

Full Length Research Paper

# Cloning of phenazine carboxylic acid genes of *Fusarium fujikuroi* antagonists bacteria

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**Bakanae disease caused by *Fusarium fujikuroi* is an important diseases on rice. *Pseudomonas fluorescens* produces the broad-spectrum antibiotic phenazine-carboxylic acid (PCA), which is active against a variety of fungal root pathogens. In this study two genes from seven gene locus of phenazine were cloned in *Escherichia coli* DH5 $\alpha$ . The contaminated rice samples were collected from infected farms of Guilan. 238 bacteria were isolated from the rhizosphere. The antagonistic ability of 12 of which, was demonstrated with dual culture method. From the biochemical and culture results, 8 isolated were identified as *P. fluorescens*. The two genes from seven gene locus of phenazine were cloned into *E. coli* DH5 $\alpha$ . We speculate that *P. fluorescens* that produce 2,4-diacetylphloroglucinol (Phl) play an important role in the natural suppressiveness of this soil to causal agent of collar and root rot of rice.**

**Key words:** *Fusarium fujikuroi*, phenazine carboxylic acid, gene cloning, antagonist bacteria.

## INTRODUCTION

There is increasing interest in applying microorganisms to control soil-borne plant pathogens. Inconsistent performance of the microorganisms, however, has hampered commercial application. Combining several modes of action against plant pathogens in one single organism by genetic modification can improve the efficacy of biological control agents (Van Loon, 1998). Bacterial secondary metabolites play critical roles in many aspects of bacterium-host interactions. Secondary metabolites that function as virulence factors play a central role in disease by altering host tissues (Kimura et al., 2001; Rahme et al., 1995). Other secondary metabolites produced by beneficial bacteria can function to prevent infection by pathogens by altering the environment and improving the bacterium's ability to compete with pathogens, by inhibiting the activity of pathogens, or by triggering host defenses (Bloemberg and Lugtenberg, 2001; Raaijmakers et al., 2002). The antibiotics phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (Phl) are major determinants of biological control of soil-borne plant pathogens by various strains of fluorescent *Pseudomonas* spp. (Raaijmakers et al., 1997). The ability to produce phenazines is limited almost exclusively to

bacteria and has been reported in members of the genera *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, and *Burkholderia* (Turner and Messenger, 1986). Particularly among fluorescent *Pseudomonas*, the production of 2,4 diacetylphloroglucinol (DAPG), Plt (pyoluteorin), Prn (pyrrolnitrin) and different derivatives of phenazine has been described (Thomashow and Weller, 1996). *Pseudomonas putida* WCS358r was modified to produce the antifungal compound phenazine-1-carboxylic acid (PCA) (Thomashow et al., 1990). One possible approach to improve biological control may be the application of combinations of biocontrol agents (Duffy and Weller, 1995). By combining microorganisms, multiple antifungal traits can be combined and one may assume that at least one biocontrol mechanism will be functional under the conditions faced by the released biocontrol agents. Moreover, combinations of biocontrol strains are expected to result in a higher level of protection (Dunne et al., 1998), have reduced variability of biological control (Guetsky et al., 2001; Guetsky et al., 2002), and have potential to suppress multiple plant diseases (Jettyanon and Kloepper, 2002). It has been demonstrated that natural suppressiveness of the Châteaurenard soil in France against *Fusarium* wilt is based on various mechanisms involving several microbial populations acting alone or together to limit the activity of the pathogen (Alabouvette,

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**Table 1.** Bacterial strains, plasmids and primers used in this study.

Strain, plasmid or primer	Description or sequence
<b>Strains</b>	
<i>Pseudomonas fluorescens</i> 2–79	Phz_ Rifr, produces PCA
<i>Pseudomonas fluorescens</i> F15	Phz, produces PCA
<i>Pseudomonas fluorescens</i> F15	Phz, produces PCA
<i>Escherichia coli</i> DH5 $\alpha$	F_ <i>traD36 proA_ proB_ lacIq lacZ</i>
<b>Plasmid</b>	
pUC - 18	ColE1 <i>bla</i>
<b>Primers</b>	
PHZ - UP	TAAGGATCCGGTAGTTCCAAGCCCCAGAAAC
PHZ - LOW	CACATTTGATCTAGATGGGTCACGGCTATTCAG

1998). Our objective is to clone entire locus of PCA from antagonist bacteria of *Fusarium fujikuroi* isolated from Guilan rice field to *Escherichia coli* DH5 $\alpha$ .

## MATERIALS AND METHODS

### Isolation of *F. fujikuroi*

Rice bakanae disease was collected from infected farming in different areas as Rasht, Lahijan, Foman, Anzaly, Talesh and Astara in the Guilan province, Iran. For isolation of *F. fujikuroi*, small pieces of infected root with bakanae disease, was washed and surface sterilized with 5% sodium hypochlorite for 10 min. The infected tissues were cultured on acidified potato dextrose agar (PDA).

The plates were incubated at room temperature 26°C for a week. The growing colonies of fungi were transferred to new plates for purification and identification.

### Isolation of antagonistic bacteria isolates and identification

Antagonistic bacteria which colonized rice rhizosphere, one gram of exercised roots were shaken at 100 rpm in 100 mL of sterile distilled water for 25 min. Fluorescent pseudomonads under UV light ( $\lambda = 356$  nm) were isolated on King's medium B (KB). According to the methodology of Schaad et al. (2001), antagonistic isolates of bacteria were identified by biochemical, physiological, biological tests and PCR.

### Screening for antifungal activity

Screening for antifungal activity was performed on PDA medium. Under this condition, fungal growth inhibition could be due to production of antifungal metabolites. Pathogen used was *F. fujikuroi*. An agar plug (5 mm diameter) taken from an actively growing fungal culture was placed on the surface of the PDA plate. Simultaneously, *P. fluorescens* strains were streaked 3 cm away from the agar plug at sides towards the edge of Petri plates. Plate inoculated with fungal agar plugs alone was used as control. The plates were incubated at 27°C until fungal mycelia completely covered the agar surface in control plate. Strains that inhibited mycelial growth of fungus were tested. Ability of antagonistic bacteria to production of volatile antibiotic, secretion of extracellular and production of diffusible antibiotic were tested according to Montealegre et al. (2003).

Results are expressed as means of inhibition (%) of the growth

of *F. fujikuroi* in the presence and absence of any bacterial isolate. Percent inhibition was calculated using the following formula (Montealegre et al., 2003).

$$\text{Inhibition (\%)} = [(1 - (\text{fungal growth} / \text{Control growth})) \times 100].$$

### Bacterial strains, plasmids and primers

The bacterial strains plasmids and primers used in this study are described in Table 1. *Pseudomonas* strains were grown at 28°C in king's B, 23 YT broth (Sambrook et al., 1989), while *E. coli* strains were grown in Luria-Bertani or 23 YT broth at 28 or 37°C.

### DNA manipulations

Standard methods were used for DNA purification, restriction enzyme digestion, agarose gel electrophoresis, and ligation (Ausubel et al., 1995). Genomic DNA was isolated and purified by a cetyltrimethylammonium bromide (CTAB) miniprep procedure. A 6.4-kb DNA probe containing the entire *phz* locus from *P. fluorescens* F15 was generated by PCR performed with oligonucleotide primers *phz*-up and *phz*-low (Table 1). The amplification was carried out by using a 50  $\mu$ l reaction mixture containing 1x eLONGase buffer (Life Technologies, Inc., Rockville, Md.), 2 mM MgSO<sub>4</sub>, 3.0% dimethyl sulfoxide, 200  $\mu$ M (each) dGTP, dATP, dTTP, and dCTP, 10 pmol of each primer, 0.7  $\mu$ l of eLONGase enzyme mixture (cinagene, Inc.), and 20 ng of purified genomic DNA from isolated strains. All amplifications were performed with a PTC- 200 thermal cycler. Amplification was performed in a thermal cycler programmed

The reaction conditions are: a initial denaturation of 94°C for 2 min followed by 37 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 10 min finishes the reaction. Amplified DNA fragments were examined by horizontal electrophoresis in 1.5% agarose gel in TBE buffer 90 mM Tris-borate, 2 mM EDTA [pH 8.3]), with 8  $\mu$ l aliquots of PCR products. Gels were stained with ethidium bromide and were photographed under UV light (312 nm).

### Transformant screening and protein expression

Recombinant cells were identified by plating on to agar medium containing ampicillin, X-Gal and IPTG.

For protein expression *E. coli* DH5 $\alpha$  harboring pUC18 was grown in LB broth to an optical density at 600 nm and induced with 0.5 mM Isopropylb- D-thiogalactopyranoside (IPTG). Cells were harvested 3 h later and total cellular protein was analyzed by electrophoresis in an SDS–10% polyacrylamide gel.

**Table 2.** Effect of antibiosis of *Pseudomonas fluorescens* isolates on radial growth of *Fusarium fujikuroi* *in vitro*.

Antibiosis (Inhibition (%))	<i>Pseudomonas fluorescens</i> isolates										
	F1	F6	F12	F15	F16	F18	F21	F25	2-79 RN	F15 (pUC-PCA)	F16 (pUC-PCA)
Daul culture	40d	41d	48c	55b	50c	49b	50c	48c	59a	58a	58.5a
Volatile antibiotics simultaneously	51d	52d	60c	65b	61c	59c	61c	59c	69a	68a	68a
Volatile antibiotics 72 h	54d	54d	63d	69b	63c	60c	63c	62c	72a	70.5a	71a
Antibiotics	58d	60d	68c	77b	70c	68c	67c	69c	80a	79a	79a
Secretion of extracellular (25% v/v)	62d	63d	73c	80b	73c	74 c	74c	75c	83a	82a	82.5a

Means followed by a common letter in a row are not significantly different according to LSD (T) test at  $P < 0.01$ .

## RESULTS

### Isolation of antagonistic bacteria

Two hundred thirty eight bacterial isolates were initially collected from the rhizoplane and rhizosphere of rice sheath blight disease in different farming of area of the Guilan province-Iran. Among them, thirteen isolates were found to inhibit growth of *F. fujikuroi in vitro*. Eight isolates; F1, F6, F12, F15, F16, F18, F21 and F25 were identified as *Pseudomonas fluorescens* biovar 3 according to the methodology of Schaad et al. (2001).

### Identification of *P. fluorescens* isolates by direct PCR

All isolates of *Pseudomonas fluorescens* were identified by specific primers PCA1 and PCA2. On agarose gel electrophoresis 2%, isolates were produced a band 1110 bp (expected size). The bands of isolates were similarly with isolate standard of 2-79 RN (Figure 1).

### Mycelium inhibition assays

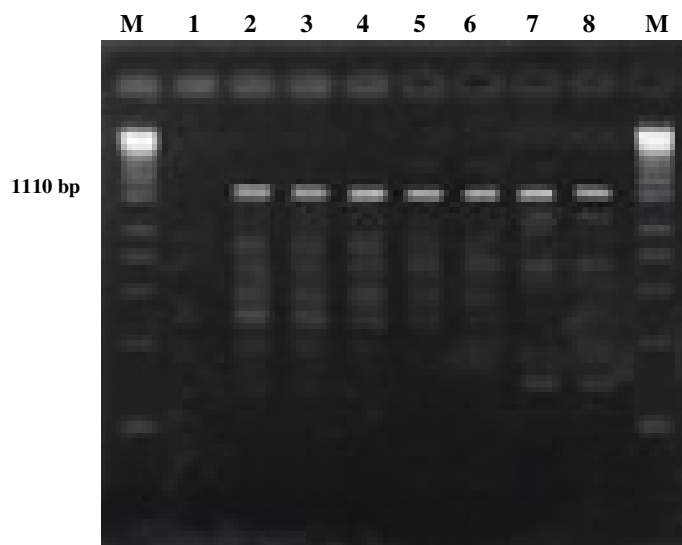
Two hundred thirty eight bacterial isolates were initially collected from the rhizoplane and rhizosphere of rice bakanae disease in different farming of area of the Guilan province-Iran. Among 268 isolates, two bacterial isolates (F15 and F16) were selected to the inhibit mycelium growth of *F. fujikuroi* greater than others (Table 2).

### Specificity of PCA primers

Primers phz-up and phz-low amplified the entire locus of *P. fluorescens* strain 2-79 RN (Figure 1). The specificity of PCA primers was reported in earlier study (Dimetri et al., 2001).

### Cloning detection

The fragment (1110 bp) was cloned into pUC18, and positive clones, were identified by standard methods



**Figure 1.** Agarose gel electrophoresis of the PCR products amplified from genomic DNA of isolated *Pseudomonas* strains with PCA1 and PCA2 primers phz-up and phz-low. Lane M, DNA 1-kb ladder marker (0.2 ng). Lane 1, negative control; Lanes 2 to 6 antagonists bacteria; Lane 7 positive control (*P. fluorescens* 2-79 RN).

(Ausubel et al., 1995). The antibiotic PCA is major determinant of biological control of soil-borne plant pathogens by strains of fluorescent *Pseudomonas* spp. (Thomashow and Weller, 1996).

## DISCUSSION

In several bioassays, strain F15 (pUC – PCA) and F16 (pUC – PCA) were able to suppress bakanae disease of rice by effectively of antifungal activity. In this study, we described variety of strains that inhibit mycelial growth of *F. fujikuroi* and screened a collection of phenazine-producing strains of *P. fluorescens*. The production of PCA increased antifungal activity of WCS358r and DAPG production resulted in an enhanced ability to inhibit growth of both fungi and bacteria. PCR and southern hybridization analysis were performed to determine the

presence of genes involved in the biosynthesis of phenazine-derivatives described in *P. fluorescens* 2-79 and *Pseudomonas aureofaciens* 30 - 84. Loci *phzC*, *phzD* (Mavrodi et al., 1998). For the presence of PCA-genes by direct PCR. Also we successfully cloned the entire locus of phenazine in *E. coli* DH5 $\alpha$  with specific primers. Results indicated that phenazine biosynthesis is highly conserved among phenazine-producing strains of *P. fluorescens*. Cloning of different fragment of the locus can described the structure and function of the biosynthetic gene clusters from the isolated strains. Characterization of phenazine regulation by strains of *P. fluorescens* F15 and F16 has revealed many complexities in the activation of phenazine production, but prior to this study, genetic screens had not identified any negative regulators. We speculate that fluorescent *Pseudomonas* spp. that produce Phl play an important role in the natural suppressiveness of bakanae disease of rice. Because phenazine production by strain 2-79 RN contributes to its capacity in biological control, we tested the ability of F15 and F16 to inhibit *F. fujikuroi*. In *in vitro* plate assays, strain F15 and F16 were better at inhibiting mycelial growth of the fungus than wild type strain 2-79 RN (Table 2). The environmental fitness of genetically modified microorganisms might be affected by the modification (De Leij et al., 1998). Future studies will determine the mechanism of PCA regulation of phenazine production and evaluate the long-term effect of the PCA mutation on bacterial colonization, persistence, and bakanae disease suppression on rice.

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