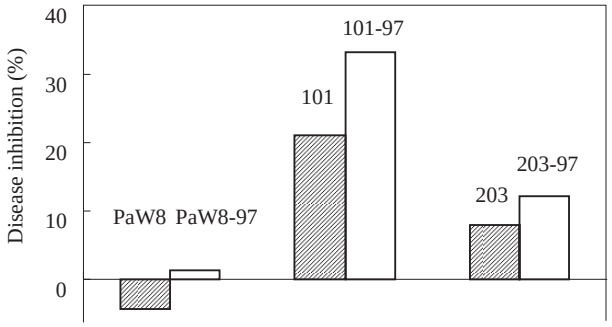

Fig. 6. Effects of wild-type and transformed *P. putida* strains on the damping-off of cucumber seedlings infected with *R. solani* AG-4

The plants were inoculated with *P. putida* using the "soak-and-drop inoculation" method. The hatched boxes represent the wild strains and the open boxes represent the strains harboring pKAC9-p07.

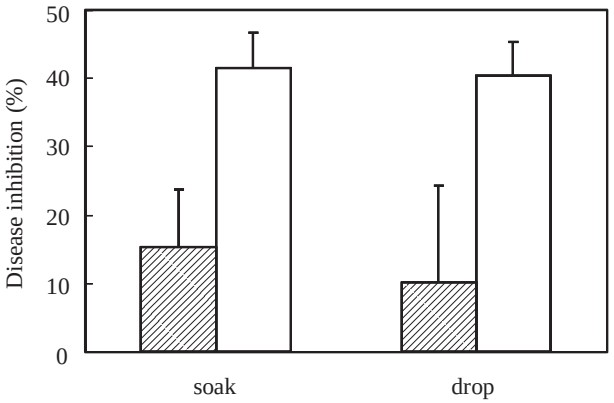

Fig. 7. Effects of *P. putida* strains 101 and 101-97 on the damping-off of cucumber seedlings infected with *R. solani* AG-4

Plants were inoculated with the bacteria using either the "soak inoculation" method or the "drop inoculation" method. The hatched boxes represent the wild strain 101 and the open boxes represent 101-97, harboring pKAC9-p07. The error bar indicates the standard deviation (n = 3).

4. Biological control of damping-off of cucumber by *P. putida* strains harboring pKAC9-p07

A bioassay was used to compare the activity of the transformed *P. putida* strains in controlling the damping-off of cucumber seedlings caused by *R. solani*. Three different inoculation methods were tested. First, to maximize the biological control activity of the inoculated strains, we performed a "soak-and-drop inoculation" method, in which the *P. putida* cultures were applied three times to the cucumber seedlings (Fig. 6). The non-transformed *P. putida* strain 101 showed some disease inhibition activity (20%), and the transformation of this strain with pKAC9-p07 significantly enhanced its activity. Among the strains tested, 101-97 exhibited the highest level of disease inhibition activity (30%) when compared with control plants that were inoculated with only the pathogen. Strains 203, 203-97, PaW8, and PaW8-97 were less effective in inhibiting *Rhizoctonia* damping-off (Fig. 6). The most effective strain (101-97) and its parental strain (101) were further tested using more practical and simpler inoculation methods, the "soak inoculation" and "drop inoculation" methods. In both cases, strain 101-97 inhibited 40% of damping-off in comparison with plants inoculated with only the pathogen (Fig. 7).

Discussion

For many soils, suppressing plant diseases has a biological origin, since sterilization frequently reduces this effect[2]. Therefore, many researchers have tried to isolate microorganisms from soils that inhibit disease, and have tested the isolates' abilities to antagonize the pathogens in laboratory assays. However, it is often the case that there is little correlation between biological control efficacy obtained in laboratory assays and in field studies[7]. For the development of effective strategies to control soil-borne diseases, it is important to use bacteria such as the bacteria used in this study, which occupy a niche similar to that of the pathogen in the environment[27]. In this study, we constructed a chitinase expression vector, pKAC9-p07, as an antipathogenic gene expression vector, and introduced it into fluorescent *P. putida* strains isolated from cucumber rhizospheres. These transformed strains were expected to compete in the same ecological niche as the pathogen, *R. solani*, and suppress *Rhizoctonia* damping-off of cucumber seedlings. The transformed *P. putida* strains 101-97 and 203-97 (strains 101 and 203 transformed with pKAC9-p07) could decompose colloidal chitin on agar plates (data not shown), and

showed significantly higher chitinase activity than their parental strains in liquid culture (Fig. 5). The chitinase activity in the culture supernatants of strains 203-97 and PaW8-97 was almost twice as high as that of strain 101-97 (Fig. 5). However, the disease inhibition activity of strains 203-97 and PaW8-97 was much lower than that of strain 101-97 (Fig. 6), even though the survival of strain 203R in the cucumber rhizosphere was almost the same as that of strain 101R. The results suggest that strain 203 cannot produce enough antifungal activity (chitinase activity) in the soil. On the other hand, the viability of PaW8R in the soil was low (data not shown), and this is a likely explanation for its low level of disease inhibition activity in the cucumber rhizosphere. Thus, we have demonstrated the construction of an effective biological control agent for a plant pathogen. The basic approach is as follows. Firstly, we should use a bacterium that occupies the same ecological niche as the target pathogen as the host for the antipathogenic vector. Secondly, the antipathogenic gene should be placed under the control of a strong promoter that functions efficiently at the time and location where disease inhibition is needed.

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