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Inhibitory Effects of a Korean Strain Gpf01 Identified as *Pseudomonas fluorescens* on *Cucumber mosaic virus*

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An antiviral producing bacterial strain was isolated from a ginseng rhizosphere in Kangwon province of Republic of Korea. In order to identify the bacterial strain, microbiological, physiological and biochemical tests were performed, along with RAPD, 16S rRNA, 16S-23S rRNA ITS (intergenic spacer region) and DNA-DNA hybridization analyses. The bacterium was found to be a strain of *Pseudomonas fluorescens*, which was designated as Gpf01. The strain was grown in Muller-Hinton (MH) broth, and the culture supernatant obtained was filtered through a 0.45 μl filter. It was further boiled at 100°C and tested in two experiments for its ability to control a yellow strain of *Cucumber mosaic virus* (CMV-Y). In the first experiment, boiled culture filtrate (BCF) was treated on one half of the leaves of *Chenopodium amaranticolor* followed by CMV-Y inoculation on both halves. In the second experiment, BCF was treated on the lower leaves of *Nicotiana tobacum* var. Xanthi-nc, with the CMV-Y mechanically inoculated onto the upper untreated leaves. In the first experiment, BCF treatment was able to considerably reduce the number of viral lesion, and in the second experiment, plants treated with BCF showed no visible viral symptoms compared to the Muller-Hinton (MH) media treated controls 15 days post inoculation (dpi), and remained symptomless throughout the study period. Thus, Gpf01, identified as *P. fluorescence*, was able to produce an antiviral component in the culture filtrate, which was found to be heat stable, non-phytotoxic and effective in local as well as systemic hosts of CMV.

Keywords : boiled culture filtrate (BCF), *Cucumber mosaic virus* (CMV-Y), local lesions, *Pseudomonas fluorescens*

Cucumber mosaic virus (CMV), belonging to the genus *Cucumovirus* (family *Bromoviridae*), is one of the economically important viruses, which causes enormous losses by infecting more than 1,000 species of plants, shrubs and trees world-wide. It is transmitted non-persistently into healthy plants by aphids, which acquire the virus during their brief probes on infected hosts or the symptomless carrier weeds in the field (Zehnder et al., 2000). Various strategies, based on the avoidance of sources of infection, control of vectors, modification of cultural practices, and the use of resistant varieties and transgenic plants have been conventionally employed to minimize the losses caused by CMV. These strategies; however, have not been effective as control measures. Many screening studies have been conducted on antiviral agents from different sources. Most of these come from plants sources, with some showing systemic control ability against a range of viruses that infect plants (Kubo et al., 1990). Comparatively, antivirals from microbial sources have been little studied. Recently, Raupach et al. (1996) showed the systemic control of CMV in cucumbers and tomatoes employing rhizosphere colonization of some bacteria by an ISR mechanism. Kim et al. (2004) used culture filtrate from *Acenetobacter* species KTB3, to systematically control some viruses in Korea.

This investigation was primarily concerned with the identification of the Gpf01 strain, with the use of biochemical and molecular methods. We have further described the antiviral activity from the heat stable culture filtrate of Gpf01 against CMV-Y, which produces local lesions in the hypersensitive host and systemically infects many important plants.

Materials and Methods

Bacterial strains and growth conditions. Soil-adhered ginseng roots were obtained from a ginseng field at Hongcheon, Kangwon province, the Republic of Korea. The roots were homogenized using demineralized water, and the homogenate was serially diluted and plated onto Mannitol-Glutamate-Yeast (MGY) agar media (Keane et al., 1970), followed by overnight incubation at 28°C. Numerous colonies, with different morphologies, were picked from the dilution plates. Each of these was assayed for antiviral activity using the half leaf method, as described

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by Kubo et al. (1990). One colony, that showing maximum antiviral activity, was selected and designated as Gpf01. This colony was stored at -70°C, using nutrient broth containing 20% glycerol, by freeze-drying in 10% skimmed milk for its long-term preservation, as described by Perry (1995). Other bacterial strains were obtained from the Korean Agricultural Culture Collection (KACC) and American Type Culture Collection (ATCC). The strains, *P. fluorescens* [(KACC 10239, KACC 10325, KACC 10003, KACC 10327)], *P. putida* [(ATCC 17426), (08891)], *P. pseudoalcaligenes* subsp. *pseudoalcaligenes* (ATCC 12815), *P. monteilii* (ATCC 700476), *P. marginalis* pv. *marginalis* (ATCC 10844), *P. agarici* (ATCC 25941), *P. viriflava* (ATCC 13223), *P. savastanoi* pv. *phaseolicola* (ATCC 13522), *P. fuscovaginae* (KACC 10676), *P. corrugata* (ATCC 29736), *P. tolaasii* (ATCC 33618), *P. cichorii* (ATCC 10857) and *P. syringae* [(PS51), (ATCC 19310)] were used for identification and comparative study of the physiological, biochemical and molecular analyses of Gpf01.

Physiological and biochemical tests. In order to identify the Gpf01 strain, thirty-six physiological and biochemical tests were carried out, as described by Schaad et al. (1988). These tests included the levan test, oxidase test, arginine dihydrolase activity, pectolytic activity, hypersensitivity response in tobacco, growth at higher and lower temperatures, nitrate reduction, PHB formation, gelatin hydrolysis, IAA production, and the acid production from various sugars and the utilization of various organic acids. Each positive and negative test result was scored as 1 and 0, respectively, and a similarity matrix created, as described by Nei and Li (1979). From this matrix, a dendrogram was plotted based on the hierarchical cluster, using NTSYS - pc software (Numerical Taxonomy System; Rolf, 1989).

Preparation of total Genomic DNA. Total genomic DNA was isolated using a lysozyme-dodecyl sulfate lysis procedure (Owen et al., 1987), modified as described previously (Leach et al., 1990). Bacterial strains were cultured in MGY broth at 28°C, with shaking at 200 rpm, and harvested by centrifugation. The cells were lysed by the addition of 10% SDS solution, followed by incubation with 100 µl RNase A. 25 µl of proteinase K solution (10 mg/ml) was added and the mixture incubated for 1h at 37°C. The DNA was then extracted using the phenol extraction method. The DNA in aqueous phase was precipitated with 95% ethanol, followed by washing with 70% ethanol. The DNA pellet was allowed to dry, then dissolved in TE buffer (pH 8.0) and stored at -20°C for further work.

PCR amplification. PCR analysis was performed with a DNA thermal cycler (Perkin-Elmer Applied Biosystems, USA). The 16S rRNA gene was amplified using fD1 and rP2 primers (Weisburg et al., 1991). Amplification was performed in a total volume of 50 µl, containing 20 pmol of each primer, 200 mM of mixture from dATP, dCTP, dGTP, dTTP (Promega Co., USA), 1× buffer, template DNA (ca. 20 ng) and 2.5 units of *taq* polymerase (Promega Co., USA), under the following reaction conditions; initial denaturation at 94°C for 4 min, then 35 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 3 min, followed by a final elongation step at 72°C for 10 min. The 16S-23S ITS region of the Gpf01 was amplified by PCR using the R16-1F and R23-3R primers (Nakagawa et al., 1994). The reaction mixtures for PCR of the 16S-23S ITS region were prepared as described above. Amplification was performed under the following conditions; initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and a final extension at 72°C for 3 min and at 72°C for 10 min.

Cloning and sequencing. The PCR products of the 16S rRNA and 16S-23S ITS regions were analyzed by electrophoretic separation in 1% (w/v) QA-agarose (Qbiogene, USA), containing 0.5 µg/L ethidium bromide. The resultant PCR products were excised from the gel and purified using a QIAquick gel extraction kit (Qiagen Inc., Germany). Purified DNAs were ligated into the pGEM-T easy vector (Promega Co., USA). Plasmids containing the 16S rRNA and 16S-23S ITS regions were then directly sequenced using an ALFred autocycle sequencing kit, with M13 forward and reverses primers. DNA homology searches were carried out with the NCBI databases, using the BLAST network service (Altschul et al., 1990).

Phylogenetic analysis. The MegaAlign software package (Window 3.88, Dnastar, Inc., USA) was used for alignment of the nucleotides of different *Pseudomonas* species. The relationship between the bacterial strains was further analyzed by a phylogenetic tree, using the MEGA program (Kumar et al., 1993; MEGA: molecular evolutionary genetic analysis, version 1.0. The Pennsylvania State University).

RAPD analysis. RAPD was performed for the Gpf01 strain, including different reference strains of *P. fluorescens* and other species, using the primers B-05, 07, 08, 09, 10, 11, 12, 15 and 16 (Operon RAPD 10-mer Kits). 50 ng of DNA of each strain was amplified by the polymerase chain reaction (PCR) using 25 µl reaction mixtures under the following conditions: 200 mM of dNTP, 1× *taq* polymerase buffer, 1.2 mM $MgCl_2$, 10 mM tris-HCl (pH 8.0), 50 mM KCl, 0.01% gelatin, 200 ng oligonucleotide primer and 2.5

unit *taq* Polymerase (Promega Co., USA). Amplifications were performed, as previously described by Weisburg et al (1991). The PCR products were subjected to electrophoresis on 5% polyacrylamide gel, and different band patterns observed (Fig. 4). These bands were scored as either 1 or 0 for their presence or absence across strains, and a binary matrix generated, which was further analyzed using the NTSYS program (Fig. 5).

DNA-DNA hybridization. 100 µl of TE buffer, 4 µl of 10M NaOH and 35 µl of 20×SSC were added to 100 µl of unlabeled total DNA (1 µg/µl) and denatured by boiling at 80°C for 10 min. Denatured DNA was applied to Hybond-N^+ nylon membranes, using slot blot apparatus. Total chromosomal DNA of *P. fluorescens* KACC 10327 was used for probe labeling. Native DNA was labeled with DIG11-dUTP using Dig-High Prime (Roche Molecular Biochemicals, Germany), prehybridized at 49°C for 3h, and then hybridized for 16h at the same temperature. Hybridization signals were detected using a DIG Luminescent Detection Kit (Roche Molecular Biochemicals, Germany), and quantified using a Densitometer (Bio-Rad, USA).

Maintenance of virus. CMV-Y was obtained from the virus culture collection of the College of Forestry Sciences, Kangwon National University, Chuncheon, Korea. The virus was inoculated into *N. tabacum* var. Xanthi-nc, and maintained on the same host throughout the period of this study. The inoculum consisted of CMV-Y systematically infected leaves ground in 0.01 M sodium phosphate buffer (SPB), pH 7.0.

BCF (boiled culture filtrate) preparation. The strain, Gpf01, was taken from glycerol stock and streaked onto a MGY agar plate. A single colony was inoculated into 100 ml MH (Muller-Hinton) broth and grown at 28°C for 48h, with shaking at 175 rpm. The culture was centrifugated at 12,000 rpm for 10 min. The culture supernatant was then filtered through a 0.45 µm filter. The filtrate obtained was boiled at 100°C for 10 min and used for the antiviral assay.

Antiviral bioassay. The antiviral activity of the BCF from Gpf01 was estimated in two experiments. In the first experiment, BCF was assayed on a hypersensitive host of CMV, *C. amaranticolor* using the half leaf method, as described previously by Kubo et al. (1990). The upper right halves of the leaves were treated with the BCF, using paintbrush, and the upper left halves were left untreated. As a control treatment, the upper right halves were treated with sterilized water, with the left halves left untreated. After one hour, the virus was inoculated onto both halves of the leaves for both treatments. Each treatment was performed in duplicate with three leaves each. The plants were kept in a green house, with 12-14 h daylight and a temperature of 30°C. The numbers of local lesions were counted after seven days, with the percentage control effects calculated using the formula: $(1\text{-}T/C) \times 100$, where, C is the number of local lesions on the control half of the leaves and T the number of local lesions on the treated half of the leaves.

In the second experiment, five to six leaved *N. tabacum* var. Xanthi-nc plants were used under each treatment. BCF was treated onto the three basal leaves, with the CMV-Y inoculated onto one upper untreated leaf after 24 h of treatment. As a control treatment, the lower leaves were treated with MH media, with the CMV-Y inoculated onto the upper untreated leaves after 24 hours. Each treatment was replicated five times.

Results and Discussion

Identification of Gpf01. Physiological and biochemical tests showed levan production, oxidase and arginine dihydrolase activity, and utilizations of 2-ketogluconate, mannitol and D-arabinose, as most *P. fluorescence* species. However, no gelatin liquefaction was observed, which is usually shown by *P. fluorescens,* such as the biovar V. The pectolytic activity, HR in tobacco and growth at higher temperatures tests were negative. The phylogenetic tree, plotted on the basis of these and 33 other test results, as shown in Table 1, indicates that the Gpf01 is closely placed with the other *P. fluorescens* strains (Fig. 1).

To further characterize the strain, phylogenetic analyses of the strain Gpf01 were performed using 16s rRNA, the 16S-23S ITS region and RAPD analyses. The 1.5 kb 16S rRNA gene was amplified from Gpf01 and its sequence compared with that of other *Pseudomonas* spp., which showed up to 97.7, 95.7, 97.0, 95.7, 96.5, 95.9, 95.4 and 97.3% identities to *P. putida*, *P. fluorescens*, *P. alcaligenes*, *P. flavescence*, *P. monteilii*, *P. nitroreducens*, *P. straminea and P. plecoglossicida*, respectively. The phylogenetic tree developed from this comparative study also indicated that the strain Gpf01 was closer to *P. fluorescens,* with an aberration to *P. putida* (Fig. 2).

Similarly, the 800bp 16S-23S ITS region was amplified from Gpf01 and its sequence aligned with the nucleotides of the 16S-23S ITS region of the other *Pseudomonas* spp., which showed up to 93.5, 75.1, 89.8, 75.1, 81.8 and 92.3% identities to *P. fluorescens I*, *P. fluorescens II*, *P. gingeri*, *P. syringae*, *P. agarici* and *P. tolaasii*, respectively (Fig. 3). The phylogenetic analysis based on the nucleotide sequences of this region also revealed the strain Gpf01 to be closer to *P. fluorescens*.

The polymorphism generated using OPB-08 RAPD across the species tested is shown in Fig. 4. The Gpf01

Table 1. Physiological and biochemical characteristics of *Pseudomonas* spp.[a]

Characterastic	Gpf01	*P. fluorescens*												*P. putida*									
		KACC					Biovar				Biovar			*P. mar*	*P. tol*	*P. aga*	*P. cic*	*P. vir*	*P. sav*	*P. syr*	*P. fus*	*P. cor*	*P. aer*	*P. chl*
		10003	10325	10327	10239		I	II	III	IV	V	A	B											
Diffusible fluorescent pigment	ND	ND	ND	ND	ND	–[b]	–	–	–	–	–	–	+	+	+	+	+	+	+	+	–	+	–
Non-diffusible pigment	ND	ND	ND	ND	ND	–	–	–	–	–	–	–	–	–	–	–	V	–	–	–	–	–	+
Levan	+	+	+	+	+	+	+	–	+	–	–	–	+	–	–	–	–	–	+	–	–	–	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	–
Pectolytic activity	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	V	–	ND	–	ND	–
Tobacco HR	–	–	–	–	–	–	–	–	–	–	–	–	V	V	+	+	+	+	+	–	ND	+	–
Growth at 37°C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+	–
Growth at 41°C	–	–	–	–	–	–	–	–	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	–
Growth at 4°C	–	–	+	–	–	+	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	+
Nitrate to N	+	+	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	+	–	ND	+	+	+
PHB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Gelatin hydrolysis	–	+	+	+	+	+	+	+	+	–	–	–	ND	ND	ND	–	+	–	V	ND	+	+	+
Ice nucleation	–	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	–	+	+	–	–	–	+	–	–	ND	ND
IAA production	–	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	–	–	–	–	–	+	–	–	–	ND	ND
Utilization of:																							
2-ketogluconate	+	+	+	+	+	+	+	+	V	+	V	+	+	+	+	+	+	+	+	–	ND	+	+
Mannitol	+	+	+	+	+	+	+	V	+	V	V	V	+	+	+	+	+	–	V	ND	+	+	+
Geraniol	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	ND	+	–
Benzoate	ND	ND	ND	ND	ND	D	V	V	+	V	V	+	–	–	+	–	–	–	–	ND	–	+	+
Cellobiose	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	ND	–	–	–
Sorbitol	–	–	+	+	–	+	+	V	+	V	–	V	+	+	–	–	+	–	+	ND	–	–	–
Trehalose	–	+	+	+	–	+	+	V	+	V	–	–	+	V	–	–	–	+	–	ND	+	–	+
Sucrose	–	–	+	–	–	+	+	–	+	V	–	V	+	–	–	–	–	–	+	+	+	–	+
Meso-tartrate	+	–	+	V	+	–	–	V	–	V	V	–	V	+	–	+	+	–	+	ND	ND	–	–
D(–)-tartrate	–	+	+	–	–	–	V	–	–	V	V	V	V	–		–	+	–	–	ND	V	–	–
D-arabinose	+	+	+	+	+	+	+	V	+	+	V	+	–	–	–	–	–	–	–	ND	+	–	–
L-rhamnose	–	–	+	–	–	–	V	V	–	V	–	–	V	ND	–	–	–	–	–	ND	–	–	–
D-aspartate	–	–	+	–	–								–	ND	ND	+	–	–	–	ND	ND	ND	ND
Growth on:																							
Nicotinate	–	–	+	+	–	–	–	–	–	V	V	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–
D-galactose	+	+	+	+	+	+	+	V	+	V	–	V	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	V
Butyrate	+	+	+	+	+	–	V	V	+	V	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+
Valerate	ND	ND	ND	ND	ND	V	V	V	–	V	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+
Azalate	ND	ND	ND	ND	ND	–	–	V	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	–
Meso-inositol	–	–	+	+	–	V	+	V	+	V	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	+
Adonitol	–	–	+	+	–	+	–	V	–	V	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–
Propylene glycol	–	–	+	–	–	–	+	V	–	V	V	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	–
Ethanol	–	–	+	–	–	–	+	V	–	V	V	V	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	V
Lecithinase	+	+	+	+	+	+	+	+	+	+	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	+
Phenyl acetate	–	–	+	–	–	–	–	V	–	V	V	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	V
Butylamine	–	–	+	–	–	–	–	–	–	V	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–
Trigonelline	+	+	+	V	+	V	V	V	–	V	V	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–

[a] *P. mar, P. marginalis*; *P. tol, P. tolaasii*; *P. aga, P. agarici*; *P. cic, P. cichorii*; *P. vir, P. viriflava*; *P. sav, P. savastanoi*; *P. syr, P. syringae*; *P. fus, P. fuscovasinae*; *P. cor, P. corrugata*; *P. aer, P. aeruginosa*; *P. chl, P. chlororaphis.*

[b] +, 80% or more strains positive; –, 80% or more strains negative; ND, not determined; V, variable

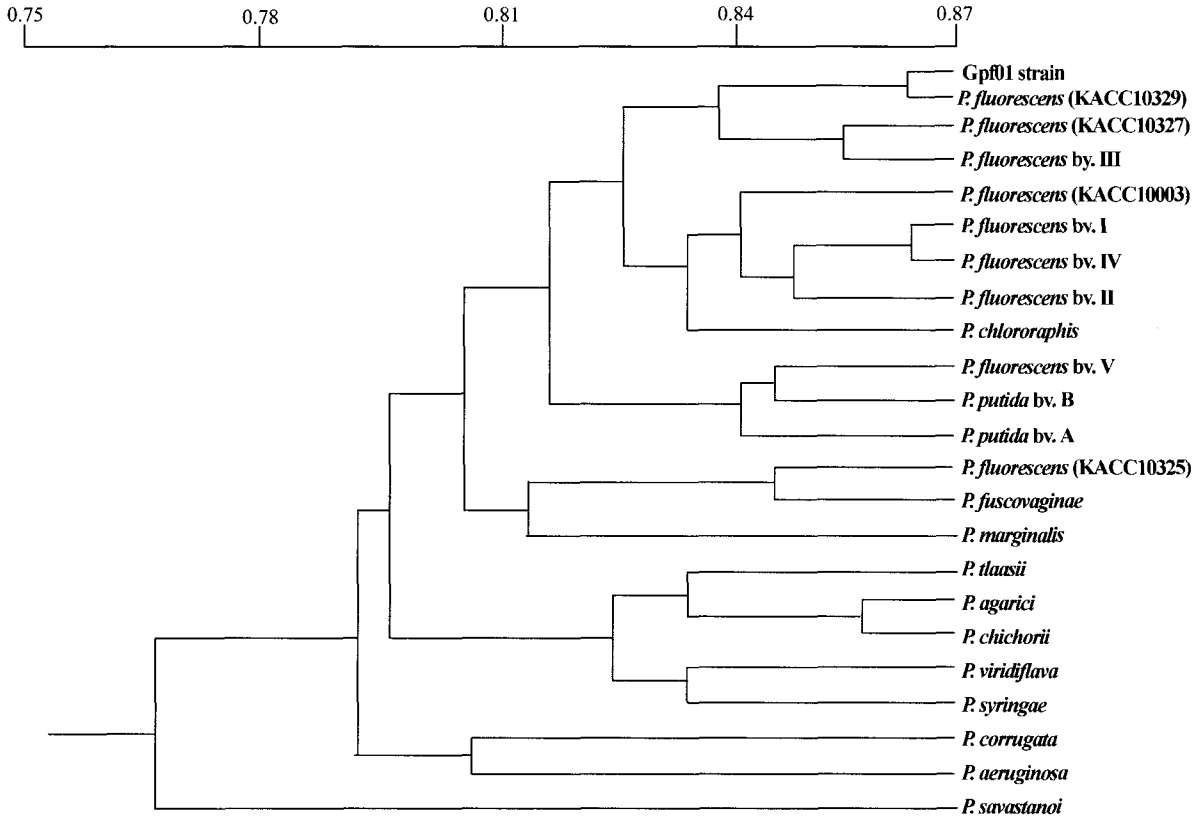

Fig. 1. Phylogenetic tree of Gpf01 and related bacteria based on physiological and biochemical tests using the NTSYS program.

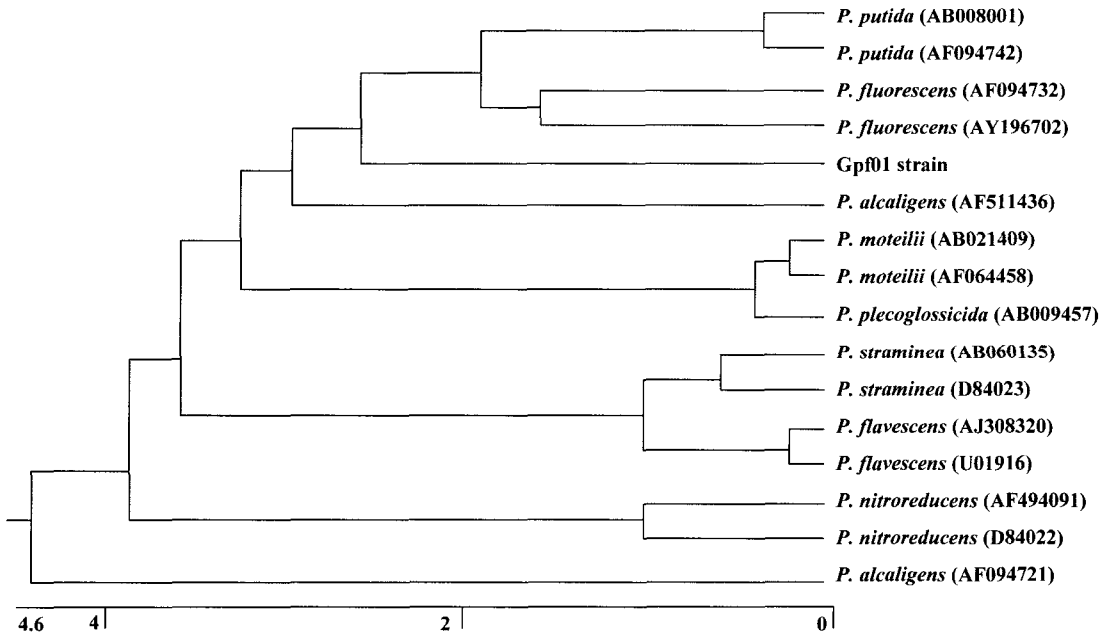

Fig. 2. Phylogenetic tree based on the16S rRNA gene sequences of Gpf01 strain and *Pseudomonas* spp. The branching pattern was generated by the neighbor-joining method. The numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets.

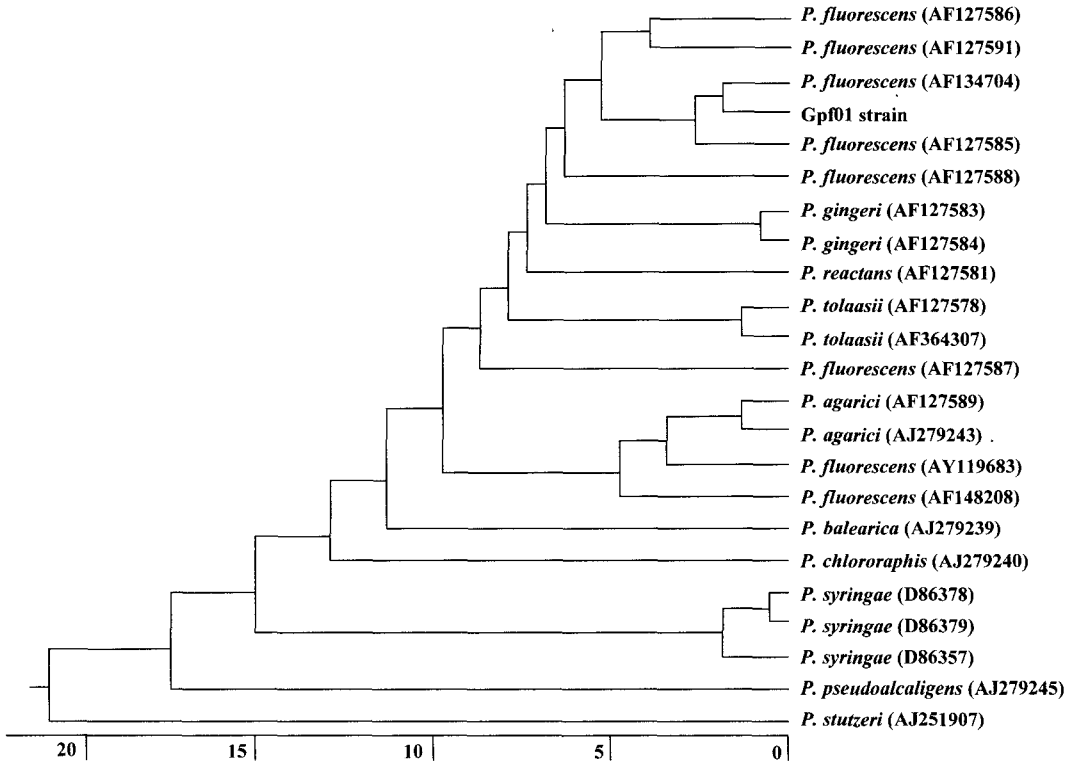

Fig. 3. Phylogenetic tree of Gpf01 and *Pseudomonas* spp. based on 16S-23S ITS region sequences obtained by the neighbor-joining method.

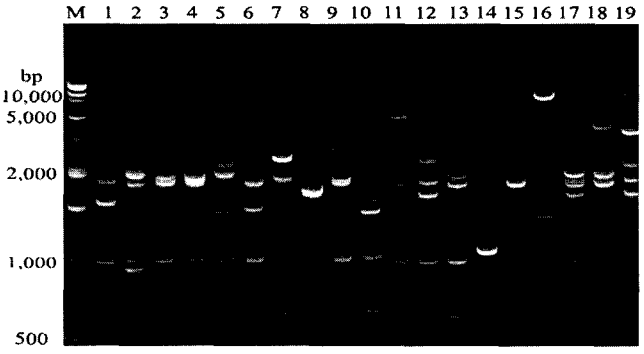

Fig. 4. RAPD profiles of Gpf01 and other related bacteria using the random primer, OPB-08 (Operon RAPD 10-mer Kits). M: 1 Kb Marker, Lane 1: Gpf01, Lane 2: KACC 10239 (*P. fluorescens*), Lane 3: KACC 10325 (*P. fluorescens*), Lane 4: KACC 10003 (*P. fluorescens*), Lane 5: KACC 10327 (*P. fluorescens*), Lane 6: ATCC 17426 (*P. putida*), Lane 7: 08891 (*P. putida*), Lane 8: ATCC 12815 (*P. pseudoalcaligenes* subsp. *pseudoalcaligenes*), Lane 9: ATCC 700476 (*P. monteilii*), Lane 10: ATCC 10844 (*P. marginalis* pv. *marginalis*), Lane 11: ATCC 25941 (*P. agarici*), Lane 12: ATCC 13223 (*P. viriflava*), Lane 13: ATCC 13522 (*P. savastanoi* pv. *phaseolicola*), Lane 14: KACC 10676 (*P. fuscovaginae*), Lane 15: ATCC 29736 (*P. corrugata*), Lane 16: ATCC 33618 (*P. tolaasii*), Lane 17: ATCC 10857 (*P. cichorii*), Lane 18: PS51 (*P. syringae*) and Lane 19: ATCC 19310 (*P. syringae*).

strain was observed to show a similar pattern with that of the other *P. fluorescens*. From the phylogenetic tree, plotted on the basis of the RAPD analysis, the Gpf01 strain was observed to be clustered with the other *P. fluorescens* (Fig. 5). In order to further characterize the strain Gpf01, DNA-DNA hybridization was performed to analyze the genetic relatedness of Gpf01 with other *Pseudomonas* spp. The strain Gpf01 showed 74% RBR (relative binding ratio) with *P. fluorescens* (KACC 10327). A very low hybridized signal was observed with other *Pseudomonas* spp. Based on this evidence, along with the biochemical and physiological analyses, and the 16s rRNA, 16s-23s ITS and RAPD analyses, it can be inferred that the strain Gpf01 is *P. fluorescens*.

Antiviral effects of Gpf01. The antiviral culture filtrate from Gpf01 showed high inhibitory activity against CMV-Y. The BCF treated part of the hypersensitive host; *C. amaranticolor* leaves showed 82.0% inhibition of the production of local lesions compared to the untreated part of the leaves. The control plants, treated with sterilized water, were unable to show inhibition of CMV-Y induced lesions (Table 2). The average numbers of local lesion in the case of the BCF treated half leaves were much lower

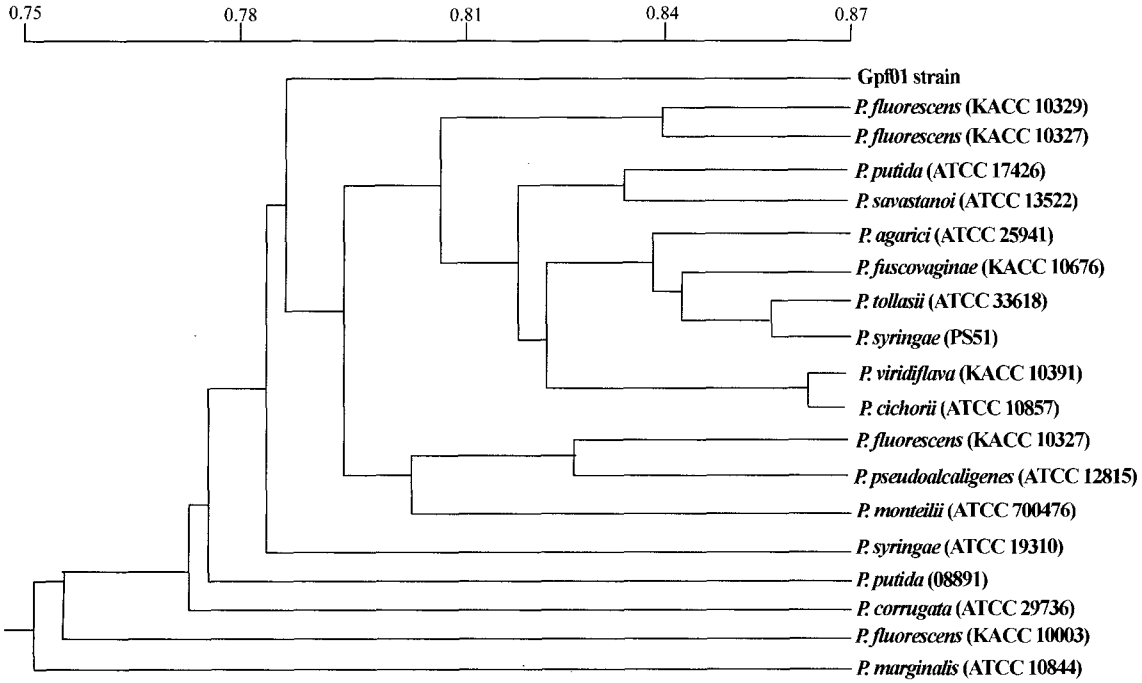

Fig. 5. Phylogenetic tree of Gpf01 and related bacteria, based on the RAPD NTSYS program.

than those of the sterilized water treated half leaves. This indicates the translocation of the antiviral effect from the BCF treated half-leaf to the untreated part of the same leaf.

When BCF was used to elucidate the systemic control effect of the Gpf01, it was found that the plants treated with BCF showed no visible viral symptoms 15 dpi (days post inoculation), and remained symptomless throughout the study period. The plants treated with MH media showed symptoms 5 dpi (Fig. 5). This reveals that the antiviral activity of BCF from Gpf01 was due to involvement of plant defense mechanism. In both the above experiments, no damage to the host plant was observed due to BCF treatment. BCF; thus, can be characterized as a non-toxic antiviral agent, which would give the necessary efficiency in combating CMV. The activity of the inhibitory agent present in the BCF obtained from Gpf01 strain was not destroyed by heating at 100°C for 10 min, indicating that the antiviral agent present in BCF is a heat stable substance. Thus, the non-toxic and heat-stable BCF induces protection against CMV in both local as well as systemic hosts.

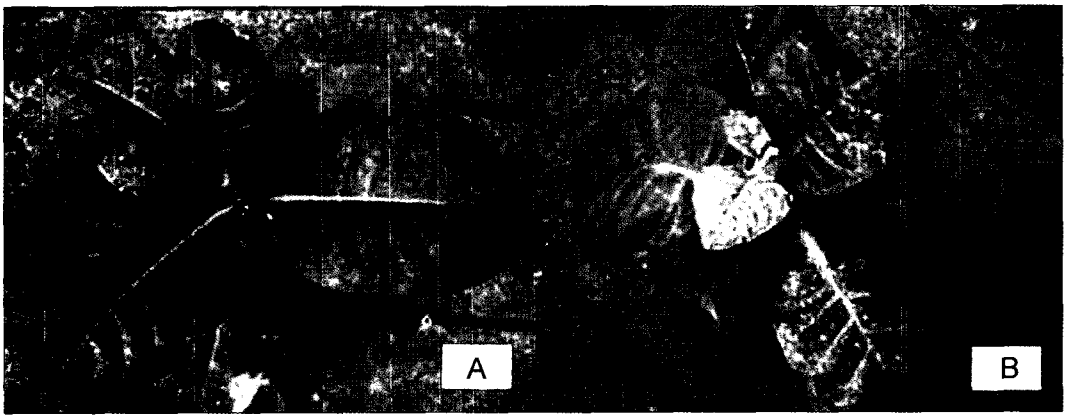

Fig. 6. Systemic control of CMV-Y in *N. tobacum* var. Xanthi-nc 15 days post inoculation. (**A**) Plants treated with BCF (boiled culture filtrate) obtained from *Pseudomonas fluorescens,* strain Gpf01, on the lower leaves of *N. tobacum* var. Xanthi-nc. After 24h, CMV-Y was inoculated onto the upper untreated leaves; (**B**) MH media, as a control, was treated onto the lower leaves of *N. tobacum* var. Xanthi-nc. After 24h, CMV-Y was inoculated onto the upper untreated leaves.

Table 2. Effect of BCF (boiled culture filtrate) of Gpf01 on the infectivity of CMV-Y in *C. amaranticolor*

Treatment	No. of local lesions/half leaf[a]		Inhibition (%)
	Treated	Untreated	
BCF[b]	1.8	9.9	82.0
Sterilized water	112.4	116.6	3.6

[a] Mean no. of local lesions on six half leaves of *C. amaranticolor*.
[b] Gpf01 culture supernatant was filtered through a 0.45 μm filter and boiled at 100°C for 10 min.

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