Biocontrol of *Phytophthora capsici* by *Serratia marcescens* F-1-1 and Analysis of Biocontrol Mechanisms Using Transposon-insertion Mutants

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Abstract

An antagonistic bacterium against *Phytophthora capsici*, the pathogen of damping-off of cucumber, was isolated from the rhizosphere of scarlet sage (*Salvia splendens* F. Sellow ex Roem. et Schult.) in Fukui Prefecture. The red-pigmented strain F-1-1 was identified as *Serratia marcescens* based on its bacteriological properties. The strain F-1-1 strongly inhibited the germination of cystospores and zoosporangia of *P. capsici* P-9-2 in *in vitro* tests, and also it suppressed the damping-off of cucumber seedlings inoculated with a cystospore suspension of *P. capsici* P-9-2 in pot tests. Moreover, all the other red-pigmented *S. marcescens* strains isolated from the rhizosphere of Japanese holly (*Ilex crenata* Thunb.) were antagonistic, unlike six non-pigmented *Serratia* spp. strains. Four mutants defective red pigment biosynthesis were obtained by transposon (Tn7) mutagenesis among 5967 transconjugant clones. These mutants simultaneously lost their antagonistic ability in *in vitro* and in pot tests. Thus, *S. marcescens* F-1-1 was found to be a potential biocontrol agent for damping-off of cucumber seedlings and its antagonistic ability was based on the production of antibiotic red pigments.

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Key words: *Serratia marcescens*, *Phytophthora capsici*, damping-off of cucumber seedlings, biocontrol, transposon mutagenesis.

INTRODUCTION

Damping-off of cucumber seedlings caused by *Phytophthora capsici* is widespread and very destructive in Japan. Control of this disease has been done by using chemicals. However, these treatments are not always effective. In addition, breeding of plants resistant to the pathogen has not been successful. Therefore, biological control is a suitable strategy for the control of damping-off. Biological control with rhizobacteria has recently been reported for more than ten genera of bacteria in relation to various soil-borne plant pathogens of crops. However, there have been no reports on antagonistic rhizobacteria for biocontrol of damping-off of cucumber seedlings.

On the other hand, molecular techniques have been used to analyze the relationship between specific toxic metabolites and disease suppression in some plant diseases\(^{1,2}\). Especially transposon mutagenesis is the most useful technique for the analysis of biocontrol mechanisms, since mutants showing an impaired production of biochemically or phenotypically distinguishable metabolites can be easily obtained by transposon insertion.

In the present study, we describe the isolation, identification and *in vitro* and *in vivo* biocontrol activity of strain F-1-1. Furthermore, we obtained useful mutants generated by transposon insertion to analyze the mechanisms of biocontrol, and we examined the role of the metabolites produced by the strain in its antagonism.

MATERIALS AND METHODS

**Bacterial strains and culture conditions** The bacterial strains used in this study are listed in Table 1. For the isolation and culture of bacteria, PSA medium (Na\(_2\)HPO\(_4\)·12H\(_2\)O 2 g, Ca(NO\(_3\))\(_2\)·4H\(_2\)O 0.5 g, peptone 5 g, sucrose 15 g, agar 15 g, 300 g of potato slices extracted in 1 liter of distilled water, pH 7.0) was used. Rifampicin-
resistant of F-1-1 strain was spontaneously obtained from cultures on modified LB medium (polypeptone 10 g, yeast extract 5 g, NaCl 15 g, distilled water 1 liter) supplemented with 20 ppm of rifampicin.

**Bacterial properties** Main bacterial properties were investigated based on the protocol of Nishiyama. For the identification of *Serratia marcescens*, API 20E kit (BIO MERIEUX S.A. Company) was used according to the protocol indicated by the company.

**Fungal inhibition assay** Inhibition of *P. capsici* P-9-2 by the bacterial strain in vitro was assayed on a glass slide. A cystospore suspension of *P. capsici* P-9-2 (10⁴ spores/ml) was mixed with an equal volume of bacterial suspension (2.3 x 10⁷ cfu/ml), and the mixture was dropped on a glass slide. After incubation for 6 hr at 28°C, the number of germinated cystospores was counted. About 5000 cystospores in total were scored in one sample.

For the inhibition assay to zoosporangia, zoosporangia were collected after incubation of cystospores under the light at 25°C. Suspension of zoosporangia (10⁴ spores/ml) was mixed with an equal volume of bacterial suspension (2.3 x 10⁷ cfu/ml), and cultured by shaking (90 rpm/min) overnight at 25°C. Thereafter, the number of germinated zoosporangia was counted.

**Damping-off suppression assay** The ability of the bacteria to protect damping-off of cucumber seedlings was determined by pot test. Pot soils of seedlings were treated with a suspension of strain F-1-1 (2.3 x 10⁴-2.3 x 10⁷ cfu/ml) and they were inoculated with a suspension of cystospores of *P. capsici* P-9-2 (10⁴ spores/ml, 20 ml/pot) after two days. Tested plants were scored for their survival 15 days after inoculation. Nine cucumber seedlings were used for one experiment (protective-treatment style).

**Transposon mutagenesis** Transposon Tn7 was used to generate mutations in chromosomal DNA of strain F-1-1. The plasmid pBPW1::Tn7 was used as a suicidal vector for introducing Tn7 into recipient cells. Strain F-1-1 was mated with *Pseudomonas syringae pv. tabaci* BR2 (pBPW1::Tn7) overnight by using the filter method described previously. The mixture was diluted and plated on modified LB agar containing rifampicin (20 ppm) and streptomycin (50 ppm) to select transconjugants (F-1-1::Tn7). Transconjugants were purified by single colony isolation and their properties such as trimethoprim (500 ppm)-resistance which was another marker of Tn7 and absence of plasmid (pBPW1::Tn7) were investigated.

**Molecular genetic technique** Plasmid DNA was extracted by the method described by Birnboim and Doly, and detected by electrophoresis at 80 mA and 80 volts in 0.8% agarose gel. Chromosomal DNA was extracted according to a slight modification of the method of Staskawicz et al. For the isolation, all the DNA were subjected to purification on CsCl-ethidium bromide gradients.

Dot blot hybridization using Tn7 DNA probe was performed as follows; *HindIII* cleavage site (1.55 and 1.75 Mdal) of Tn7 was selected as a probe. The DNA fragment was isolated from DNA bands appearing by low melting agarose gel electrophoresis by using a standard method. The DNA fragments were labeled with photobiotin by using the photobiotin-labeling and detection kit of BRESA company.

The labeling and colorimetric detection of the biotinylate probe were performed according to the protocol of the company. Dot blot hybridization was carried out as follows; Five µl of DNA solution (250 ng and 25 ng of DNA) of each sample was spotted on nitrocellulose followed by baking (80°C, 2 hr). The DNA probe was denatured by incubation at 95°C for 5 min. The biotinylated probe concentration in the hybridization solution was ca. 50 ng/ml.

### RESULTS

**Isolation of bacteria**

Over 1000 bacterial strains were collected from the rhizosphere and rhizoplane of various plants and from a mushroom, and were screened for the ability to inhibit the germination of cystospores of *P. capsici* P-9-2 and to protect cucumber seedlings from damping-off. Among them, several potential strains for biocontrol were detected. Especially one red-pigmented strain designated as F-1-1 which was isolated from the rhizosphere of scarlet sage (*Salvia splendens*) in Fukui Prefecture was selected for this study. Thereafter, seventeen red-pigmented bacterial strains designated as T1-T17 were

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains used in the present study</th>
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<tbody>
<tr>
<td><strong>Bacterial strain</strong></td>
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<tr>
<td><strong>Serratia marcescens</strong></td>
</tr>
<tr>
<td>F-1-1</td>
</tr>
<tr>
<td>F-1-1rIF</td>
</tr>
<tr>
<td>F-1-1rTn7 W15</td>
</tr>
<tr>
<td>F-1-1rTn7 W18</td>
</tr>
<tr>
<td>S-1-1-1</td>
</tr>
<tr>
<td>JCM 1239</td>
</tr>
<tr>
<td>T1-T17</td>
</tr>
<tr>
<td><strong>Serratia spp.</strong> (6 species)</td>
</tr>
<tr>
<td><strong>Pseudomonas syringae pv. tabaci</strong> BR2</td>
</tr>
<tr>
<td>(pBPW1::Tn7)</td>
</tr>
</tbody>
</table>

a) Red⁺, red-pigmented colony; Red⁻, non-red pigmented (white) colony; Rif⁻, Resistant to rifampicin (20 ppm); SM⁺, Resistant to streptomycin (50 ppm); TP⁺, Resistant to trimethoprim (500 ppm).

b) Isolated from the rhizosphere of scarlet sage (*Salvia splendens*).

c) Japan Collection of Microorganisms (RIKEN).

d) Isolated from the rhizosphere of Japanese holly (*Ilex crenata*).
isolated from only the rhizosphere and rhizoplane of Japanese holly (Ilex crenata) in Fukui Prefecture but not from any other samples including 21 species of plants. These strains were used for comparative tests of F-1-1 strain.

**Bacteriological properties**

Strain F-1-1 consisted of Gram-negative rods was motile with peritrichous flagella (Plate I-1) and formed red-pigmented colonies (Plate I-5). It showed a positive reaction in the following tests; Voges-Proskauer test, gelatin hydrolysis, DNase activity, catalase activity, H2S production, utilization of citrate and acetate, lysine and ornithine decarboxylase; but negative for oxidase activity, MR test, indole production, and utilization of arginine and malonate. These properties were almost the same as those of JCM 1239 of S. marcescens used as a control strain except for the MR test.

These data strongly suggested that F-1-1 strain belongs to S. marcescens. Furthermore, to confirm this assumption, the bacterial properties of F-1-1 strain were investigated by using an API 20E identification kit. As shown in Table 5, the profile index, which is expressed based on 27 biochemical properties, was 530776157 and it coincided with those of S. marcescens. From these results, F-1-1 strain was identified as S. marcescens. Other red-pigmented strains, T1-T17, showed similar results in main bacteriological properties using the API 20E kit (data not shown). These strains, therefore, also were identified as S. marcescens.

**Fungal inhibition assay**

S. marcescens F-1-1 and other Serratia spp. strains were assayed for in vitro inhibition of the germination of cystospores of P. capsici P-9-2. When the cystospores of P. capsici P-9-2 were mixed with various concentrations of F-1-1 strain, they displayed a red color 2 hr after mixing, and their germination was strongly inhibited (Plate I-3,4). The inhibition of germination by F-1-1 strain was observed even when the bacterial suspension was diluted at 1/16 (Table 2). S. marcescens JCM1239 and S-1-1-1 also showed a similar fungal inhibition ability, although their antagonistic ability was lower than that of F-1-1 strain. Both strains lost their antagonistic ability when diluted at 1/8, whereas, other species of Serratia with white colonies did not affect the germination of P. capsici P-9-2. However, S. plymuthica JCM1244 of which shows pinkish white colonies exhibited a slight inhibition ability. Thus, F-1-1 strain was the most antagonistic strain against P. capsici P-9-2.

Next, the effect of other red-pigmented strains of S. marcescens isolated from the rhizosphere of Japanese holly (I. crenata) on the germination of cystospores and zoosporangia of P. capsici P-9-2 was investigated (Table 3 and Plate I-2). To compare the strength of antifungal activity among bacterial strains including F-1-1, germination rates of cystospores and zoosporangia of P. capsici P-9-2 inoculated with each bacterial suspension of "1/4 dilution" were determined according to Duncan's multiple range test. As a result, the strength of antifungal activity varied with the bacterial strains according to Duncan's multiple range test. Strain F-1-1 exhibited the most powerful ability to inhibit the germination of cystospores (B rank against E rank of control) and zoosporangia (A rank against D rank of control).

**Damping-off suppression assay**

Effect of treatments with a bacterial suspension of S. marcescens F-1-1 on the suppression of damping-off of cucumber seedlings in pots was investigated. When roots of seedlings were treated with a high population of bacterial suspension (2.3 × 10^7 cfu/ml), the disease was...
clearly suppressed. Even when the roots were treated with a diluted bacterial suspension (1/10 to 1/100), some degree of suppression was observed (Table 4).

**Transposon mutagenesis**

The relationship between the production of red pigments by a strain of *S. marcescens* F-1-1 and its antagonism was investigated. Mutants defective in red pigment biosynthesis (Red-) were obtained by Tn7 mutagenesis. Transconjugants (rifampicin 20 ppm, streptomycin 50 ppm resistant) were recovered at a frequency of $1.0 \times 10^{-4}$ per recipient cell. Of these 9567 transconjugants, four Red- clones were detected in several matings. The mutants without red pigments still displayed the original bacteriological properties of their parent strain F-1-1, when investigated using APE 20E, the identification kit of enterobacteriaceae. Moreover, they formed white colonies, with a morphology very similar to that of F-1-1 strain except for the color. These mutants acquired two new markers of streptomycin (50 ppm) and trimethoprim (500 ppm) resistance which were double markers of Tn7, whereas their parent strain was sensitive to both antibiotics (Table 5 and Plate 1-5). It was also confirmed that the plasmid pBPW1:: Tn7 was not detected in these mutants, indicating that the plasmid acted as a suicidal vector. Since strain F-1-1 has no indigenous plasmid, a foreign plasmid introduced into the strain can be easily detected. Moreover there is no possibility of spontaneous mutation, since the mutation frequency of double markers should be less than at least $10^{-14} (10^{-7} \times 10^{-7})$. Furthermore, we could not detect any spontaneous mutants with white colonies among more than 20,000 clones. These results suggest that the mutants were induced by transposon insertion into the chromosomes of F-1-1 strain.

To confirm the insertion of the transposon into chromosomes by molecular analysis, the following experiments were conducted. Total DNA was extracted from a wild type, a rifampicin-resistant strain and two Red- mutants (W15 and W18) of strain F-1-1, respectively, and assayed for dot blot hybridization. As a result, typical colorimetric detection of the biotinylated probe was confirmed in chromosomal DNAs from two Red- mutants and pBPW1:: Tn7 DNA of a positive control (Table 5), whereas both DNAs from strains F-1-1 (wild type) and F-1-1$rif$ did not hybridize to Tn7 DNA probe. Thus, Tn7 insertion into the genome of strain F-1-1 was confirmed.

**Antagonistic assay of transposon mutants**

*In vitro* antagonistic assay of transposon mutants was investigated. None of the three Tn7 mutants inhibited the germination of cystospores of *P. capsici* P-9-2. Although a slight inhibition activity was observed in W18 and W21 strains, it could be clearly distinguished from the strong activity of the F-1-1 wild type (Table 6).

In the pot test, also, no signs of suppression against damping-off disease of cucumber seedlings by transposon mutants were observed even 15 days after the treatment with a diluted bacterial suspension (1/10 to 1/100). Some degree of suppression was observed (Table 4).

### Table 4. Suppression of damping-off of cucumber seedlings by treatment with *Serratia marcescens* F-1-1 strain

<table>
<thead>
<tr>
<th>Protective treatment</th>
<th>Disease incidence (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5 days $^{a}$</td>
</tr>
<tr>
<td><em>P. capsici</em> $^{b}$+F-1-1 (1×) $^{a}$</td>
<td>0.0 A</td>
</tr>
<tr>
<td><em>P. capsici</em> + F-1-1 (10×)</td>
<td>2.1 B</td>
</tr>
<tr>
<td><em>P. capsici</em> + F-1-1 (100×)</td>
<td>2.5 B</td>
</tr>
<tr>
<td><em>P. capsici</em> + F-1-1 (1000×)</td>
<td>2.3 B</td>
</tr>
<tr>
<td><em>P. capsici</em> only (cont.)</td>
<td>3.2 C</td>
</tr>
</tbody>
</table>

a) Days after inoculation of F-1-1 strain to cucumber seedlings.
b) Twenty ml of cystospore suspension (10⁴ spores/ml) per pot was applied.
c) Non-diluted bacterial suspension was applied at a concentration of $2.3 \times 10^7$ cfu/ml. 10×:10 fold dilution.
d) Values followed by the same letter are not significantly different ($p=0.05$), according to Duncan's multiple range test (suppression-grade; A>B>C).

## Table 5. Characterization of Tn7-insertion mutants of *Serratia marcescens* F-1-1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pigment $^{a}$</th>
<th>API 20E $^{b}$</th>
<th>SM $^{c}$</th>
<th>TP $^{d}$</th>
<th>Tn7-Hybr. $^{e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-1-1 Wild Type</td>
<td>Red</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F-1-1 $^{rif}$</td>
<td>Red</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F-1-1$rif$::Tn7 W15</td>
<td>White</td>
<td>530776157</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>F-1-1$rif$::Tn7 W18</td>
<td>White</td>
<td>530776157</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>F-1-1$rif$::Tn7 W21</td>
<td>White</td>
<td>530776157</td>
<td>+</td>
<td>+</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

a) Color of colonies observed on modified LB media.
b) Profile indexes obtained by API 20E *Enterobacteriaceae* identification kit.
c) Resistant to streptomycin (50 ppm) which is one of Tn7 markers.
d) Resistant to trimethoprim (500 ppm) which is one of Tn7 markers.
e) Dot blot hybridization against chromosomal DNA with Tn7 DNA-probe.

## Table 6. Effect of inoculation of Tn7-insertion mutants of *Serratia marcescens* F-1-1 on germination of cystospores of *Phytophthora capsici* P-9-2

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Cystospores germination of <em>P. capsici</em> P-9-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 $^{a)$</td>
</tr>
<tr>
<td>F-1-1$rif$::Tn7 W15</td>
<td>4 $^{b}$</td>
</tr>
<tr>
<td>F-1-1$rif$::Tn7 W18</td>
<td>2 $^{c}$</td>
</tr>
<tr>
<td>F-1-1$rif$::Tn7 W21</td>
<td>2 $^{d}$</td>
</tr>
<tr>
<td>F-1-1 Wild type</td>
<td>-</td>
</tr>
<tr>
<td>Water (control)</td>
<td>4 $^{e}$</td>
</tr>
</tbody>
</table>

a) Non-diluted bacterial suspension was applied at a concentration of $2.3 \times 10^7$ cfu/ml. 1/2-1/32 showed dilution of bacterial suspension.
b) Germination rate: -, 0 (%); +, 1-10 (%); 2+, 11-49 (%); 3+, 50-79 (%); 4+, 80-100 (%).
inoculation, whereas in the case of the wild type strain, suppression was evident (Plate I-6).

Thus, red pigment-deficient mutants completely lost their antagonism against *P. capsici* P-9-2.

**DISCUSSION**

In this study, it was shown that *S. marcescens* F-1-1 displays an antibiotic activity *in vitro* and in pots tests and could therefore become a potential biocontrol agent for damping-off of cucumber seedlings. The finding of the *S. marcescens* acted as a biocontrol agent against this pathogen, *P. capsici*, was reported for the first time, although the bacterium had been known to be antagonistic against other pathogens such as *Sclerotium rolfsii* (Stacey, G. and Keen, N. eds.), Chapman and Hall, New York, 1992, 15. Thomashow, L.S. and Weller, D.M. (1996). A rapid alkaline DNA extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.


Plate I
York, USA, pp. 187-235.


Explanation of plates

Plate I

1. Electron micrograph of the antagonistic bacterium S. marcescens F-1-1 (bar represents 1 μm).

2. Suppression of germination of zoosporangia and cystospores of P. capsici P-9-2 by treatment with S. marcescens F-1-1 (bar represent 50 μm).


4. Suppression of germination of cystospores of P. capsici P-9-2 by treatment with S. marcescens F-1-1. Cystospores with red staining are observed (bar represent 30 μm).

5. Colonies of S. marcescens F-1-1 (W) and its transposon (Tn7) mutants (M1: F-1-1rif:: Tn7, Red+ and M2: F-1-1: Tn7, Red-) observed on modified LB medium with or without antibiotics, streptomycin (50 ppm) or trimethoprim (500 ppm). C: LB medium (All strains grow well but Tn7-mutant, M2, can not produce red pigments.). S: LB medium supplemented with streptomycin (only Tn7-mutants, M1 and M2, can grow). T: LB medium supplemented with trimethoprim (Only Tn7-mutants, M1 and M2, can grow).

6. Suppression of damping-off disease of cucumber seedlings by treatment with S. marcescens F-1-1 or its transposon mutant W18 at 15 days after inoculation. A: Treatment with red pigment-deficient mutant W18 induced by Tn7 (severe damping-off symptoms are observed). B: Treatment with water (control) (severe damping-off symptoms are observed). C: Treatment with F-1-1 wild type (no symptoms are observed).