

## Molecular detection of antibiotic related genes from *Pseudomonas aeruginosa* FP6, an antagonist towards *Rhizoctonia solani* and *Colletotrichum gloeosporioides*

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**Abstract:** Despite the importance of antibiosis in biological control, little is known about the genes involved in antifungal activity. Therefore, the present study was aimed at identifying the location of the antagonistic gene(s) in *Pseudomonas aeruginosa* FP6 towards *Rhizoctonia solani* and *Colletotrichum gloeosporioides* using a PCR-based approach. A new bacterial strain, designated as FP6, was isolated from rhizospheric soil and identified as a member of *Pseudomonas aeruginosa* based on 16S rRNA analysis. The secondary metabolites produced by this strain have shown broad-spectrum antifungal activity against *Rhizoctonia solani* and *Colletotrichum gloeosporioides*. Antifungal metabolites of a nonenzymatic nature were found to be responsible for the antagonism. Hyphal malformation was also observed in both of the fungal pathogens. PCR analysis of the genomic DNA with antibiotic specific primers detected phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol, and pyoluteorin. Plasmid isolation and plasmid curing of the strain exhibited varied antagonistic activity. A chromosomal gene was found to be involved in the production of a fungal antagonistic compound as demonstrated by the gel elution technique using antibiotic gene-specific primers. The study speculates that antimetabolites play an important role in the control of phytopathogens.

**Key words:** Fluorescent pseudomonads, PCR, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, plasmid curing

### 1. Introduction

In commercial agriculture, crop protection against phytopathogens relies heavily on chemical pesticides. Use of commercial fungicides offers an effective control strategy, but this approach is not only expensive but also poses adverse effects to human health and the environment and is lethal to other beneficial rhizosphere bacteria. At this juncture, environmentally friendly control of fungal pathogens is a pressing need for sustainable agriculture (1). Biological control of soil-borne diseases by plant growth promoting rhizobacteria is a well-established phenomenon and antibiotics have been shown to play a major role in the suppression of several plant pathogens (2).

The control of phytopathogenic fungi by biocontrol agents depends on a wide variety of traits, such as the production of antifungal metabolites and lytic enzymes, induction of systemic resistance, and high competitive maintenance in the rhizosphere, which are considered to be important prerequisites for the optimal performance of plant growth-promoting rhizobacteria (PGPR) towards plant pathogens (3–5). Bacterial secondary metabolites play a critical role in bacterium–host interactions. Secondary metabolites produced by beneficial bacteria

prevent infection 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 (6,7).

In recent years, several plant root-colonizing *Pseudomonas* spp. have been shown to be a potent biocontrol agent in various plant–pathogen systems (8). The production of antifungal secondary metabolites such as 2,4-diacetylphloroglucinol (2,4-DAPG / DAPG or PhI), pyoluteorin (PLT), hydrogen cyanide (HCN), phenazines, or pyrrolnitrin (PRN) is a prominent feature of many biocontrol fluorescent *Pseudomonas* spp. (9–11).

Recently, genetic analysis of several *Pseudomonas* strains has also established a positive correlation between antibiotic production and disease suppression (12,13). However, success in the field is limited due to variable results. Isolation and characterization of the genes from microbes antagonistic to the different phytopathogens will enable us to understand the molecular mechanism involved in biological control approaches. Therefore, the present study was aimed at understanding the genes involved in antagonism to fungal pathogens so as to develop the strain *Pseudomonas aeruginosa* FP6 into an efficient biocontrol agent.

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## 2. Materials and methods

### 2.1. Bacterial strain

The bacterium *P. aeruginosa*, isolated from rhizospheric soil samples from in and around Bangalore, was used in this study. The isolate was identified as *Pseudomonas aeruginosa*. The nucleotide sequence of the 16S rRNA of *Pseudomonas aeruginosa* FP6 was deposited in the GenBank database under the accession number JN861778.

### 2.2. Fungal pathogens

*Rhizoctonia solani* (MTCC 4233) was procured from the Microbial Type Culture Collection Centre (IMTECH, Chandigarh, India) and *Colletotrichum gloeosporioides* (OGC1) was kindly provided by the Indian Institute of Horticultural Research (IIHR), Bangalore. Phytopathogens were maintained on potato dextrose agar (PDA) medium.

### 2.3. Antagonistic activity

Antagonistic activity of *P. aeruginosa* FP6 was tested against *R. solani* and *C. gloeosporioides* by dual culture technique. The percentage inhibition was then calculated (14).

*P. aeruginosa* was grown in King's B medium at 37 °C and 120 rpm for 48 h. The supernatant was separated by centrifugation at  $11,200 \times g$  for 10 min. Supernatant was concentrated by lyophilization and sterilized by filtration through a 0.45- $\mu$ m filter (Millipore, India). Test plates were prepared by mixing 9 mL of molten PDA and 1 mL of concentrated supernatant. In an actively growing mycelial disk of 9 mm in diameter, *R. solani* and *C. gloeosporioides* were inoculated in the center of the petri plate. The plates were incubated for 5 days at 28 °C and the results were expressed as means of percentage inhibition. Plates inoculated with fungal agar plugs alone were used as the control. Similarly, the extracts of the metabolites DAPG, phenazine-1-carboxylic acid (PCA), and PLT were also checked for their antagonistic activity (15).

### 2.4. Sensitivity of *P. aeruginosa* culture supernatant to proteolytic enzymes, surfactants, heat, and pH

To investigate the stability of the antifungal metabolites, concentrated crude supernatant (15  $\mu$ L) of *P. aeruginosa* FP6 was subjected to treatments for 2 h at 37 °C (for enzymes) or room temperature (for surfactants). The proteolytic enzymes (Sigma) were used at a final concentration of 1 mg/mL in 10 mmol<sup>-1</sup> potassium phosphate buffer, pH 7.0. The concentrated crude supernatant in buffer without enzymes as well as the enzyme solutions were exposed to the same conditions. The surfactants used were sodium dodecyl sulfate (SDS), Tween 80, and SDS and urea, at a final concentration of 1% (v/v) and incubated at 37 °C for 5 h. All surfactants (Sigma) were prepared at 10% in water and filter-sterilized before use. For the heat treatment, the preparations were incubated at 25 °C or heated at 37 °C and 60 °C and autoclaved (121 °C, 15 min). For pH stability, 10  $\mu$ L of concentrated supernatant was mixed with an equal

volume of citric acid-Na<sub>2</sub>HPO<sub>4</sub> buffer to achieve different pH values lower than 6.0, and with Tris-HCl buffer for pH 8.0 and 10.0. Antifungal activities were checked before and after all treatments on test plates containing *R. solani* and percentage inhibitions were calculated.

### 2.5. Effect of crude supernatant on the hyphal morphology and spore germination of *R. solani* and *C. gloeosporioides*

For the testing, 100  $\mu$ L of spore suspensions ( $10^4$  spores/mL) of *R. solani* and *C. gloeosporioides* were allowed to germinate on a glass slide under high humidity in the presence of 10  $\mu$ L of extract. The germination of spores was allowed at 28 °C for 24–48 h and the change in morphology was monitored microscopically.

### 2.6. Screening of putative antimetabolites by polymerase chain reaction

Detection of the genes that encode for the production of antibiotics such as DAPG, PCA, phenazine-1-carboxamide (PCN), PRN, and PLT was done by polymerase chain reaction (PCR) using gene-specific primers. Oligonucleotide primers were synthesized by Chromous Biotech Pvt. Ltd. (Bangalore, India). The primer sets and the amplification conditions for the screening of gene-encoding antibiotics are listed in Table 1.

Genomic DNA from *P. aeruginosa* FP6 was isolated using the Chromous Bacterial Genomic DNA Isolation Kit and was used in PCR analysis. The PCR reaction (50  $\mu$ L) contained 50 pmol of primer, 50 ng of genomic DNA, 1X Taq DNA polymerase buffer, 0.5 U of Taq DNA polymerase, 0.2 mM of each dNTP, and 1.5 mM MgCl<sub>2</sub>. Amplification was performed in a DNA thermal cycler (Corbett Thermocycler). Aliquots of 10–15  $\mu$ L of each amplification product were electrophoresed on a 0.8% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 50 V for 1–3 h and stained with ethidium bromide, and the PCR products were visualized under a UV transilluminator. Following this, 1-kb and 5-kb ladders (Chromous Biotech Pvt. Ltd.) were used as size markers.

### 2.7. Plasmid curing

Curing of the plasmid from the *P. aeruginosa* FP6 strain was done by exposing the overnight-grown culture of *P. aeruginosa* to ethidium bromide (500  $\mu$ g/mL). The derivatives of *P. aeruginosa* that were cured of the plasmid were selected on the basis of their inability to grow on the culture media supplemented with kanamycin (1000  $\mu$ g/mL), unlike the parent strain, which is kanamycin-resistant. The treated culture of *P. aeruginosa* was initially plated on a complete medium without antibiotic kanamycin. Colonies of *P. aeruginosa* obtained were picked and spotted on medium containing kanamycin. Colonies that were unable to grow on the medium containing kanamycin were derivatives of *P. aeruginosa* that were cured of the plasmid and were designated as *P. aeruginosa* PC 1–100. The plasmid cured strains were

**Table 1.** Primer and amplification conditions for the different PCR based screenings of genes that encode for antibiotics.

Gene and primer set	Primer sequence	Amplification conditions	Reference
PCA PHZ1 PHZ2	5'-GCGACATGGTCAACGG-3' 5'-CGGCTGGCGGCGTATTC-3'	Initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 60 s, 58 °C for 45 s, and 72 °C for 60 s; final extension at 72 °C for 10 min	16
PCN PhzH- up PhzH-low	5'-CGCACGGATCCTTTCAGAA TGTTC-3' 5'-GCCACGCCAAGCTTCACGC TCA-3'	Initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 60 s, 67.2 °C for 45 s, and 72 °C for 60 s; final extension at 72 °C for 10 min	17
DAPG Phl2a Phl2b	5'-GAGGACGTCGAAGACCAC CCA-3' 5'-ACCGCAGCATCGTGTATGA G-3'	Initial denaturation at 94 °C for 90 s min; 35 cycles of 94 °C for 35 s, 64.9 °C for 30 s, and 72 °C for 45 s; final extension at 72 °C for 10 min	18
PRN Prncf Prncr	5'-CCACAAGCCCCGCCAGG AGC-3' 5'-GAGAAGAGCGGGTCGATG AAGCC-3'	Initial denaturation at 94 °C for 2 min; 30 cycles of 94 °C for 1 min, 58 °C for 45 s, and 72 °C for 1 min; final extension at 72 °C for 10 min	18
PLT PLTC1 PLTC2	5'-ACAGATCGCCCCGGTACA GAACG-3' 5'-GGCCCGGACACTCAAGAA ACTCG-3'	Initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 2 min, 67 °C for 1 min, and 72 °C for 1 min; final extension at 72 °C for 10 min	15

also screened for their antagonistic activity (dual plate culture), siderophore production (19), HCN production (20), indole-3-acetic acid production (21), and phosphate solubilization (22).

### 2.8. Plasmid and genomic DNA isolation

The presence of plasmid in the plasmid-cured strains of *P. aeruginosa* PC 1 through *P. aeruginosa* PC 100 and the parent strain (*P. aeruginosa* FP6) was detected by alkaline lysis method (23). The purified plasmid DNA was visualized by resolving on 0.8% agarose gel electrophoresis. Genomic DNA was isolated from *P. aeruginosa* using a genomic DNA isolation kit (Chromous Biotech Pvt. Ltd.).

### 2.9. Gel extraction of DNA

After agarose gel electrophoresis and visualization, both genomic DNA and plasmid DNA fragments of the parent strain were excised and eluted from the gel using a gel elution kit (Chromous Biotech Pvt. Ltd.) according to the manufacturer's protocol. Eluted plasmid and genomic DNA were PCR-amplified for antifungal antimetabolites using the gene-specific primers as mentioned above.

## 3. Results and discussion

Antibiotic production by fluorescent *Pseudomonas* species is now recognized as an important factor in disease suppression. Performance of biocontrol microorganisms in the field is variable. Elucidation of the mechanism behind this phenomenon will contribute to defining the traits required for robust biocontrol strains and therefore enhanced performance.

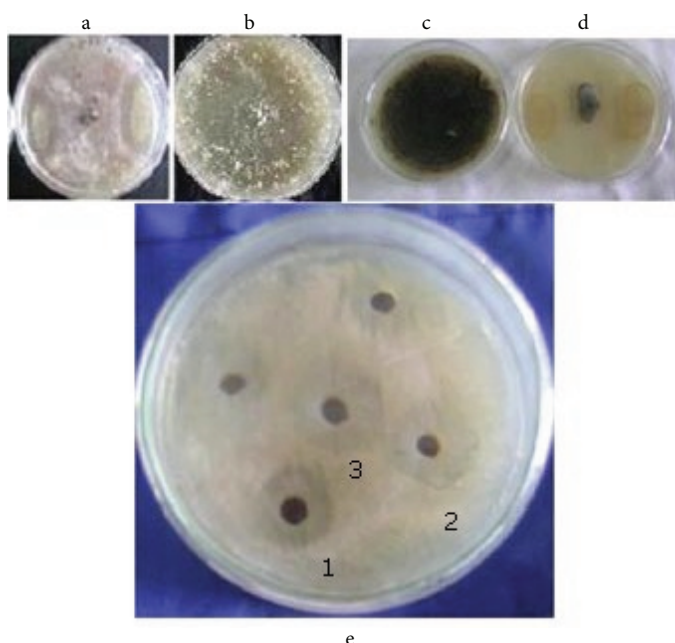
In the present study *P. aeruginosa* significantly ( $P < 0.01$ ) inhibited the growth of both *R. solani* (52%) and *C. gloeosporioides* (49%) when compared to the control by dual culture method (Figure 1; Table 2). In dual cultures with rhizospheric bacteria, soil-borne pathogens *R. solani*, *M. phaseolina*, and *S. sclerotiorum* (24,25) were reported to be inhibited. The antimicrobial activity of *P. fluorescens* was reported against numerous fungi (26).

### 3.1. Sensitivity of *P. aeruginosa* FP6 culture supernatant to proteolytic enzymes, surfactants, heat, and pH

The cell-free culture supernatant of *P. aeruginosa* FP6 exhibited a maximum antifungal activity in all the treatments and was found to be highly stable at extreme pH levels and temperatures, and also after treatments with pepsin, trypsin, and different detergents (Table 3). These results clarify the role of secondary metabolites in *R. solani* and *C. gloeosporioides* inhibition and the adaptation capability of the antagonist.

### 3.2. Study on hyphal morphology

Light microscope investigation revealed that the extract induced morphological abnormalities in fungal structures. It had a significant effect on spore germination and hyphal morphology after 24 h of incubation at room temperature. Sclerotial germination inhibition and immature, thin hyphae were observed in extract-treated *R. solani* (Figure 2). Swelling in the hyphal tips, slimming of hyphae, and occurrence of bubbles and vacuoles in hyphae were observed in the extract-treated *C. gloeosporioides*.



**Figure 1.** Antagonistic activity test by dual culture assay: a and d) inhibition of *R. solani* and *C. gloeosporioides* by *P. aeruginosa* FP6; b and c) control plate of *R. solani* and *C. gloeosporioides*; e) inhibition of *R. solani* by *P. aeruginosa* FP6 metabolites pyoluteorin (1), phenazine-1-carboxylic acid (2), and 2,4-diacetylphloroglucinol (3).

**Table 2.** Antifungal activity of *Pseudomonas aeruginosa* (FP6) isolate towards fungal pathogen.

Culture	Control mycelium ( diameter in cm)	Test diameter (cm)	Percent inhibition of mycelium by antagonist	P-value
<i>R. solani</i>	7.3	3.53	51.52	0.0006
<i>C. gloeosporioides</i>	3.3	1.7	48.63	0.025

Results were the means of triplicates. Data were analyzed using Student's t-test.

The mycelial malformation observed was due to the toxic effect of antibiotic substances interfering with the normal growth processes (27). *P. aeruginosa* is known to produce a wide range of secondary metabolites such as DAPG, PRN, and phenazine, which influence the growth and morphology of fungal mycelium (28). Antibiotic production by *P. aeruginosa* has been suggested to be the mode of disease control (12,29,30). The vacuolar appearance of *C. gloeosporioides* mycelium may be due to the antibiotic metabolites produced by the bacteria, which may penetrate and cause protoplasmic dissolution and disintegration (31).

### 3.3. Detection of antimetabolite genes by polymerase chain reaction

When total DNA of the strain *P. aeruginosa* FP6 was tested by PCR using gene-specific primers, the strain amplified the DNA fragment of 745 bp of DAPG, 1408 bp of PCA, and 438 bp of PLT (Figure 3). The presence of secondary metabolites confirms that secondary metabolites do have a role in disease suppression. The presence of PLT

and DAPG genes supports the co-occurrence of their biosynthetic pathways and is very specific only in certain group of DAPG producers (17).

### 3.4. Plasmid curing

In order to determine whether the antagonistic genes were located on plasmid or chromosomal DNA, plasmid curing was carried out. Antibiotic susceptibility tests of the wild strain showed sensitivity to most of the antibiotics except kanamycin (1000 µg/mL) and rifampicin (500 µg/mL); therefore, kanamycin was used as a marker (32). A total of 100 colonies were obtained, of which 88 colonies were able to grow on Luria-Bertani broth (LB) medium, but not on LB supplemented with kanamycin. Thus, those colonies that grew on LB were selected for further studies. The plasmid curing efficiency was 88%. When plasmid cured strains were checked for loss of antagonistic activity against *R. solani* and *C. gloeosporioides*, they showed variation in their antagonistic potential. By dual plate assay it was shown that 25 (28%) of the cured strains inhibited the growth of *R. solani* and *C. gloeosporioides*. Another 44

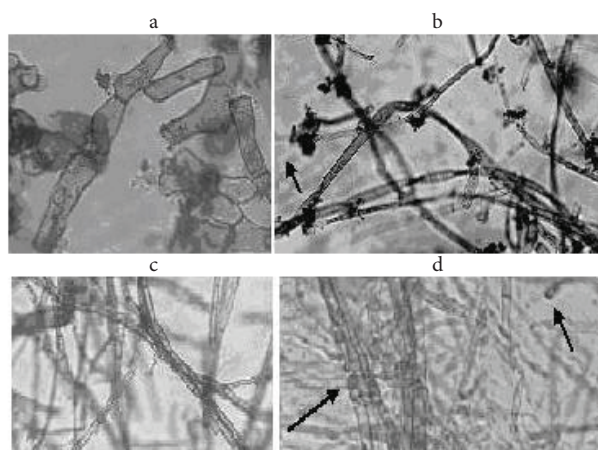
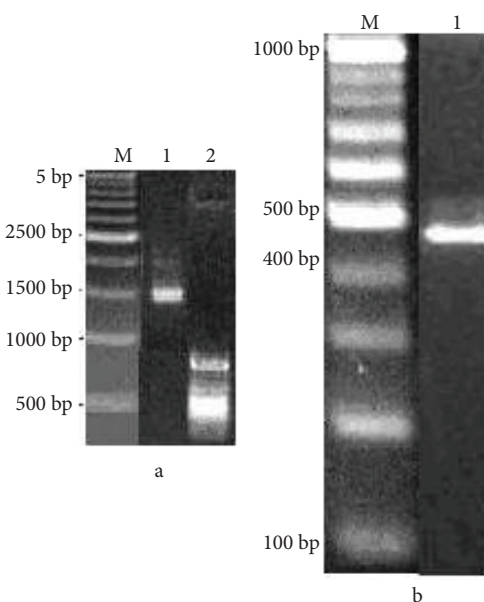


**Table 3.** Sensitivity of *P. aeruginosa* FP6 culture supernatant to heat, pH, proteases, and surfactants.

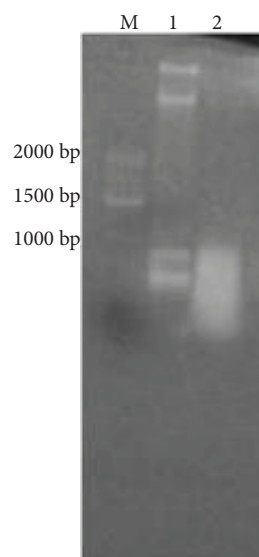
Treatments	Percentage inhibition
Heat	
25 °C for 30 min	99.16 ± 0.089
37 °C for 30 min	99.98 ± 0.014
60 °C for 30 min	99.95 ± 0.044
Autoclaving (121 °C for 20 min)	99.93 ± 0.064
pH	
2.0	99.99 ± 0.002
4.0	99.99 ± 0.009
6.0	97.96 ± 0.035
8.0	99.94 ± 0.019
10.0	99.97 ± 0.009
Enzyme	
Trypsin	99.71 ± 0.03
Surfactant	
SDS	98.25 ± 0.083
Tween 80	99.79 ± 0.041
Urea	99.65 ± 0.008
Dialysis	99.60 ± 0.039

strains (50%) showed weak antagonistic activity, i.e. the colonies were not overgrown by the fungus. The remaining 19 strains (22%) lost antagonistic property against the fungal species tested.

Cured strains when further screened for other PGPR traits (indole-3-acetic acid, phosphate solubilization, siderophore, and HCN) showed no loss except the loss of

**Figure 2.** Effect of *P. aeruginosa* FP6 on *R. solani* and *C. gloeosporioides*: a) Control mycelia showing mature, melanized sclerotia with thick hyphae in *R. solani*; b) treated mycelia showing immature, nonmelanized sclerotia with thin hyphae in *R. solani*; c) normal hyphae of *C. gloeosporioides*; d) occurrence of bubbles, vacuoles, and swelling in hyphal tips in the treated mycelia of *C. gloeosporioides*.**Figure 3.** a) Agarose gel electrophoresis of the PCR products amplified from DNA of *P. aeruginosa* FP6 with primers PHZ1 and PHZ2 (lane 1) and with primers Phl2a and Phl2b (lane 2). b) Agarose gel electrophoresis of the PCR products amplified from DNA of *P. aeruginosa* FP6 with primers PLTC1 and PLTC2 (lane 1). M: DNA molecular marker, 100- and 500-bp ladder.

phosphate solubilization efficiency. Plasmid analysis of the wild strain showed the presence of 4 bands suggesting the presence of 1 or more plasmids in this strain (Figure 4, lane 1). Cured clones showed the loss of plasmid bands that were present in the original wild strain, which clearly

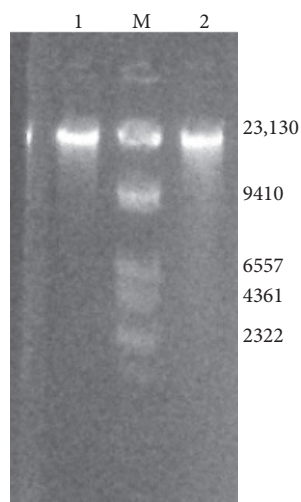
**Figure 4.** Photograph showing absence of plasmid in *Pseudomonas aeruginosa* PC strain (lane 2) as compared to wild *Pseudomonas aeruginosa* FP6 (lane 1) and 500-bp ladder (lane M).

demonstrates the relationship between the loss of plasmid and the loss of kanamycin resistance and phosphate solubilizing efficiency. El-Hamshary et al. showed the presence of 5 plasmids in *P. aeruginosa* (33). Since our isolate showed resistance to lead and cadmium metals (data not shown), these plasmids may be coding for these genes.

Since plasmid curing studies have not conclusively proven the role of plasmid in antagonism, gene(s) located on the chromosome cannot be ruled out. *Pseudomonas* species are also known to harbor megaplasms, and these plasmids have several sets of genes involved in mobilization, genes for nodulation, and biocontrol traits (34). Furthermore, by the alkaline lysis method, when chromosomal DNA and proteins are precipitated by SDS and NaCl (5 M), megaplasms do precipitate with

them. Therefore, an alternate method was carried out to isolate the megaplasms DNA. The preparation, however, showed the presence of only genomic DNA in both the preparations (Figure 5), which also amplified the genes coding for antimetabolites, indicated by the PCR products (of expected size) that were obtained with genomic DNA preparation (Figure 6) and proving that chromosomal genes have role in fungal antagonism.

In the present investigation, there was variation in the ability of cured strains in their antagonistic potential towards fungal pathogens, owing either to phase variation, which regulates biocontrol traits (production of antifungal metabolites and biosurfactants) as reported for *Pseudomonas* spp. strains (35), or to mutation in the chromosomal genes as a result of incubation in the presence of the curing agent.

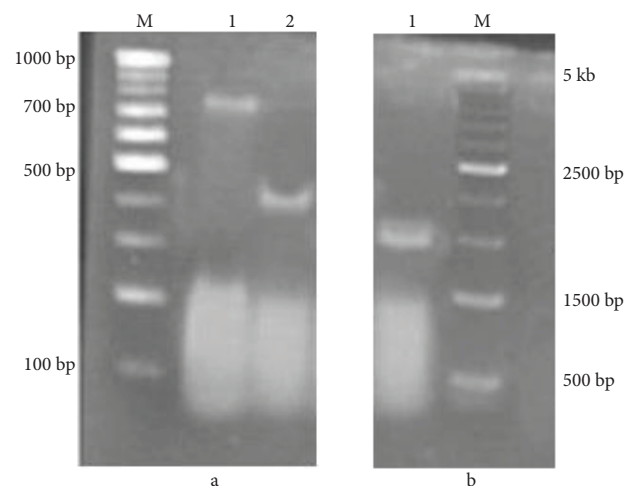


**Figure 5.** Agarose gel electrophoresis of genomic DNA (lane 1) and plasmid DNA (lane 2). DNA marker:  $\lambda$  DNA HindIII Digest (lane M).

Antifungal antibiotics of microbial origin, which are synthesized biologically, have been demonstrated not only to have specific activity against the target pathogens but also to be generally biodegradable, thus overcoming the concerns about residual effects of synthetic fungicides. The present study demonstrated the presence of common, well-characterized antibiotic biosynthetic genes in our strain located on the chromosome. Knowledge of the genes that harbor specific biocontrol traits will contribute in improving the efficacy of existing biocontrol agents.

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**Figure 6.** PCR amplification of antibiotic genes from genomic DNA: a) Phl2a and Phl2b genes (lane 1), PLTC1 and PLTC2 (lane 2); b) PCR amplification of PHZ1 and PHZ2 genes (lane 1). Lane M: DNA molecular marker of 1-kb and 5-kb ladder.

A better understanding of the properties of beneficial agents, and more specifically of the genes conferring the biocontrol potential, would prove useful in both facilitating requirements for registration of such agents and also for improving their salient properties.

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