Identification of some molecular traits in fluorescent pseudomonads with antifungal activity

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Abstract
We assessed a collection of 47 fluorescent *Pseudomonas* spp., some with known biological control activity against certain soil-borne phytopathogenic fungi such as, *Macrophomina phaseolina, Rhizoctonia solani, Phytophthora nicotianae* var. *parasitica, Pythium* sp. and *Fusarium* sp. *in vitro* and the potential to produce known secondary metabolites such as, siderophore, HCN and protease. The results indicated that 66%, 40.42%, 63.82%, 48.94% and 27.65% of strains revealed antagonistic activity against *R. solani*, *M. phaseolina*, *Pythium* sp., *P. nicotianae* and *Fusarium* sp., respectively. Among the 47 strains, 76.59%, 97.87% and 17% produced protease, siderophore and HCN, respectively. In this survey, the detection of *phlD* and *phlA* genes was evaluated with a PCR-based assay. We detected *phlD* in strains P-5, P-32, P-47, and *phlA* in strains P-5, P-18, P-34 and P-35. Strain CHA0 was used as positive control for the detection of both genes. Overall, there was no obvious link between inhibition of fungal growth *in vitro* and production of the antifungal metabolites or existence of *phlD* and *phlA* genes. Characterization of fluorescent pseudomonads with potential to produce of 2, 4-diacetylphloroglucinol will further enhance our knowledge of their function in the suppression of root diseases.
Keywords: Fluorescent pseudomonads, soil-borne pathogens, antifungal metabolites, *phlA*, *phlD*, PCR

INTRODUCTION

The plant growth-promoting rhizobacteria (PGPR) improve plant growth either directly via production of plant growth regulators such as auxine and cytokinines and by increasing the plant uptake of some micro and macro elements in the rhizosphere (Glick, 1995; Lugtenberg *et al*., 1991) or indirectly, through biological control of pathogens or induction of host defense mechanisms (O'Sullivan and O'Gara, 1992; Thomashow and Weller, 1996; Van Loon *et al*., 1998; Pieterse *et al*., 2000). Production of antifungal secondary metabolites, such as 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (PLT), pyrrolnitrin (PRN), phenazines, hydrogen cyanide (HCN), siderophore and lytic enzymes (protease), is a prominent feature of many biocontrol fluorescent pseudomonads (Fenton *et al*., 1992; Lemanceau *et al*., 1992; Dowling and O'Gara, 1994; Thomashow and Weller, 1996). Fluorescent pseudomonads that produce the polyketide antibiotic 2, 4-DAPG are an important group of PGPR that are effective against a broad spectrum of soil-borne plant pathogenic fungi (Shanahan *et al*., 1992; Harrison *et al*., 1993; Nowak-Thompson *et al*., 1994; Duffy and Defago, 1997; Sharifi-Tehrani *et al*., 1998). In *Pseudomonas fluorescens* strain Q2-87, genes responsible for the synthesis of 2, 4-DAPG have been cloned and sequenced. A total of five complete open reading frames (ORFs) and one partial ORF, within the 6.8 kb segment of DNA, is responsible for the biosynthesis of 2,4-DAPG (Dwivedi and Johri, 2003). The biosynthetic locus includes *phlA*, *phlC*, *phlB*, and *phlD*, which are transcribed as an operon from the promoter upstream of *phlA* (Bangera and Thomashow, 1999). *phlD* is responsible for the production of the monoacetylphloroglucinol (MAPG), and *phlA*, *phlC*, *phlB* are involved in the conversion of MAPG to 2,4-DAPG. The biosynthetic operon is flanked on either side by *phlE* and *phlF*, which code respectively for putative efflux and repressor proteins (Bangera and Thomashow, 1999; Mavrodi *et al*., 2001). The limited distribution of *phlD* among

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microbes makes it an ideal marker gene for investigation of genetic diversity of 2, 4-DAPG-producing fluorescent *Pseudomonas* spp. (McSpadden Gardener *et al*., 2001). Probes and primers specific for sequences in *phlD* have been used in combination with colony hybridization and polymerase chain reaction (PCR) to quantify population size of 2, 4-DAPG-producers in the rhizosphere environment (Raaijmakers *et al*., 1997; Raaijmakers and Weller, 1998; Picard *et al*., 2000). Studies of the genotypic diversity of 2, 4-DAPG producers by different techniques, including BOX-PCR, ERIC (enterobacterial repetitive intergenic consensus)-PCR, ARDRA (amplified ribosomal DNA restriction analysis), RAPD (random amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) analysis, have revealed a substantial number of distinct groups (Keel *et al*., 1996; Sharifi-Tehrani *et al*., 1998; McSpadden Gardener *et al*., 2000; Picard *et al*., 2000). Phylogenetic comparisons based on either sequencing or ARDRA of 16S ribosomal DNA genes have revealed three distinct lineages (phylogenetic groups) (Keel *et al*., 1996; McSpadden Gardener *et al*., 2000). These groups, originally designated as ARDRA groups 1, 2, and 3 by Keel and co-workers (1996), correspond to groups A, B, and C as defined by McSpadden Gardener and co-workers (2000). Group 1 (A) contains all strains that produce 2,4-DAPG, HCN, and PLT. Strains that produced DAPG and HCN but not PLT are clustered in Group 2 (B) and Group 3(C). The latter group consists of only two strains, F113 and P12.

The investigation of the genetic diversity of 2, 4-DAPG-producing fluorescent pseudomonads is regarded with special importance. The first step required to investigate the genetic diversity of these bacteria is the detection of the genes involved in the biosynthesis of this antibiotic.

The objectives of the current study were the detection of *phlD* and *phlA* genes within fluorescent pseudomonads by a PCR-based assay with specific primers, and comparison of phenotypic and genotypic characteristics of fluorescent pseudomonads with proven biocontrol potential against certain soil- borne phytopathogenic fungi.

MATERIALS AND METHODS

Source of microorganisms and maintenance

Fluorescent *Pseudomonas* spp. strains (n=46) were assessed as potential bicontrol agents in this research (Table 1). These strains were isolated during previous studies from the rhizosphere of wheat, bean, onion,

Table 1. Origin and hosts of fluorescent *Pseudomonas* spp.

Strains	Host	Location
CHA0	Tobacco	Switzerland
P- 5	Onion	Gharre tappe
P- 6	Chickpea	Karaj
P- 7	Chickpea	Karaj
P- 9	Wheat	Karaj suburbs
P-10	Wheat	Karaj suburbs
P-11	Wheat	Karaj suburbs
P-12	Wheat	Karaj suburbs
P-13	Wheat	Karaj suburbs
P-14	Wheat	Karaj suburbs
P-15	Wheat	Karaj suburbs
P-16	Wheat	Karaj suburbs
P-17	Wheat	Karaj suburbs
P-18	Wheat	Karaj suburbs
P-19	Wheat	Karaj suburbs
P- 20	Wheat	Karaj suburbs
P- 21	Wheat	Karaj suburbs
P- 22	Wheat	Karaj suburbs
P- 23	Wheat	Karaj suburbs
P- 24	Wheat	Karaj suburbs
P- 26	Wheat	Karaj suburbs
P- 28	Wheat	Karaj suburbs
P- 29	Wheat	Karaj suburbs
P- 30	Wheat	Karaj suburbs
P- 32	Wheat	Karaj suburbs
P- 33	Wheat	Karaj suburbs
P- 34	Wheat	Karaj suburbs
P- 35	Wheat	Karaj suburbs
P- 36	Wheat	Karaj suburbs
P- 37	Wheat	Karaj suburbs
P- 42	Wheat	Karaj suburbs
P- 44	Bean	Karaj suburbs
P- 45	Bean	Karaj suburbs
P- 46	Bean	Karaj suburbs
P- 47	Bean	Karaj suburbs
P- 48	Bean	Karaj suburbs
P- 49	Bean	Karaj suburbs
P- 50	Bean	Karaj suburbs
P- 51	Bean	Karaj suburbs
P- 52	Bean	Karaj suburbs
P- 53	Bean	Karaj suburbs
P- 54	Bean	Karaj suburbs
P- 55	Bean	Karaj suburbs
P- 56	Bean	Karaj suburbs
P- 57	Bean	Karaj suburbs
P- 58	Bean	Karaj suburbs
P- 59	Onion	Gharre tappe

and chickpea, belonging to different areas of Iran. *P. fluorescens* strain CHA0 was originally obtained from the Swiss Federal Institute of Technology. For short-term storage, the bacteria were cultured on nutrient agar (NA) in tubes, after 24h of growth, the cultures were over layered by double-distilled paraffin and stored at 4ºC. The bacteria were also stored in 0.1 M magnesium sulfate solution ($MgSO_4$, $7H_2O$) at room temperature.

The fungi *Macrophomina phaseolina* (soybean charcoal rot), *Phytophthora nicotianae* var. *parasitica* (pistachio gummosis), *Rhizoctonia solani* (bean damping off), *Pythium* sp. (pepper damping off) and *Fusarium* sp. (cucumber wilt), were obtained from the Department of Plant Protection at Tehran University, where their pathogenicities in respective hosts, had previously been identified and proven.

Fungal inhibition assay

In the plate procedure, the bacteria were spotted over the surface of potato dextrose agar (PDA) plates and after 4 days of incubation at 25ºC, the plates were inoculated with PDA plugs containing 48h old mycelia from each fungus. Control plates were inoculated only by fungi. Plates were incubated at 25ºC and examined for evidence of fungal growth inhibition. Any positive response was recorded by measuring the distance between the edges of the bacterial colony and the fungal mycelium and scored on a 0-3 scale [(0, none; 1, inhibition zone 5-10 mm; 2, inhibition zone 10-15 mm; 3, inhibition zone ≥ 15 mm)] (Table 2).

Production of antifungal metabolites

Siderophore: Evaluation of siderophore production was semi-quantified, using Chrome Azurol S (CAS) medium as described by Schwyn and Neilands (1987). The diameter of the orange halo around colonies after incubation at 27ºC for 48h to 72h was an indication of the level of siderophore production.

HCN: Production of HCN was assessed on King' S B medium (KB) containing 4.4g/l of glycine with indicator paper (whatman soaked in 0.5% (w/v) picric acid and 2% (w/v) sodium carbonate) and plates incubated at 27ºC for 48h to 72h. Any positive response caused the indicator paper to turn from yellow to cream, light brown, dark brown and brick (Alstrom and Burns, 1989) and scored on a 0-2 scale (0, none; 1, little; 2, strong).

Protease: Production of extracellular protease was tested as described by Maurhofer and co-workers (1995). Bacterial strains were spotted on plates of Skim Milk Agar (SMA). Semi quantification of protease was carried out by measuring a halo zone around the bacterial colonies. The experiments were performed in triplicate.

Statistical analysis: The data were transformed using $\sqrt{x+0.5}$ transformation and then analyzed by ANOVA using SAS (V6.12). Differences between treatments were determined by Duncan's Multiple Range Test at 5% significant level.

PCR experiments

Preparations of bacterial templates for detecting the *phlD* gene: Preparation of bacterial templates was carried out as described by Wang and co-workers (2001). Two bacterial colonies obtained from KB agar plates after a 48h incubation period at 27ºC were transferred into 100 µl of lysis solution (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% (v/v) Tween 20). The resulting suspension was centrifuged at 1200 ×*g* for 1 min and incubated for 10 min at 99ºC in a GP001 thermal cycler (Corrbet Research). The heat-lysed bacterial suspension was frozen at -20ºC during a 30 min period. After thawing, 4 µl volumes of the supernatant were carefully taken and used for PCR.

Preparation of bacterial templates for detecting the *phlA* gene: Preparation of bacterial templates was carried out as described by Rezzonico and co-workers (2003). Cells were obtained after growth at 27ºC and shaking for 14 to 16 h (log cells) in 500 µl KB broth. Five µl of cell suspensions were heated for 10 min at 99ºC with 95 µl of lysis buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% (v/v) Tween 20) in a GP001 thermal cycler.

PCR amplification

PCR amplification of *phlD* was performed using the forward primer phl2a (5′-GAG GAC GTC GAA GAC CAC CA -3′) and reverse primer phl2b (5′- ACC GCA GCA TCG TGT ATG AG -3′), which were developed from the *phlD* sequence of *P. fluorescens* Q2-87 (Raaijmakers *et al*., 1997). PCR amplification was carried out in 20 µl reaction mixtures containing 4 µl of lysed bacterial suspension, 10X PCR buffer, 2 mM $MgCl_2$, bovine serum albumin (BSA) (20 mg/ml), 5% (v/v) dimethyl sulfoxide (DMSO), 0.4 mM dNTPs, 0.4 pmol of each primer and 1.5 U of Smar *Taq* DNA polymerase. Amplification was performed with a GP001 thermal cycler. PCR cycling program was used as

Table 2. *In vitro* antifungal activities of fluorescent pseudomonads against soil-borne fungi.

Strain	*Rhizoctonia solani*	*Macrophomina phaseolina*	*Pythium sp.*	*Phytophthora nicotianae*	*Fusarium sp.*
CHA0	1	0	1	3	2
P-5	2	3	1	2	2
P-6	2	1	3	2	0
P-7	1	1	3	1	0
P-9	1	0	0	0	0
P-10	1	0	1	0	0
P-11	1	0	0	0	0
P-12	0	1	1	1	1
P-13	2	0	0	0	0
P-14	3	3	1	2	0
P-15	1	2	3	1	1
P-16	0	0	1	0	0
P-17	2	1	3	2	1
P-18	0	2	0	1	2
P-19	1	2	1	0	0
P-20	0	0	0	0	0
P-21	1	0	0	0	0
P-22	2	1	1	1	0
P-23	1	1	0	0	0
P-24	1	0	3	2	0
P-26	1	0	1	0	0
P-28	1	0	1	1	1
P-29	1	0	2	1	1
P-30	3	2	1	0	0
P-32	1	0	2	1	0
P-33	0	1	0	1	0
P-34	2	0	1	0	0
P-35	3	3	2	2	1
P-36	1	1	1	1	0
P-37	0	0	0	0	1
P-42	0	0	0	0	0
P-44	0	0	0	0	0
P-45	0	0	1	1	0
P-46	0	0	2	1	0
P-47	1	1	1	0	0
P-48	0	0	2	3	0
P-49	1	0	1	0	0
P-50	1	1	3	2	2
P-51	2	3	2	1	2
P-52	0	1	2	0	0
P-53	1	0	0	0	0
P-54	2	0	2	0	0
P-55	2	0	0	0	0
P-56	0	0	0	0	0
P-57	0	0	0	0	0
P-58	0	0	0	0	0
P-59	0	0	0	1	1

Each number is mean of three replicates. 0, none; 1, inhibition zone 5-10 mm; 2, inhibition zone 10-15 mm; 3, inhibition zone ≥15 mm.

described by Wang and co-workers (2001), but with modification of the annealing temperature. The cycling program included an initial denaturation at 94ºC for 2 min followed by 30 cycles of 94ºC for 30s, 65ºC for 30s, 72ºC for 60s, and then a final extension at 72ºC for 10 min.

The specific primers phlA-1f (5′- TCA GAT CGA AGC CCT GTA CC- 3′) and phlA-1r (5′- GAT GCT GTT CTT GTC CGA GC-3′) were used to amplify a 418 bp fragment of *phlA* as described by Rezzonico and co-workers (2003). These primers are able to amplify *phlA* sequences from fluorescent pseudomonads belonging to the ARDRA group 1 (e.g., CHA0) and 3 (e.g., F113) but not from strains of ARDRA group 2 (e.g., Q2-87). PCR amplification was carried out in 20 µl reaction mixtures containing 4 µl of cell lysate, 10x PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 1.5U of Smar*Taq* DNA polymerase, 4 pmol of each primer. Five percent (v/v) DMSO was used as PCR enhancer. PCR cycling program was used as described by Rezzonico and co-workers (2003), with some modification. The cycling program including an initial denaturation at 94ºC for 5 min was followed by 25 cycles of 94ºC for 30s, 62ºC for 30s, 45s at 72ºC, and then 5 min at 72ºC for the final extension. All of the materials used in PCR reaction mixtures, with the exception of the primers, were obtained from CinnaGen Inc, Iran. Primers were synthesized by MWG Biotech (Germany).

The amplification products were electrophoresed in a 1% (w/v) agarose gel with 1x TBE buffer at 80V at room temperature, stained with ethidium bromide, and photographed under UV light by Gel-Documentation (IMAGO, B & L system). The 1- kbp and 100-bp DNA ladders (MBI Fermentas, Germany) were used as molecular size markers. Strain CHA0 and lysis buffer were used as positive and negative controls respectively, in each PCR experiment.

Table 3. Ranking of the fluorescent *Pseudomonas* spp. on the basis of their effect spectrum against the fungi.

Groups	Strains
Group I	P-5, P-15, P-17, P-35, P-50, P-51
Group II	P-6, P-7, P-12, P-14, P-22, P-28, P-29, P-36, CHA0
Group III	P-18, P-24, P-30, P-47, P-32, P-19
Group IV	P-10, P-23, P-26, P-34, P-45, P-46, P-48, P-52, P-54, P-59, P-33, P-49
Group V	P-9, P-11, P-13, P-16, P-21, P-37, P-53, P-55

Groups I, II, III and IV inhibited mycelial growth of 5, 4, 3 and 2 fungi, respectively. Groups only inhibited the growth of one fungi.

RESULTS

Comparative analysis of antifungal activity

The efficacy of each bacterial strain for *in vitro* antifungal activity is shown in Table 2. Strains P-5, P-15, P-17, P-35, P-50 and P-51 inhibited mycelial growth of each the above five mentioned fungi. Strains P-20, P-42, P-44, P-56, P-57 and P-58 could not inhibit mycelial growth in any of these fungi. We clustered the fluorescent *Pseudomonas* spp. into five groups, on the basis of their effectiveness against these fungi. Groups I, II, III, and IV included strains that inhibited mycelial growth of five, four, three and two fungi, respectively. Group V only inhibited the growth of one fungus (Table 3).

Table 4. Correlation coefficients between antagonistic activities of the fluorescent *Pseudomonas* spp. against the fungi.

Variable	Correlation coefficient (r)
Antagonistic activity against *Rhizoctonia solani* and *Pythium* sp.	0.31
Antagonistic activity against *R. solani* and *Phytophthora nicotianae*	0.21
Antagonistic activity against *R. solani* and *Fusarium* sp.	0.08
Antagonistic activity against *R. solani* and *Macrophomina phaseolinae*	0.51*
Antagonistic activity against *M. phaseolina* and *Pythium* sp.	0.26
Antagonistic activity against *M. phaseolina* and *P. nicotianae*	0.37
Antagonistic activity against *M. phaseolina* and *Fusarium* sp.	0.45
Antagonistic activity against *Pythium* sp. and *P. nicotianae*	0.57*
Antagonistic activity against *Pythium* sp. and *Fusarium* sp.	0.21
Antagonistic activity against *P. nicotianae* and *Fusarium* sp.	0.51*

* Numbers above 0.5 indicate significant correlation.

The results of correlation coefficients between antagonistic activities of strains against the fungi showed that the only significant correlations were found between antagonistic activities of strains against *Rhizoctonia solani* and *M. phaseolina* (0.51), and also between antagonistic activities of strains against *P. nicotianae* var. *parasitica* and *Pythium* sp. (0.57) or *Fusarium* sp. (0.51) (Table 4).

Production of secondary metabolites

Siderophore: Siderophore production**,** as determined by the change in the color of Chrome Azurol S (CAS) in an agar medium, was highly variable. All of the strains produced siderophore on CAS blue agar, with the exception of strain P-18, which was not able to change the color of the CAS medium from blue to orange. The diameter of the orange halo around colony of strain CHA0 was the largest (Table 5).

HCN: Eight strains including CHA0, P-5, P-10, P-12, P-18, P-21, P-32 and P-57 were able to produce different quantities of HCN. Other strains did not display HCN production in KB medium supplemented with glycine (Table 5). We can indicate that the majority of the strains that produced HCN were isolated from wheat.

Protease: Extracellular protease can contribute to the ability of bacteria to suppress fungal diseases and here, 36 of the 47 strains displayed proteolyctic activity in SMA medium. The diameter of the halo zone around strain P-23 was the largest (Table 5). The smallest of the halo zones related to strains P-10 and P-30.

The results of secondary metabolites production showed that there was no relationship between production of siderophore, HCN, protease and the origin or hosts from which the fluorescent *Pseudomonas* spp. were isolated.

***In vitro* detection of 2, 4-DAPG-producing *Pseudomonas* spp. with gene-specific primers:** The results of the PCR analysis with primers phl2a and phl2b indicated that a DNA fragment approximately 745 bp in size was obtained in three strains P-5, P-32 and P-47 (Fig. 1A), as predicted from the known *phlD* sequence (Bangera and Thomashow, 1999; Ramette *et al*., 2001). The results of the PCR analysis with primers phlA-1f and phlA-1r indicated that a DNA fragment approximately 418 bp in size was obtained in four strains P-5, P-18, P-34 and P-35 (Fig. 1B). For comparative purposes, strain CHA0 was used as a positive control for the detection of both *phlD* and *phlA* genes.

Table 5. Production of metabolites by the fluorescent *Pseudomonas* spp.

Strains	[a]Protease		[b]Siderophore		[c]HCN	
CHA0	11	BC	10	A	2	A
P-5	7	EF	8	EF	2	A
P-6	9	CDE	6	BCD	0	C
P-7	11	BC	5	CDEF	0	C
P-9	0	I	1	H	0	C
P-10	3	H	2	GH	2	A
P-11	6	FG	2	GH	0	C
P-12	0	I	1	HI	2	A
P-13	0	I	3	EFG	0	C
P-14	5	G	3	EFG	0	C
P-15	10	BCD	3	EFG	0	C
P-16	0	I	1	H	0	C
P-17	12	B	3	FG	0	C
P-18	8	DE	0	I	1	B
P-19	6	FG	2	GH	0	C
P-20	10	BCD	1	H	0	C
P-21	0	I	1	HI	1	B
P-22	9	CDE	4	DEF	0	C
P-23	15	A	1	H	0	C
P-24	8	DE	2	GH	0	C
P-26	9	CDE	2	GH	0	C
P-28	0	I	3	EFG	0	C
P-29	9	CDE	2	GH	0	C
P-30	3	H	3	EFG	0	C
P-32	11	BC	5	CDE	2	A
P-33	0	I	1	HI	0	C
P-34	8	DE	3	EFG	0	C
P-35	6	FG	2	GH	0	C
P-36	8	DE	5	CDEF	0	C
P-37	8	DE	5	CDEF	0	C
P-42	0	I	1	HI	0	C
P-44	10	BCD	1	H	0	C
P-45	0	I	1	HI	0	C
P-46	0	I	1	H	0	C
P-47	9	DE	7	BC	0	C
P-48	6	FG	2	GH	0	C
P-49	7	EF	1	H	0	C
P-50	6	FG	4	DEF	0	C
P-51	6	FG	2	GH	0	C
P-52	10	BCD	3	EFG	0	C
P-53	10	BCD	3	EFG	0	C
P-54	6	FG	1	HI	0	C
P-55	8	DE	1	HI	0	C
P-56	5	G	1	H	0	C
P-57	10	BCD	2	GH	2	A
P-58	0	I	1	HI	0	C
P-59	11	BC	6	BCD	0	C

Each number is mean of three replicates. Means with the same letter are not significantly different (P = 0.5).
a. Diameter of halo zone around the bacterial colonies (mm)
b. Diameter of orange halo around the bacterial colonies (mm)
c. number 2: color change of indicator paper to dark brown, number 1: color change of indicator paper to light brown

Correlation analysis was used to identify whether there was a link statistically between inhibition of fungal mycelial growth on plates and production of the

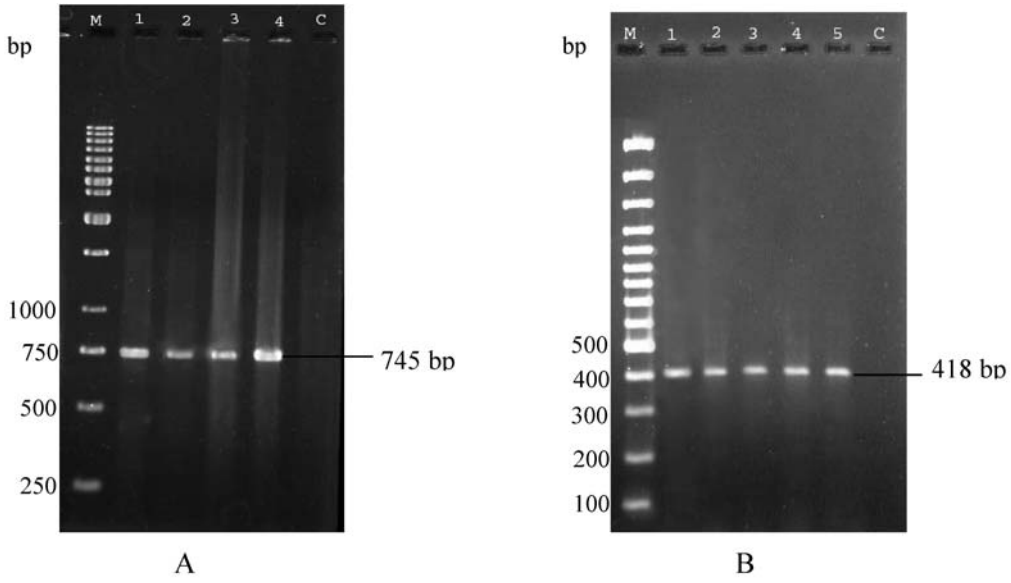

Figure 1. (A)PCR amplification of the *phlD* gene in fluorescent *Pseuodomonas* spp. Lanes M, 1-kb ladder; 1, P-5; 2, P-32; 3, P-47; 4, CHA0; C, negative control (lysis buffer). (B) PCR amplification of the *phlA* gene in fluorescent *Pseuodomonas* spp. Lanes M, 100-bp ladder ; 1, P-5 ; 2, P-18; 3, P-34; 4, P-35; 5, CHA0; C, negative control (lysis buffer).

antifungal metabolites by the fluorescent *Pseudomonas* spp. or the existence of *phlD* and *phlA* genes in these bacteria. The results revealed that, collectively there was no obvious link between them, but in some strains such as CHA0 and P-5 a correlation observed between the presence of *phl* genes and antifungal activities.

DISCUSSION

Antibiotic-producing PGPR have been studied intensively during the last decade, and special attention has been given to 2, 4-DAPG-producing *Pseudomonas* spp. because of their ability to control a wide variety of soil-borne plant pathogens (Fenton *et al*., 1992; Tamietti *et al*., 1993; Pierson and Weller, 1994; Cook *et al.*, 1995; Duffy and Defago, 1997; Sharifi-Tehrani *et al*., 1998). Probes and primers for sequences within *phlD* have been used to monitor the population dynamics of 2,4-DAPG producers in take-all decline disease suppressive and conducive soils (Raaijmakers *et al*., 1997; Raaijmakers and Weller, 1998) and in the rhizosphere of maize (Picard *et al*., 2000).

In this report, we used PCR analysis to detect 2,4-DAPG-producing *Pseudomonas* populations based on the amplification of *phlD* and *phlA* genes sequences. Occurrence of the *phlD* gene was demonstrated through the detection of a 745 bp DNA fragment in three strains P-5, P-32 and P-47 from Iran as well as the control strain CHA0, using a PCR assay with primers phl2a and phl2b. These results corresponded to those of Wang and co-workers (2001). We were also able to demonstrate the occurrence of the *phlA* gene in a DNA fragment of approximately 418 bp in four bacterial strains P-5, P-18, P-34 and P-35 from Iran as well as the control strain CHA0, using primers phlA-1f and phlA-1r, the results corresponded to those of Rezzonico and co-workers (2003). Primers phlA-1f and phlA-1r enabled the amplification of *phlA* sequences from fluorescent pseudomonads belonging to ARDRA group 1 (e.g., CHA0) and 3 (e.g., F113) but not from strains belonging to ARDRA group 2 (e.g., Q2-87). The strains of the ARDRA-2 group do produce phloroglucinol (hence they have the *phlA* gene), but due to differences in the *phlA* sequence at the annealing sites of the phlA-1f and phlA-1r primers, no amplification can be achieved under the conditions described by Rezzonico and co-workers (2003). Because of a partial deletion of *phlD* or a part of the operon, it is possible that we might have positive *phlA* amplification but no *phlD* amplification.

The results revealed that 66%, 40.42%, 63.82%, 48.94% and 27.65% of the strains showed antagonistic effects against *R. solani*, *M. phaseolina*, *Pythium* sp., *P. nicotianae* var. *parasitica* and *Fusarium* sp., respectively. *R. solani* was recognized as the most vulnerable fungus. The interaction analysis indicated that the only

significant correlations were found between antagonistic activities of the strains against *R. solani* and *M. phaseolina* (0.51), and also between antagonistic activities of the strains against *P. nicotianae* var. *parasitica* and *Pythium* sp. (0.57) or *Fusarium* sp. (0.51). Therefore, it is not possible to assign a specific fungus as a suitable criterion for screening antagonistic strains.

In this study, on the basis of the effect spectrum of bacterial strains against fungal pathogens *in vitro*, we clustered these strains into five groups, with group I showing the widest spectrum of activity. This group consisted of six strains P-5, P-15, P-17, P-35, P-50 and P-51 that inhibited the mycelial growth of each of the five mentioned fungi. Among the 47 strains which were used in this investigation, 76.59%, 97.87% and 17% of the strains produced protease, siderophore and HCN, respectively.

Overall, correlation analyses did not reveal significant links between inhibition of fungal growth on plates and production of the antifungal metabolites by the fluorescent *Pseudomonas* spp. or the existence of *phlD* and *phlA* genes in these bacteria. In some strains such as P-5 and CHA0, we saw a link between the existence of *phl* genes and antifungal activities, whereby theses two strains inhibited the mycelial growth of five and four fungi, respectively. These two strains also produced siderophore, protease and HCN. One important factor that may account for the lack of this relationship is the fact that more than one type of bacterial trait can contribute to biocontrol. For instance, CHA0 produces several antifungal metabolites in addition to 2,4-DAPG, noticeably siderophore(s), HCN and PLT (Sharifi-Tehrani *et al*., 1998). In fluorescent Pseudomonad*s* and other gram-negative bacteria, the expression of genes involved in the production secondary metabolites e.g. antibiotics is controlled by a global regulatory system known as GacA/GacS (Heeb and Haas, 2001). GacS, a member of the sensor kinas family, is greatly influenced by environmental signals, most of which have not been identified so far. Furthermore, production of the antifungal metabolites is influenced by environmental conditions. Additionally, the appearance of spontaneous mutations in the GacA/GacS system may result in loss of the ability to produce 2, 4-DAPG, however, the occurrence of such mutants in natural systems has not been reported yet. It is also important to note that the confirmation of the presence of *phl* genes does not always reflect the antibiosis properties of bacteria. There is correlation between population size of 2, 4-DAPG-producing pseudomonads and suppressive soils. Ramette and co-workers (2003) indicated that there was no difference in the population size of total pseudomonads of the rhizosphere and roots of tobacco in both suppressive and conducive soil, but the percentage of phl^+ pseudomonad strains was significantly higher in the rhizosphere and roots of plants grown in the suppressive soils compared with conducive soils. Therefore, the presence of *phl* genes can be sued as a suitable marker for screening and selection of bacteria with potential biocontrol activity, *in vitro* and *in situ* conditions.

Acknowledgements

The authors are grateful to F. Rezzonico for sending the *Pseudomonas fluorescens* strain CHA0, and his technical assistance. We thank the Iran National Science Foundation for financial assistance. This study was also partly supported by the Biological Center of Excellence, Tehran University.

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