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Assessment of the Effects of Genetically Modified *Pseudomonas* spp. Expressing Chitinase on the Soil Microbial Community in the Cucumber Rhizosphere

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Abstract

In our previous study, a genetically-modified (GM) rhizosphere bacterium, *Pseudomonas putida* strain 101-97 harboring the chitinase-expression vector pKAC9-p07, showed over-production of chitinase and suppressed the damping-off of cucumber seedlings caused by the plant-pathogenic fungus *Rhizoctonia solani*. To assess the effects of the GM bacterium on other soil microorganisms, we monitored the microbial population in the cucumber rhizosphere after inoculation with the GM bacterium by culture-dependent and -independent methods. In the culture-dependent analysis, total bacterial and fungal populations in the cucumber rhizosphere did not differ after inoculation with either the GM bacterium or its parental strain, *P. putida* 101R. In the culture-independent analysis, we determined changes in the microbial communities by PCR-denaturing gradient gel electrophoresis (DGGE). The DGGE profiles of bacterial 16S rDNA genes differed significantly depending on whether they had been inoculated with the GM bacterium or its parental strain, whereas the profiles of fungal 18S rDNA genes remained similar.

Discipline: Biotechnology
Additional key words: biological control agent, fluorescent pseudomonads, PCR-DGGE, *Pseudomonas putida*

Introduction

The biological control of plant diseases using microorganisms with antipathogenic activities is one way to reduce the application of agricultural chemicals for sustainable agriculture. Consequently, disease-suppressive soils, which possess biological activities that suppress plant diseases, have attracted interest[7]. Some forms of disease suppression may be caused by bacteria of the genus *Pseudomonas*, which aggressively colonize root surfaces[19]. Several 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[5, 15, 19]. To develop effective strategies to control soil-borne diseases, it is important to use bacteria that occupy a niche similar to that of the pathogen in the environment[25]. In a previous study, we screened fluorescent *Pseudomonas putida* strains from the rhizosphere of cucumber plants and used them as host strains to construct a genetically-modified (GM) biological control agent, which harbored an antipathogenic gene expression vector. To achieve high antifungal activity in the biological control agent, we screened for a strong promoter in *Pseudomonas putida* strain PaW8 to control the expression of the *chi9* chitinase gene[8] from the marine bacterium *Alteromonas* sp. strain 79401 in the antipathogenic gene expression vector, pKAC9-p 07[14]. Chitinases are useful in the biological control of soil-borne fungal pathogens because they degrade chitin, a major component of fungal cell walls[3]. The GM bacteria were tested for activity against the plant-pathogenic fungus *Rhizoctonia solani*, which causes the damping-off of crop seedlings. The pathogen lives in the rhizosphere of many plants and has significant effects on crop production, both in the field and under greenhouse conditions[4, 23]. The GM *P. putida* strain harboring pKAC 9-p07, which could decompose colloidal chitin on agar

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plates, showed significantly increased chitinase activity, and suppressed the growth of hyphae of *R. solani* as well as the development of damping-off[14].

Although biological control agents with antipathogenic activities may have fewer negative impacts on human health than traditional agrochemicals, concerns have been expressed about the potential environmental effects of GM bacteria on rhizosphere microorganism communities. The aim of this study was to assess the effects of the GM biological control agent on microbial diversity in the soil. We used quantitative methods based on colony isolation on selective agars, and monitored the 16S rDNA and 18S rDNA DGGE (PCR-denaturing gradient gel electrophoresis) profiles of rhizosphere soil samples from cucumber roots.

Materials and methods

1. Bacterial strains, plasmids, culture conditions, and soils

The bacterial strains and plasmids used in this study are listed in Table 1. Cells were grown at 30°C on a Luria-Bertoni (LB) medium. If necessary, kanamycin (50 μg/mL final concentration) and/or rifampicin (100 μg/mL) were added to the medium. For the selection and growth of rifampicin mutant strains of *P. putida*, cells were grown in a 1/3 KMB medium[20] containing rifampicin. To remove contaminant fungi from unsterilized soil samples, we added 100 μg/mL cycloheximide to the medium if needed. The fertilized granulated soil was bought from Kureha Corporation (Tokyo Japan), and field soil was collected from a field at the National Institute for Agro-Environmental Sciences (NIAES) Japan.

2. Population dynamics of *P. putida* strains in sterile soil

Strain PaW8[24] is a commonly used laboratory strain of *P. putida*, while strain 101 had been isolated from cucumber rhizosphere in our previous study[14]. The GM *P. putida* strains harboring the pKAC9-p07 plasmid, PaW8-97 and 101-97, showed chitinase production and suppressed the damping-off of cucumber seedlings in soils infected with *Rhizoctonia solani* AG-4[14]. To monitor the population dynamics of the biological control agents in soil after inoculation, we used the spontaneous rifampicin-resistant mutants *P. putida* PaW8R and 101R as recipients for the chitinase expression vector pKAC9-p07. Three milliliters of suspensions (approximately 1×10^8 CFU/mL) of *P. putida* strains 101R-97 or PaW8R-97 carrying pKAC9-p07 were added to sterilized vials containing 3 g of soil. The vials were then kept in 28°C. One hour, and 1, 3 and 7 days respectively after inoculation, 5 mL of sterile water was added to each vial, and the contents were mixed with a vortex mixer. The soil suspensions were then diluted and spread on plates of 1/3 KMB with 100 μg/mL rifampicin to select rifampicin-resistant colonies, or with 100 μg/mL rifampicin and 50 μg/mL kanamycin to select chitinase plasmid-harboring colonies. Plates were incubated at 30°C for 48 h, before the colonies on the plates were counted to estimate the *P. putida* populations.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant properties and remarks	References
Pseudomonas putida isolates		
PaW8	A plasmid-cured strain of *P. putida* PaW1	Worsey & Williams[24]
101	Isolated from cucumber rhizosphere	Ohno *et al*.[14]
PaW8R	Spontaneous rifampicin-resistant mutant of PaW8	Ohno *et al*.[14]
101R	Spontaneous rifampicin-resistant mutant of 101	Ohno *et al*.[14]
PaW8-97	PaW8 harboring pKAC9-p07	Ohno *et al*.[14]
101-97	101 harboring pKAC9-p07	Ohno *et al*.[14]
PaW8R-97	PaW8R harboring pKAC9-p07	This study
101R-97	101R harboring pKAC9-p07	This study
Plasmids		
pKT230	Km^r, Sm^r	Bagdasarian *et al*.[2]
pKAC9-p07	Km^r, *chi9*	Ohno *et al*.[14]

Km^r, kanamycin-resistant; Sm^r, streptomycin-resistant; *chi9*, chitinase gene from *Alteromonas* sp. 79401[8];

3. Monitoring the colonization of the cucumber rhizosphere by the GM *P. putida*

Germinating cucumber seeds (*Cucumis sativus* L., cv. Shimoshiradzu) were soaked in suspensions of *P. putida* 101R-97 (approximately 1×10^8 CFU/mL) for 1 hour, and grown in pots with 270 g fertilized granulated soil (Kureha Corporation) in a closed system greenhouse at 25°C. One hour and 1, 3 and 7 days respectively after inoculation, the plants were excavated, and after shaking off loosely adhering soil, the tightly adhering soil was collected as rhizosphere soil samples. Bulk soil control samples were taken at distances greater than 7 cm from any roots. The soil suspensions were diluted and spread on plates of 1/3 KMB with 50 μg/mL kanamycin and 50 μg/mL cycloheximide to select kanamycin-resistant bacteria (plasmid-containing bacteria) in the rhizosphere, or with 100 μg/mL rifampicin, 50 μg/mL kanamycin, and 50 μg/mL cycloheximide to select kanamycin- and rifampicin-resistant bacteria (plasmid-containing strains) in the samples. To assess the potential of bacteria to be transformed with DNA derived from the GM biological control agents, we inoculated soils with DNA (26 μg/g dry soil) extracted from *P. putida* 101R-97.

4. Analysis of bacterial and fungal population dynamics in the rhizosphere after inoculation with GM bacteria

Germinating cucumber seeds were soaked in suspensions of strains 101R and 101R-97 (approximately 1×10^8 CFU/mL) for 1 hour, and grown in pots with 270 g field soil in a closed system greenhouse at 25°C. One hour and 2, 7 and 12 days respectively after inoculation, the plants were excavated, and after shaking off loosely adhering soil, tightly adhering soil was collected as rhizosphere soil samples. To characterize the population dynamics of microorganisms, viable bacteria colonies were counted by a dilution plating technique on a PTYG[1] medium with 100 μg/mL cycloheximide. Total fungal CFUs were determined by dilution plating on Martin's rose Bengal medium[11].

DNA was extracted from soil samples (400 mg) using the FastDNA Spin kit for soil (Bio101, Vista, CA, USA) according to the manufacturer's recommendations, except that 16 mg skim milk was added to the soil slurry. Skim milk had to be used as a competitor during DNA extraction from certain types of Andisol because of its high DNA adsorption to clay particles[9]. Bacterial 16S rRNA genes and fungal 18S rRNA genes were amplified from soil DNA by PCR for DGGE. Amplification of the DNA was performed with an iCycler™ (Bio-Rad laboratories, Hercules, CA, USA). The bacterial 16 S rRNA genes were amplified with the primers GC-338 F[16]/907R[10], and the fungal 18S rRNA genes were amplified with the primers FF390/GC-FR1[22]. The PCR mixtures (50 μL) contained 0.2 μmol/L of each primer, 200 μmol/L of each dNTP, 1.5 mmol/L of $MgSO_4$, 5 μL of 10 × PCR buffer, 1.0 U of KOD-plus DNA polymerase (TOYOBO, Osaka Japan), 100 ~ 200 ng of template soil DNA, and sterile water. The PCR program was as follows: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 68°C for 1 min. The PCR products were separated by electrophoresis on 1.0% agarose gels to ascertain their size and quality.

DGGE was performed using the D code system (Bio-Rad), based on the method of Muyzer *et al*[13]. PCR products were loaded on 6 % (w/v) polyacrylamide gels (37.5:1, acrylamide: bisacrylamide) in a TAE buffer. Gels had a denaturing gradient ranging from 45 to 65 % and from 30 to 55 % for bacterial 16S rDNA and fungal 18S rDNA, respectively (where 100% denaturant contains 7 M urea and 40 % formamide). Electrophoresis was carried out at 60°C and 65 V for 21 h. Gels were stained with SYBR Green I (1:10,000 dilution; FMC Bioproducts, Philadelphia, PA, USA) for 30 min, photographed, scanned, and analyzed with a Molecular Imager FX (Bio-Rad). Banding patterns of DGGE profiles were analyzed to compare changes of microbial communities after the inoculation of *P. putida* strains. The presence or absence of bands was converted into binary data (1 and 0 respectively for the presence and absence of each band). Multidimensional scaling (MDS) maps were generated using the R software (ver. 2. 4. 1)[18] and we generated dissimilarity matrices based on the Dice index[6]. Clustering was performed using UPGMA (unweighted pair group method using averages) and the DARwin software 5.0[17]. Bootstrap analyses were performed using 1,000 replicates.

Results

1. Survival and plasmid stability of GM *P. putida* strains in soil

In order to demonstrate biological control activity in the field, it is necessary for GM bacteria carrying a vector with an antipathogenic gene to survive in the environment and maintain the vector with high stability. In our previous study, we screened the rhizosphere of cucumber plants for fluorescent pseudomonads as potential vector hosts. In the present study, we compared the survival rates and plasmid stabilities of the GM *P. putida* strain 101R-97 and the typical laboratory strain PaW8R-97 in sterile soil (Fig. 1). Over 1 week following inocu-

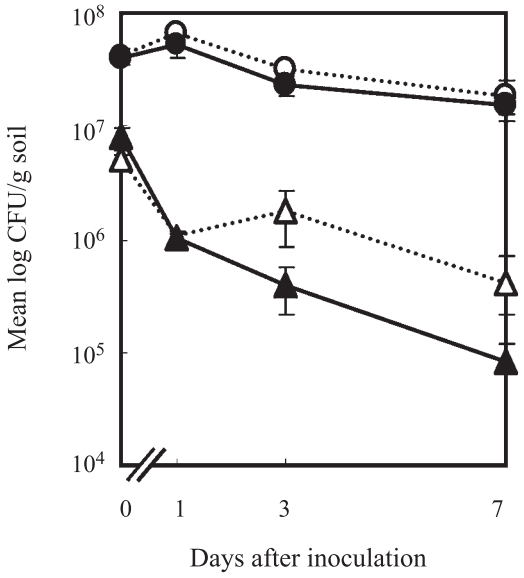

Fig. 1. Population dynamics of rifampicin-resistant bacteria (open symbols) and of bacteria resistant to both rifampicin and kanamycin (solid symbols) after inoculation with strain PaW8R-97 (triangles) or with strain 101R-97 (circles)

Error bars represent standard deviations (n=3).

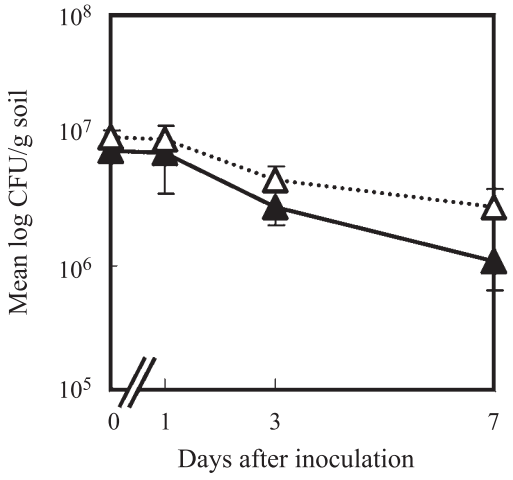

Fig. 2. Detection of kanamycin-resistant (open triangles) and rifampicin- and kanamycin-resistant bacteria in the rhizosphere (solid triangles) after inoculation with strain 101 R-97

Error bars represent standard deviations (n=3).

lation, the population level of strain 101R-97 remained at approximately 10^7 CFU/g soil, whereas that of strain PaW8R-97 declined from 10^7 to 10^5 CFU/g soil, indicating the improved adaptability of strain 101R-97 to the soil environment compared to the laboratory strain PaW 8R-97. The proportion of plasmid-cured colonies was given by the number of rifampicin-resistant colonies minus the rifampicin- and kanamycin-resistant colonies, while the plasmid-curing rate in strain 101R-97 was significantly lower than that in strain PaW8R-97 (Fig. 1).

In the cucumber rhizosphere, the population level of strain 101R-97 declined from 8×10^6 to 1×10^6 CFU/g soil within 7 days, indicating that more than 10% of the bacteria had survived (Fig. 2). Control samples taken at a distance of more than 7 cm from any root contained undetectable levels of GM bacteria, which evidently did not colonize the soil or transmit genetic information during the experimental period (data not shown). After inoculation of the soil with free DNA extracted from strain 101R-97, no increase of the number of kanamycin-resistant colonies was observed (data not shown).

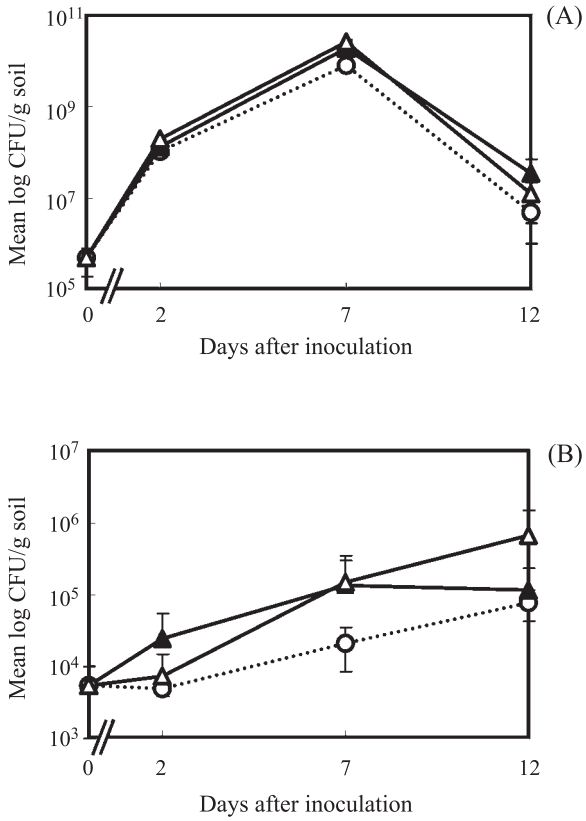

Fig. 3. (A) Bacterial and (B) fungal population dynamics in the rhizosphere after inoculation with P. putida strains 101R-97 (solid triangles) 101R (open triangles), and without inoculation (open circles)

Error bars represent standard deviations (n=3).

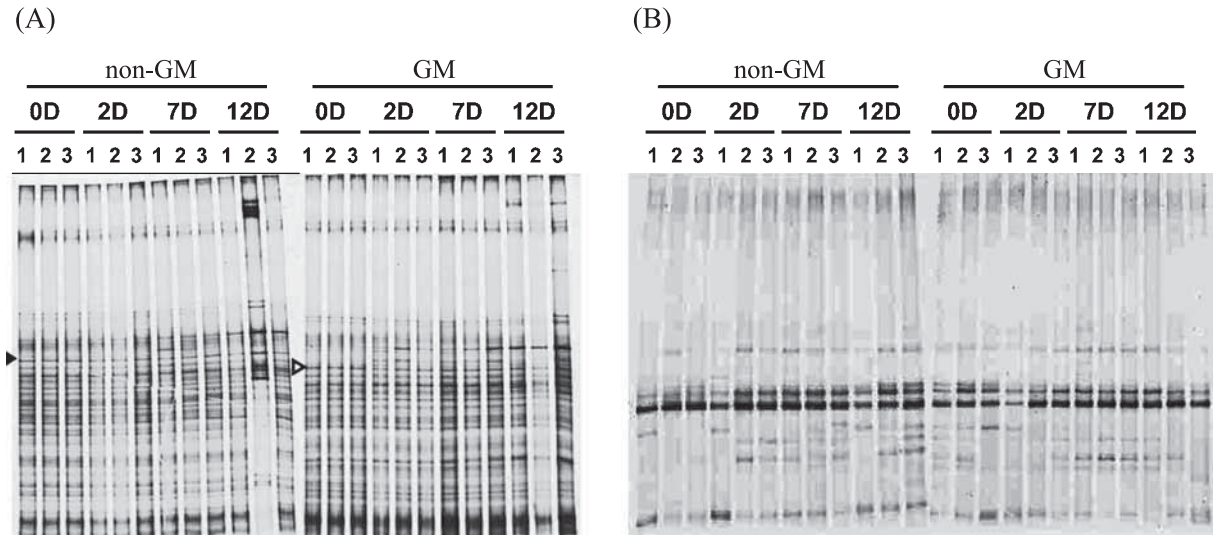

Fig. 4. Changes in the DGGE profiles of bacterial 16S rDNA (A) and fungal 18S rDNA (B) collected from rhizosphere soil after inoculation with *P. putida* strains

The bands of strains 101R and 101R-97 are indicated by black and white arrows, respectively. Non-GM, inoculation with strain 101R; GM, inoculation with strain 101R-97. 0D, immediately after inoculation; 2D, 2 days after inoculation; 7D, 7 days after inoculation; 12D, 12 days after inoculation. Numbers on lanes indicate the trial number.

2. Changes of microbial populations in the rhizosphere

Bacterial and fungal CFUs per gram of rhizosphere soil showed no significant differences when the soil had been inoculated with *P. putida* 101R as opposed to strain 101R-97 (Fig. 3). The populations of bacteria in the rhizosphere increased for 7 days after inoculation with *P. putida*, and then decreased after 12 days. In contrast, the population of fungi generally increased during the entire experimental period. We also characterized changes in the soil communities following inoculation with the two *P. putida* strains by PCR-DGGE analysis based on bacterial or fungal ribosomal DNA genes, a method that does not require cultivation of these communities (Fig. 4). The banding patterns obtained were transformed to numerical data for further statistical analysis. Two-dimensional plots of MDS scores for 16S and 18S rDNA are shown in Figs. 5A and 5C, respectively. The closer together the points, the more similar the DGGE banding patterns they represent. In the MDS map of the 16S rDNA profiles, the samples from rhizosphere soils inoculated with strain 101R and those inoculated with strain 101R-97 were separate (Fig. 5A). In contrast, their positions in the map of 18S rDNA profiles were not clearly distinguished (Fig. 5C). In the UPGMA dendrogram based on 16S rDNA, samples of the plots inoculated with strains 101R and 101R-97 were clearly separated into two major clusters (Fig. 5B), but this was not the case in the dendrogram based on 18 S rDNA (Fig. 5D).

Discussion

In sterile soil, strain 101R-97 showed a higher survival rate than strain PaW8R-97 (Fig. 1). Strain 101 (a parental strain of 101R and 101R-97) is an environmental isolate from the rhizosphere of cucumber plants, whereas strain PaW8 (a parental strain of PaW8R and PaW8R-97) is a laboratory strain. Thus, the strains derived from 101 might be better adapted to soil environments than derivatives from the laboratory strain PaW8. Obviously, biological control agents need to survive in the soils to which they are applied. To efficiently suppress the damping-off of cucumber seedlings caused by *Rhizoctonia solani*, strain 101R-97 must survive in cucumber rhizosphere for almost one week after inoculation, because cucumber seedlings are most sensitive to the pathogen in the early growth stages. In our experiments, about 10% of the inoculated strain 101R-97 survived for one week, as opposed to only 1% of the laboratory strain PaW8R-97 (Fig. 1). In addition, the plasmid (pKAC9-p07) stability in strain 101R-97 (the ratio of kanamycin- and rifampicin-resistant CFUs compared to rifampicin-resistant CFUs) was higher than in strain PaW8R-97 (Fig. 1). Presumably for these reasons, strain 101R-97 previously showed significant disease-suppression activity[14]. We conclude that strain 101 is a promising material for the creation of efficient biologi-

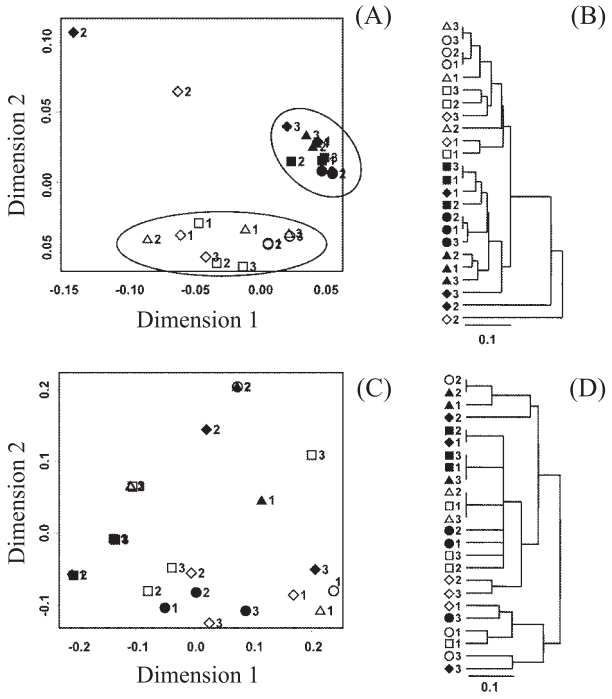

Fig. 5. Two-dimensional plots of MDS analyses of the DGGE profiles of 16S rDNA (A) and 18S rDNA (C), and dendrogram cluster analyses (UPGMA) of the profiles of 16S rDNA (B) and 18S rDNA (D)

Open circles, immediately after inoculation with strain 101R; open triangles, 2 days after inoculation with strain 101R; open squares, 7 days after inoculation with strain 101R; open diamonds, 12 days after inoculation with strain 101R; closed circles, immediately after inoculation with strain 101R-97; closed triangles, 2 days after inoculation with strain 101 R-97; closed squares, 7 days after inoculation with strain 101R-97; closed diamonds, 12 days after inoculation with strain 101R-97. Numbers next to the symbols indicate the trial number. Scale bars under the dendrograms indicate the distance between data points.

cal control agents.

Conversely, to minimize the effects on the natural environment, long-term survival of GM bacteria is not desirable. Kanamycin- and rifampicin-resistant bacterial populations in sterilized soil declined constantly to 10^6 CFU/g soil (1 % of the initial population) at 38 days after inoculation (data not shown). Strain 101R-97 did not diffuse easily in soil, as kanamycin- and rifampicin-resistant bacteria in bulk soil (at a distance of 7 cm from cucumber seedlings) were not detected during the incubation period (data not shown). Furthermore, the potential for genetic transformation in the soil environment seemed very low (data not shown).

The effect of the inoculation of strain 101R-97 on the community structure of bacteria measured by PCR-DGGE differed from that of fungi. In PCR-DGGE analyses targeting bacterial 16S rDNA, the profiles in the soil inoculated with the strain 101R-97, differed from that with strain 101R (Fig. 5). Conversely, no significant differences in terms of bacterial CFUs per gram of rhizosphere soil were observed by plating (Fig. 3), suggesting that the inoculation of strain 101R-97 changed only the member of bacteria in the rhizosphere soil. Chitinase produced by strain 101R-97 might change the nutritional conditions in the rhizosphere soil by supplying nutrients derived from chitinous compounds (fungal mycelia and arthropod exoskeletons contained in the field soil), a condition to which the community structure of bacteria might respond sensitively. Further analyses are necessary to understand the mechanism of the change in the bacterial community structure by inoculation with the GM bacterium.

In contrast to the case of bacteria, no differences were observed between the rhizosphere soil inoculated with strain 101R-97 and that with strain 101R, in either the fungal CFUs or the PCR-DGGE patterns of the fungal community, despite the cloned gene in strain 101R-97 being for an antifungal enzyme. These observations indicated that chitinase produced by strain 101R-97 may not be strong enough to affect the biodiversity and total biomass of the fungi in the rhizosphere, and/or the fungal community in the soil may not be so sensitive to the change of nutrient condition as bacteria. In the previous study, however, strain 101R-97 successfully suppressed the damping-off of cucumber seedlings in soils infected with the plant-pathogenic fungus *Rhizoctonia solani*[14]. Strain 101R-97 may block the colonization of the pathogen to the cucumber root. Analogous results were reported in the case of another GM biocontrol agent *P. fluorescens*. This agent producing an antifungal compound, phenazine-1-carboxylic acid (PCA) could suppress the damping-off caused by *Pythium* spp. No structural changes in the microbial community were observed following inoculation with the agent, whereas the inoculation had only a transient impact on mycorrhizal associations in established plant communities[21].

The data presented in this work indicate that the impact of strain 101R-97 on the environment was relatively small. However, this strain showed a higher survival rate than that of a laboratory strain, indicating that further genetic manipulation, such as the introduction of suicidal genetic elements[12], might be required.

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References

1. Atlas, R. M. (2004) *Cellulomonas* PTYG medium. In: Parks L.C. (ed.), *Handbook of Microbiological Media 3rd edition*. CRC Press Inc., Ann Arbor, Michigan. pp. 324−325.
2. Bagdasarian, M. et al. (1981) Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSF1010-derived vector, and a host-vector system for gene cloning in *Pseudomonas*. *Gene*, **16**, 237−247.
3. Bartnicki-Garcia, S. (1968) Cell wall chemistry, morphogenesis and taxonomy of fungi. *Annu. Rev. Microbiol*., **22**, 87−108.
4. van Beneden, S. et al. (2009) Characterisation of fungal pathogens causing basal rot of lettuce in Belgian greenhouses. *Eur. J. Plant Pathol*., **124**, 9−19.
5. Choudhary, D. K. et al. (2009) Insights of the fluorescent pseudomonads in plant growth regulation. *Curr. Sci*., **97**, 170−179.
6. Dice, L. R. (1945) Measurement of the amount of ecologic association between species. *Ecology*, **26**, 297−302.
7. van Elsas, J. D. et al. (2008) The metagenomics of disease-suppressive soils - experiences from the METACONTROL project. *Trends in Biotechnol*., **26**, 591−601.
8. Hirayae, K. et al. (1996) In vitro growth inhibition of plant pathogenic fungi, *Botrytis* spp., by *Escherichia coli* transformed with a chitinolytic enzyme gene from a marine bacterium, *Alteromonas* sp. strain 79401. *Ann. Phytopathol. Soc. Jpn*., **62**, 30−36.
9. Takada-Hoshino, Y. & Matsumoto, M. (2004) An improved DNA extraction method using skim milk from soils that strongly adsorb DNA. *Microbes Environ*., **19**, 13−19.
10. Lane, D. J. et al. (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proc. Natl. Acad. Sci. USA*, **82**, 6955−6959.
11. Martin, J. P. (1950) Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. *Soil Sci*., **69**, 215−232.
12. Molin, S., et al. (1993) Suicidal genetic elements and there use in biological containment of bacteria. *Annu. Rev. Microbiol*., **47**, 139−166.
13. Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol*., **59**, 695−700.
14. Ohno, M. et al. (2011) Biological control of *Rhizoctonia* damping-off of cucumber by a transformed *Pseudomonas putida* strain expressing a chitinase from a marine bacterium. *JARQ*., **45**, 91−98.
15. O'sullivan, D. J. & O'gara, F. (1992) Traits of *fluorescent Pseudomonas* spp involved in suppression of plant-root pathogens. *Microbiol. Rev*., **56**, 662−676.
16. Øvereås, L. et al. (1997) Distribution of bacterioplankton in meromictic Lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol*., **63**, 3367−3373.
17. Perrier, X. & Jacquemoud-Collet, J. P. (2006) DARwin software. http://darwin.cirad.fr/darwin.
18. R Development Core Team (2006) R: A language and environment for statistical computing. http://www.R-project.org.
19. Schroth, M. N. & Hancock, J. G. (1982) Disease-Suppressive Soil and Root-Colonizing Bacteria. *Sci*., **216**, 1376−1381.
20. Simon, A. & Ridge, E. H. (1974) The use of ampicillin in a simplified selective medium for the isolation of fluorescent pseudomonads. *J. Appl. Bact*., **37**, 459−460.
21. Timms-Wilson, T. M., Kilshaw, K. & Bailey, M. J. (2004) Risk assessment for engineered bacteria used in biocontrol of fungal disease in agricultural crops. *Plant and Soil*, **266**, 57−67.
22. Vainio, E. J. & Hantula, J. (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycol. Res*., **104**, 927−936.
23. Watanabe, B. & Matsuda, A. (1966) Studies on the grouping of *Rhizoctonia solani* Kühn pathogenic to upland crops. *Shitei shiken [Plant Dis. Insect Pest]*, **7**, Agriculture, Forestry and Fisheries Research Council, Tokyo, pp. 1−131 [In Japanese with English summary].
24. Williams, P. A. & Murray, K. (1974) Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida (arvilla)* mt-2: evidence for the existence of a TOL plasmid. *J. Bacteriol*., **120**, 416−423.
25. Yin, B. et al. (2004) Identifying microorganisms which fill a niche similar to that of the pathogen: a new investigative approach for discovering biological control organisms. *Plant and Soil*, **259**, 19−27.