J. Microbiol. Biotechnol. (2004), **14**(4), 653–657

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Improved Plant Growth from Seed Bacterization Using Siderophore Overproducing Cold Resistant Mutant of *Pseudomonas fluorescens*

KATIYAR, VANDANA AND REETA GOEL*

Department of Microbiology, College of Basic Sciences and Humanities, G.B. Pant Univ. of Ag. & Tech., Pantnagar-263145, Uttaranchal, India

Received: October 28, 2002
Accepted: June 18, 2004

Abstract The cold resistant mutants of *P. fluorescens* strain PRS_0 and ATCC13525 were developed which could grow equally well at 28°C and 10°C. All the mutants were tested for siderophore production, of which $CRPF_0$ (ATCC13525 mutant) was selected, as there was a 16.8-fold increase when compared to its wild-type. Under *in vitro* conditions, $CRPF_0$ showed better growth promotion both in wheat (29.1% increase in root length) and mung bean (51.5% increase in root length) at 10°C. Greenhouse trials showed a significant increase in root (13.84 cm) and shoot (15.0 cm) length of $CRPF_0$-treated mung bean seeds, indicating increased rhizocompetence of the mutant. Ferric citrate was a better iron source than ferric hydroxide for plant growth.

Key words: Cold resistant mutant, *P. fluorescens*, plant growth promotion, siderophore

The term rhizosphere was introduced by Hiltner in 1904, and is defined as a volume of soil surrounding plant roots in which bacterial growth is stimulated [18]. The rhizosphere is populated by a diverse range of microorganisms, and the bacteria colonizing this habitat are known as rhizobacteria [17]. Plant root colonizing bacteria can function as harmful, deleterious rhizobacteria (DRB) or beneficial, plant growth promoting rhizobacteria (PGPR). Plant growth promotion by rhizobacteria can occur either directly or indirectly [4, 15]. PGPR promote plant growth directly in several ways, viz., by solubilization of minerals such as phosphorus, fixation of atmospheric nitrogen, production of plant growth regulators (phytohormones) as auxins and cytokinins, and/or production of siderophores that solubilize and sequester iron. Indirect plant growth promotion occurs when PGPR promote plant growth by improving growth restricting conditions [5]. This happens directly by producing antagonistic substances, or indirectly by inducing resistance to pathogens [4].

The plant growth promoting activity of PGPR resides in the siderophores, which are low molecular weight compounds with high affinity for iron. Under conditions of iron limitation, fluorescent yellow-green siderophores, named pyoverdines (PVDs) or pseudobactins, are produced and excreted by *Pseudomonas* sp. [9]. PVDs are composed of a conserved dihydroxyquinoline chromophore, a variable peptide chain comprising of 6 to 12 amino acids, depending on the producing strain, and a side chain, generally a dicarboxylic acid or a dicarboxylic acid amide [1, 9]. PVDs produced by different strains of fluorescent pseudomonads exhibit extreme diversity in the peptide chain attached to the chromophore [11]. Different strains of fluorescent pseudomonads can be differentiated from each other by isoelectric focusing of partially purified siderophores, as the different peptides result in PVDs with different pIs that can be visualized directly under UV light [11].

Uptake of PVDs occurs via a Ton B-dependent iron-repressed outer membrane protein (IROMP) that acts as a specific receptor for the siderophore. Some *P. putida* or *P. fluorescens* strains have a remarkable capacity to use a broad spectrum of heterologous PVDs, whereas their PVDs cannot be used by competitive *Pseudomonas* [12, 14]. The production of PVDs contributes to the biocontrol capacity of the fluorescent pseudomonads. Siderophores provide the organism a competitive advantage under conditions of iron limitation by scavenging iron from the environment. In this way, siderophores provide the organism an advantage of becoming rhizocompetent.

Establishment of a threshold population of viable inoculant is an important prerequisite for plant-microbe interactions like growth enhancement and biocontrol by bacteria. The soil persistence of bacteria is influenced by many factors, of which temperature is one [19]. While considerable attention has been given to the potential use of fluorescent pseudomonads as biocontrol agents, which

*Corresponding author
Phone: 91-5944-233341; Fax: 91-5944-233473;
E-mail: rg55@rediffmail.com

exclude pathogenic microorganisms from the rhizosphere [21], little is known about the direct effect of competitive root-colonizing pseudomonads on a plant in the absence of plant pathogenic microorganisms. The objective of this study was to screen cold resistant mutants of *P. fluorescens* for siderophore production and to study the effect of select mutant on plant growth attributes.

MATERIALS AND METHODS

The Organism and Culture Conditions

P. fluorescens strain PRS_9, originally isolated from the rhizosphere of *Pisum sativum* [16], was obtained from departmental culture collection. *P. fluorescens* strain ATCC13525 was obtained from IMTECH, Chandigarh, India. Both the strains were maintained on modified King's B medium containing (g or ml/l): proteose peptone, 20; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.4; glycerol, 8 ml; pH 7.0± 0.1 at 28±2°C.

Chemical Mutagenesis and Selection

P. fluorescens ATCC13525 and PRS_9 were mutagenized with nitrosoguanidine as described by Mishra and Goel [13]. Following mutagenesis, the diluted cells were immediately plated onto solid Tryptone Soya Broth (TSB) medium containing (g/l): soya peptone, 5.0; peptone, 15.0; and NaCl, 5.0 (pH 7.0±0.2) and incubated at 10°C for colony development. The confirmation of mutants was done by ARDRA [6].

Siderophore Estimation

Siderophore was quantitated by the method of Meyer and Abdallah [10]. Absorbance of culture supernatant was recorded at 400 nm, and siderophore was calculated using extinction coefficient.

Gnotobiotic Root Elongation Assay

P. fluorescens strains ATCC13525 and PRS_9 and their respective mutants $CRPF_5$, $CRPF_6$, $CRPF_7$, $CRPF_8$, and $CRPF_9$, grown to the log phase at 28°C in the TSB medium, were used for seed bacterization of wheat var. Sonalika and mung bean var. PM-4 for a paper towel assay, and incubated at 25°C and 10°C as carried out as mentioned previously [13]. Non-bacterized carboxymethyl cellulose coated seeds served as a control. Standardizing the inoculum size, bacterized seeds were sampled and colony-forming units (cfu) were counted on TSB medium. The bacterial suspension was adjusted to 1.90×10^8 CFU per seed.

Root Colonization

The mung bean seeds bacterized as above were sown in pots (3 seeds per pot) containing local sterile and unsterile field soil (pH 7.2) and reared in a greenhouse at 25°C. Each treatment had four replications. The growth in terms of root and shoot length was recorded after three weeks.

Effect of Different Iron Sources

To evaluate the effect of different iron sources, quartz was used instead of soil. An appropriate amount of quartz was placed in a tray, and after preliminary washing by tap water, it was flooded with 17% (w/v) HCl and kept overnight. This process was repeated three times. Subsequently, quartz was washed with deionized water until the pH reached 6.5. The bacterized seeds were sown in pots containing quartz and reared in the greenhouse at 25°C as above. Three different sets, two for different iron sources (ferric citrate and ferric hydroxide) and a control set without iron, were designed using the above culture treatments. Different iron sources were supplied in Hoagland's solution. Ferric hydroxide was supplemented with the same concentration of ferric citrate as in normal Hoagland's solution [7]. The growth parameters as above were recorded after three weeks. The data were subjected to ANOVA and showed significance at the 5% level.

RESULTS AND DISCUSSION

Development and Selection of Mutants

The importance of fluorescent *Pseudomonas* in soil nutrient cycling and their ability to colonize aggressively make them the preferred choice for ecofriendly studies. Considerable information is available with respect to their use in the natural environment, and one fact is that low temperature habitats require strains with a greater adaptability to such temperature. Therefore, in this study, cold resistant mutants of *P. fluorescens* ATCC13525 and PRS_9 were developed, which function equally well at 10°C. The mutants had a longer generation time (Table 1), but eventually a similar cell density could be achieved. PCR

Table 1. Generation time of *P. fluorescens* strains ATCC 13525 and PRS_9 and their mutants at 10°C, 25°C, and 30°C.

Strain	Generation time[1,2]		
	10°C	25°C	30°C
ATCC13525 (wt)[3]	isg	42.77	42.27
$CRPF_8$	77.36	55.93	49.13
$CRPF_9$	71.29	52.69	48.48
PRS_9 (wt)[3]	isg	40.85	39.95
$CRPF_5$	61.62	55.09	46.02
$CRPF_6$	64.92	56.81	43.28
$CRPF_7$	84.55	52.39	54.19

[1]Mean of three replicates.
[2]Time in minutes.
[3](wt) represents wild-type strains and the rest are mutants.
isg=insignificant growth.

Table 2. Siderophore level of wild-types and their respective mutants.

Strain	Siderophore production[a,b]
ATCC13525 (wt)[c]	1.54
$CRPF_8$	12.66
$CRPF_9$	25.86
PRS_9 (wt)[c]	1.24
$CRPF_5$	8.40
$CRPF_6$	2.36
$CRPF_7$	7.85

[a]Mean of three replicates.
[b]µg/ml of siderophore.
[c](wt) represents wild-type strains and the rest are mutants.

amplification using universal primers [8] and a subsequent RFLP pattern further confirmed the authenticity of the mutants [6].

For screening, quantitative estimation of siderophore production was carried out using *P. fluorescens* strains PRS_9 and ATCC13525 and their cold resistant mutants. Invariably all the mutants showed an increase in siderophore production when compared to the respective wild-types (Table 2). For further studies, $CRPF_9$ was selected as it exhibited the highest increase in siderophore production. Gnotobiotic root elongation assay was performed to see the effect of the mutant on plant growth. This assay is independent of all other variable factors affecting plant growth in the soil system. Moreover, to see the effect of mutant on two different plant systems, growth promotion of wheat and mung bean was studied. Growth promotion after treatment with both the mutant and wild-type was compared with an untreated control, and it was found that the mutant was significantly more effective in promoting the growth of both the crops ($p<0.05$) both at 25°C and 10°C. An increase of 29.1% in root length of wheat seedlings and 51.5% increase in root length of mung bean seedlings at 10°C treated with $CRPF_9$ were observed (Table 3). Sun and co-workers [19] found stimulation of canola seedling root length by *P. putida* GR12-2 at 25°C and 5°C [19].

Effect of Mutant on Plant Growth Attributes

Because of the higher effectiveness of the mutant at low temperatures in gnotobiotic conditions, it was tested further in soil. A persistent and statistically significant increase in all the variables used to measure growth (root length 13.84 cm and shoot length 15.0 cm) is evident in the plants whose seeds were bacterized with $CRPF_9$ (Fig. 1). The wild-type ATCC13525 did not show significant plant growth promotion in unautoclaved soil, but it showed a significant increase (8- 10%) in growth parameters in autoclaved soil that clearly suggests the role of siderophore in outcompeting pathogenic soil microorganisms. Similar studies with a siderophore overproducing mutant of *P. putida* was done by Vandenbergh, P. A. and C. F. Gonzalez in 1984 (U.S. patent No. 4 479 936) where they found that

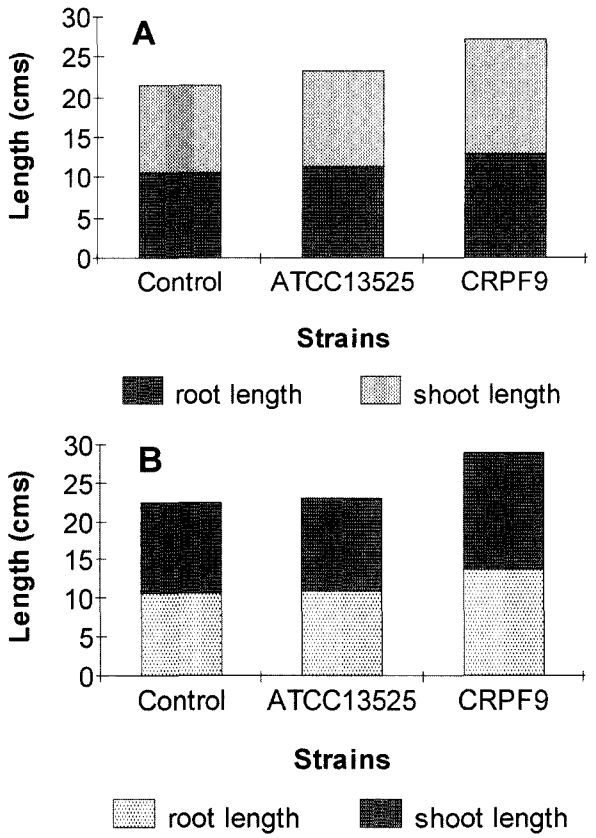

Fig. 1. Effect of wild-type and mutant on root and shoot length of mung bean in autoclaved (A) and unautoclaved (B) soil under greenhouse conditions.

Table 3. Root length of wheat and mung bean in gnotobiotic conditions as influenced by wild-type and mutant.

Strain	Wheat		Mung bean	
	25°C[1,2]	10°C[1,2]	25°C[1,2]	10°C[1,2]
Control	8.92	7.13	4.9	3.1
ATCC 13525	9.5 (6.0%)	7.21 (1.0%)	5.25 (7.1%)	3.32 (7.0%)
$CRPF_9$	10.15 (13.7%)	9.21 (29.1%)	5.4 (10.1%)	4.7 (51.5%)

[1]Length in cm.
[2]Each value is the mean of 30 replicates.
Values in the parentheses indicate % increase over control.

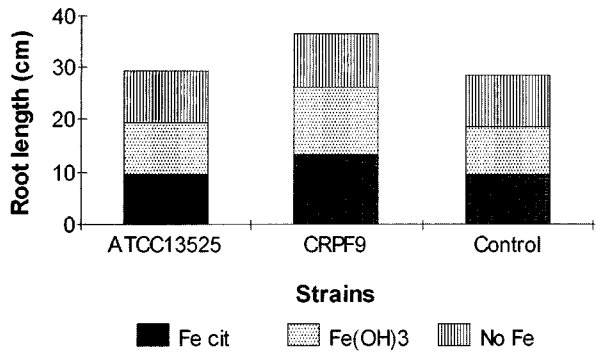

Fig. 2. Effect of wild-type and mutant on root length of mung bean in the presence of different iron sources under greenhouse conditions.

the mutant was more effective in controlling a strain of *Fusarium oxysporum*. It was interesting to note, in this study, that the effect of $CRPF_9$ was higher in unsterilized soil than in sterilized soil. This might be due to the higher siderophore production, which made the mutant a good rhizocompetitor (Fig. 1). There are reports that suggest a number of plants have mechanisms for binding the bacterial iron-siderophore complex, transporting it through the plant, and then reductively releasing the iron from the siderophore so that it can be used by the plant [3, 20].

Furthermore, to elucidate the effect of different forms of iron on siderophore production and its effect on plant growth, an experiment in quartz was set up using ferric citrate, ferric hydroxide, and no iron source. It was revealed that $CRPF_9$ significantly enhanced plant growth ($p<0.05$) in ferrric citrate and then in ferric hydroxide. The least growth was when there was no iron treatment, with the result of a slight yellowing of leaves (Fig. 2). This suggests that $CRPF_9$ synthesizes siderophore in iron-limiting conditions, and that this has a direct effect on plant growth. To confirm the synthesis of siderophores by PGPR in the rhizosphere, a study was done in which monoclonal antibodies were used to quantify the amount of siderophore from a fluorescent pseudomonad that was present in a barley rhizosphere sample [2]. This study indicates it is possible to select physiologically efficient strains of *P. fluorescens* through mutagenesis and that the resultant mutant strain with greater adaptability and rhizocompetence can be used to improve crop productivity at lower temperatures.

Acknowledgment

The senior author acknowledges the financial assistance she received from CSIR during her course of this study as a Senior Research Fellowship.

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