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In vitro evaluation of antagonistic properties of *Pseudomonas corrugata*

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Diffusible compounds;
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Hydrolytic enzymes

Summary

Pseudomonas corrugata, a soil bacterium originally isolated from a temperate site of Indian Himalayan Region (IHR) is examined for its antagonistic activities against two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum*. Although the bacterium did not show inhibition zones due to production of diffusible antifungal metabolites, a reduction in growth between 58% and 49% in both test fungi, *A. alternata* and *F. oxysporum*, was observed in sealed Petri plates after 120 h of incubation due to production of volatile antifungal metabolites. Reduction in biomass of *A. alternata* (93.8%) and *F. oxysporum* (76.9%) in Kings B broth was recorded after 48 h of incubation in dual culture. The antagonism was observed to be affected by growth medium, pH and temperature. The reduction in fungal biomass due to antagonism of bacteria was recorded maximum in the middle of the stationary phase after 21 h of inoculation. The production of siderophore, ammonia, lipase and chitinase in growth medium by *P. corrugata* were considered contributing to the antagonistic activities of the bacterium.

Introduction

Control of phytopathogens by biological means is environmentally advantageous in comparison to chemical control (Nautiyal, 2001). Several *Pseudomonas* species have been extensively used for biological control against many soil-borne plant pathogens (Weller, 1988; Whipps, 2001). The biocontrol properties of the bacteria belonging to the genus *Pseudomonas* are considered superior because of their adaptive metabolism and their ability to produce an array of compounds inhibiting the growth of several fungal pathogens (Thomashow and Weller, 1990). Production of antibiotics, siderophores and

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a variety of enzymes has been implicated as mechanism(s) used by biocontrol agents to limit the damage to plants by phytopathogens (Glick and Bashan, 1990; Bowen and Rovira, 1999). These mechanisms are not mutually exclusive, and the overall biocontrol activity is due to the synergistic effects of different modes of antagonism (O'Sullivan and O'Gara, 1992).

In the present investigation, *Pseudomonas corrugata*, a well-adapted antagonistic species of the colder regions (Pandey and Palni, 1998) is evaluated for its antagonistic properties against two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum*, to determine the mechanism(s) of biocontrol involved.

Materials and methods

Microorganism

Pseudomonas corrugata, used in the present investigation, is an isolate from temperate soils and has been found positive for N fixation, phosphate solubilization and production of antifungal metabolites against a range of fungi (Pandey and Palni, 1998). *P. corrugata* cultures were maintained on *Pseudomonas* agar slants stored at 4 °C. The isolate has been deposited by the Agricultural Research Culture Collection, International Depository Authority, Illinois, USA (Accession no. NRRL B-30409). The pathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum,* were isolated from soil samples collected in temperate forests of Uttaranchal Himalaya. The test fungi are known to cause diseases (*Alternaria alternata* – leaf spot and leaf blight, *Fusarium oxysporum* – *Fusarium* wilt and rots) in various plant species. Fungal cultures were maintained on potato dextrose agar (PDA) slants at 4 °C. The fungal isolates have been deposited in the Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi (Accession nos. ITCC 4215.2k and ITCC 4219.2K, respectively).

Qualitative evaluation of antagonism due to diffusible and volatile compounds

For examining the antagonism due to diffusible compounds, a lawn of the test fungus was grown on PDA plates. A disc (7 mm diameter) from the well-grown lawn of the fungus was cut and inoculated on potato carrot agar (PCA) plate. A sterilized paper disc (5 mm diameter), dipped in bacterial suspension raised in tryptone yeast extract (TY) broth (10^8 cfu ml^{-1}) was inoculated about 2.0–2.5 cm away from the fungal disc on the same agar plate. The plates were incubated in inverted position at 28 °C in the dark. The observations were recorded at intervals of 24 h for 120 h by measuring the growth of the fungus around the bacterial colony.

The antagonism due to volatile compounds was evaluated by preparing a bacterial lawn on TY agar plates. After incubation for 24 h, the lid was replaced by a plate containing an agar block (7 mm diameter) of the test fungus grown on PCA. The two plates were sealed together with parafilm. Control sets were prepared in a similar manner, without bacteria in the bottom plate. Such sealed sets of Petri dishes were incubated at 28 °C, and the observations were recorded at intervals of 24 h for 120 h. Growth inhibition of the test fungus was calculated in % using the formula: $(r1-r2/r1) \times 100$, where $r1$ (a control value) represents the radial growth of the fungus in control sets without, and $r2$ with bacteria.

Quantitative evaluation of antagonism in different growth media

A volume of 1 ml of bacterial culture grown in TY broth for 24 h (containing 10^8 cfu ml^{-1}) and a disc of test fungus (5 mm) from a well-grown fungal colony on PDA plates were inoculated in 50 ml broth of six different media (potato dextrose, tryptone yeast, Sabouraud dextrose, potato carrot, Kings B and Miller 9 broth) in 250 ml conical flasks at 25 °C on a rotary shaker. Broth inoculated only with fungi served as control. The differences in dry weights between the fungus and the bacterium or the control cultures (without bacterium) were recorded by passing 48 h grown dual cultures through preweighed filter paper (Whatman No. 1). The filter papers were dried for 24 h at 70 °C and weighed. The % reduction in weight of the test fungus was calculated using the formula: $(w1-w2/w1) \times 100$, where $w1$ represents the weight of the test fungus in control flasks without and $w2$ with the bacteria.

Influence of pH and temperature on antagonism of *P. corrugata*

The effect of pH on antagonism of *P. corrugata* was determined by measuring the fungal biomass in broth (M9)-based dual culture, adjusting the pH of the medium with NaOH or HCl to 0.5 unit intervals between pH 3.5 and 9.0. To determine the effect of temperature, the flasks containing dual cultures in M9 broth were incubated at 7, 14, 21, 28 and 35 °C. For the control experiment, fungi alone were

inoculated. Reduction in fungal biomass was determined as described previously.

Enumeration of antagonized fungi

An agar block 4 mm diameter with fungal growth showing inhibition on Petri dishes in ''volatile'' sets was taken from the antagonized and control plates after 120 h incubation. The agar block with the fungus was serially diluted and plated on PDA. The plates were incubated at 28 °C and counts were recorded after 3 days.

Microscopic observations

Smears were made of the antagonized portions of the test fungi from the sealed Petri dishes and of the fungal cultures from each flask used for broth based assays. The smears were then stained with lactophenol cotton blue and observed under a Nikon inverted microscope.

Inhibition of fungal biomass in relation to growth phase of *P. corrugata*

Growth curves of *P. corrugata* were prepared by inoculating 1 ml of a 24 h grown culture of the bacterium into 25 ml M9 broth. Optical density (OD_{595}) of the growing culture was recorded at 1 h intervals. Simultaneously, an experiment for biomass inhibition of *F. oxysporum* was performed as described earlier. Fungal weight (in control and dual cultures) was recorded at intervals of 1 h for 24 h and % weight reduction was determined by the formula previously described.

Production of siderophore, ammonia and hydrocyanic acid (HCN)

Production of siderophore was estimated on chrome-azurol S-agar medium (CAS), (Schwyn and Neilands, 1987). The bacterium was spot inoculated on CAS agar medium and the plates were incubated at 28 °C for 48 h. Development of orange colour around the bacterial colony indicated siderophore production. To determine the involvement of siderophore in the antagonism between *A. alternata* or *F. oxysporum* and *P. corrugata*, the reduction in fungal biomass was checked by adding 100 μM $FeCl_3$ in KB broth. Ammonia production was determined according to Dye (1962). The bacterial isolates were grown in peptone water in 30 ml tubes and incubated at 25 °C for 4 days. Afterwards, 1 ml of Nessler's reagent was added to each tube. Development of a faint yellow colour was indicative of weak reaction and deep yellow to brownish colour was indicative of strong reaction. HCN production of the isolate was detected by the method of Bakker and Schippers (1987). *P. corrugata* was inoculated individually on Petri dishes containing tryptone soya agar supplemented with 4.4 g l^{-1} glycine. Filter paper discs (9 cm diameter, Whatman No. 1) soaked in 0.5% picric acid in 2% sodium carbonate were placed in the lid of each Petri dish. Uninoculated controls were used for comparison. The plates were sealed with parafilm and incubated at 25 °C for 4 days. Change in colour from yellow to light brown and reddish brown was indicative of moderate and strong production of HCN by the bacterium, respectively. No change in colour indicated negative reaction. Quantitative estimations of siderophore and salicylic acid (SA) were carried out following the methods described in Nagarajkumar et al. (2004).

Evaluation of production of extracellular hydrolytic enzymes by *P. corrugata*

The activities of extracellular hydrolytic enzymes were detected on plate-based assays by streaking *P. corrugata* on the medium containing enzyme substrate and measuring the zone of degraded substrate formed around the colony after an incubation period of 5 days at 25 °C. Amylase activity was studied using soluble starch as substrate, lipase using tributyrin, protease using milk agar, (Benson, 1990); cellulase using carboxymethyl cellulose, pectinase using citrus pectin (Aneja, 1996), and chitinase using colloidal chitin (Basha and Ulaganathan, 2002). Quantitative estimation of chitinase was carried out following the method described in Nagarajkumar et al. (2004). All experiments were carried out in triplicate.

Results and discussion

In Petri dish-based assays carried out for the determination of diffusible antifungal metabolites, *P. corrugata* did not show any clear zone of inhibition in growth of the test fungi, *A. alternata* or *F. oxysporum* (Fig. 1A and B). After 120 h of incubation, the fungi overgrew the bacterial colony in both cases. However, *P. corrugata* produced volatile antifungal compound(s), as evident from the growth inhibition of both test fungi in sealed Petri dishes (Fig. 1C–F; Table 1). The inhibitory effect was observed to increase with time. Maximum inhibition in fungal growth (58% in *A. alternata* and 49% in *F. oxysporum*) was observed after 120 h of incubation. The normal colour of *A. alternata*

(black) and *F. oxysporum* (pink) changed to white due to the effect of volatile metabolites. The production of volatile antifungal compounds by *P. cepacia* and fluorescent Pseudomonads has been reported by Jayaswal et al. (1993) and Tripathi and Johri (2002), respectively. In the cited studies, the effect of inhibitory volatile metabolite(s) received less importance than the inhibitory diffusible metabolite(s). In the present investigation, it was demonstrated that the volatile metabolite(s) produced by *P. corrugata* had a predominant inhibitory role in the antagonism of the test fungi, *A. alternata* and *F. oxysporum*, and the diffusible metabolite(s) played only a subsidiary or no role in the antagonism.

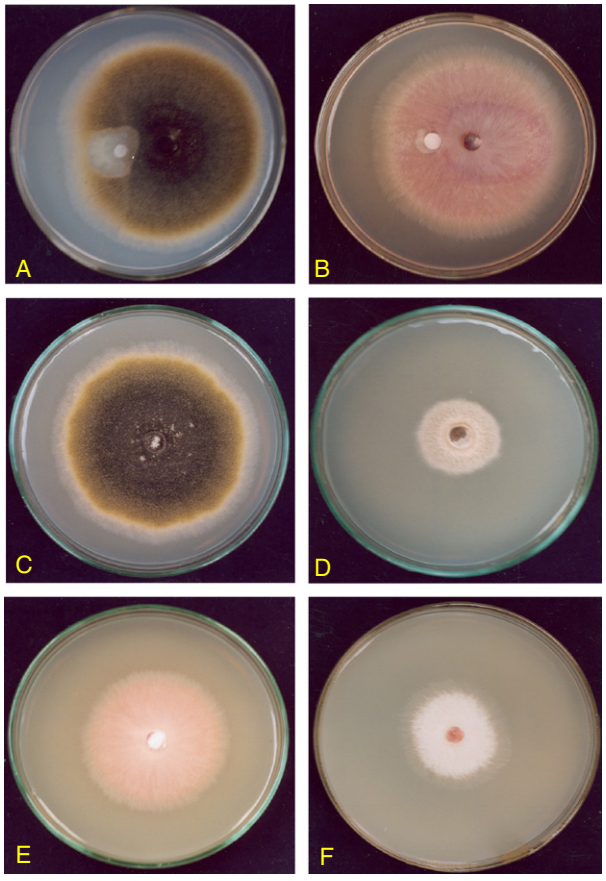

Figure 1. Antagonism of *P. corrugata*. (A, B) Inhibition of *A. alternata* and *F. oxysporum*, respectively, due to diffusible metabolites produced by *P. corrugata*. (C–F) Inhibition of *A. alternata* and *F. oxysporum* due to volatile metabolite(s) produced by *P. corrugata* – (C, E) controls (*A. alternata* and *F. oxysporum*, respectively); (D, E) inhibition of *A. alternata* and *F. oxysporum*, respectively.

P. corrugata showed biomass reduction in both test fungi in broth based dual cultures using six different growth media (Fig. 2). Reduction in fungal biomass varied from 93.8% (KB) to 57.9% (M9) for *A. alternata*, whereas for *F. oxysporum* it varied from 76.9% (KB) to 41.0% (SD). Determination of in vitro antagonistic activities by estimating the reduction in fungal biomass has been used by several workers (Broekaert et al., 1990; Knot et al., 1996; Basha and Ulaganathan, 2002). Variable responses in reduction of fungal biomass in different broths were indicative of the effect of nutritional factors on *P. corrugata*-induced antagonism.

The antagonistic activities were also found to be influenced by pH of the medium and incubation temperature. *P. corrugata* showed no antagonistic activity in M9 broth at pH 8.5 or above (Fig. 3). The inhibition of both test fungi was greater at acidic pH than at alkaline. Maximum reduction in weight for both test fungi was observed at pH 5.5. Observations on biomass reduction at different temperatures showed a broad temperature range (7–35 °C) of *P. corrugata* for the production of antifungal compounds (Fig. 4). Maximum reduction, 68.0% and 52.3%, for *A. alternata* and *F. oxysporum*, respectively, was observed at 21 °C. It was interesting to note that *P. corrugata* exerted its antagonistic effects at lower temperatures. The species has already been reported as a psychrotroph (Pandey et al., 2002). The effect of various physiological parameters like nutrients, pH and

Table 1. In vitro effect of volatile metabolites of *P. corrugata* on inhibition of *A. alternata* and *F. oxysporum*

Incubation period (h)	Colony diameter (mm)			
	A. alternata		*F. oxysporum*	
	Control	Antagonized	Control	Antagonized
24	35	20 (42)	32	24 (25)
48	48	23 (52)	45	27 (40)
72	60	27 (55)	55	34 (38)
96	73	32 (56)	67	38 (43)
120	81	34 (58)	76	39 (49)

Figures in parentheses denote percent inhibition of fungal growth vis-à-vis its control.

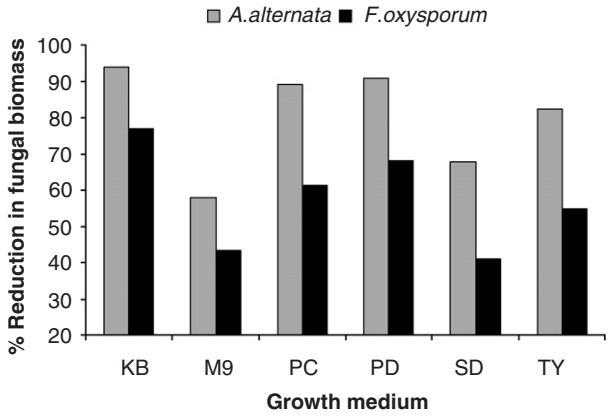

Figure 2. Reduction in fungal biomass due to antagonism of *P. corrugata* in different growth media (KB = Kings B; M9 = Millers 9; PC = potato carrot; PD = potato dextrose; SD = Sabouraud dextrose; TY = tryptone yeast).

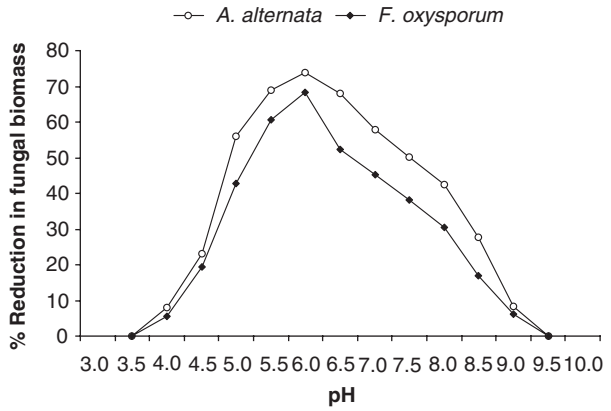

Figure 3. Effect of pH on reduction in fungal biomass caused by antagonism of *P. corrugata*.

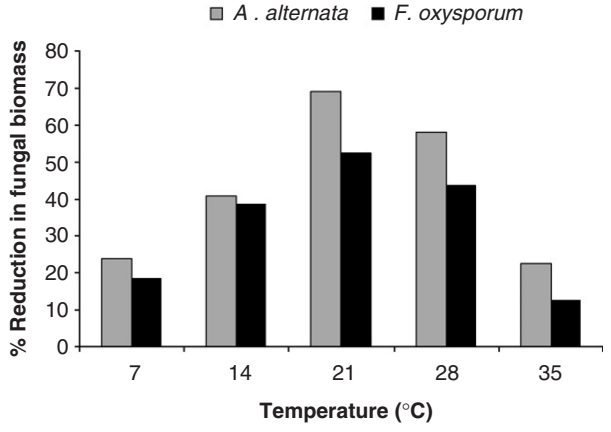

Figure 4. Effect of temperature on reduction in fungal biomass caused by antagonism of *P. corrugata*.

temperatures on the production of antifungal compounds by biocontrol agents has been reported (Jayaswal et al., 1990; Upadhyay et al., 1991).

Antimicrobial compounds may act on phytopathogenic fungi by inducing fungistatis, inhibition of germination, lysis of fungal mycelia, or by exerting fungicidal effects (Gloud, 1990). When the inoculum was taken from antagonized portions of either, *A. alternata* or *F. oxysporum*, and plated on fresh PDA, normal growth of fungi was obtained. Also, plating of the fungi taken from the broth based dual assay gave plate counts similar to that of the control. Microscopic examination of the antagonized fungi did not show any structural deformities. This indicated fungistatic effects of *P. corrugata* on *A. alternata* and *F. oxysporum*. Our results are in contrast to those reported by Upadhyay and Jayaswal (1992) who suggested the induction of morphological abnormalities and inhibition of conidiation in phytopathogenic fungi by another species of *Pseudomonas*, i.e., *P. cepacia*, as a possible mechanism of biological control.

Antagonistic activity of *P. corrugata* in relation to its growth phase was determined in terms of ''time course'' of reduction in fungal biomass (Fig. 5). No reduction in biomass of *F. oxysporum* was observed in the early stage of growth of *P. corrugata*. Antagonism started in the mid-exponential phase that became maximum (76.0%) in stationary phase, after 21 h of incubation. Antagonistic properties are generally mediated by the production of secondary metabolites, many of which are produced in the stationary growth phase (Vining, 1990). Carson et al. (1992) reported that organic acids such as malate, citrate and hydroxamate are produced during the late logarithmic or early stationary phase by different root nodulating bacteria.

Results on production of chemicals and enzymes, which may be involved in the antagonistic activities of *P. corrugata*, are presented in Table 2. Formation of orange zones around the bacterial colony on CAS medium indicated the production of siderophores. The bacterial isolate produced 8.6 μmol benzoic acid ml^{-1} of siderophore after 48 h of incubation in KB broth. In $FeCl_3$-supplemented KB broth, *P. corrugata* showed no reduction in biomass of the test fungi. Siderophores are not produced in the presence of iron (Meyer and Abdallah, 1978), and thus the loss in antagonistic property of the bacterium may be due to the absence of siderophore in iron-containing medium. The role of siderophore in biocontrol of several fungal phytopathogens has been reported (Scher and Baker, 1982; Kumar Dileep, 1998). The isolate was found to produce 17.7 μg ml^{-1} of SA in vitro. Siderophores and SA are known to induce systemic acquired resistance (SAR) (Enyedi et al., 1992; Leeman et al., 1996).

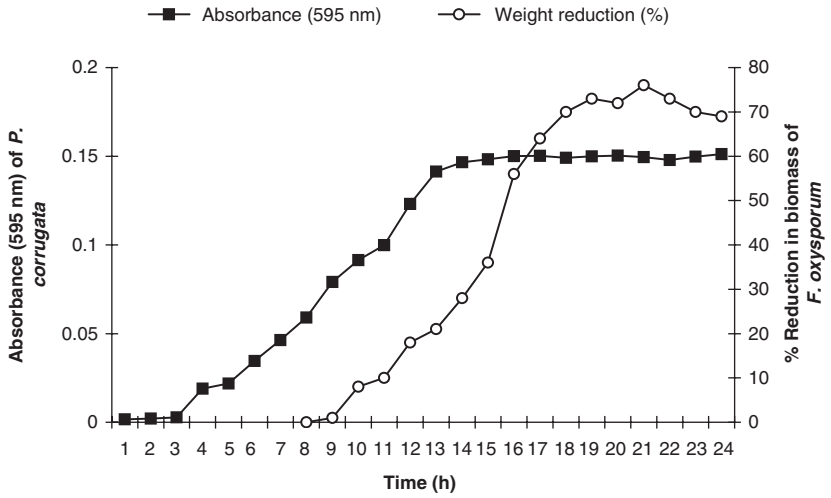

Figure 5. Growth curve of *P. corrugata* in relation to inhibition of *F. oxysporum*.

Table 2. Detection of antagonistic properties of *Pseudomonas corrugata*

Antagonistic properties	Reaction
Production of	
Siderophore	+
Ammonia	+
HCN	−
Production of hydrolytic enzymes	
Chitinase	+
Lipase	+
Amylase	−
Cellulase	−
Pectinase	−
Protease	−

+, Positive; −, negative.

Volatile compounds such as ammonia and hydrogen cyanide are produced by a number of rhizobacteria and are reported to play an important role in biocontrol (Brimecombe et al., 2001). No change in the colour of filter paper after 4–5 days of incubation showed that *P. corrugata* does not produce HCN in vitro. Presence of deep yellow colour after the addition of Nessler's reagent to peptone water cultures of *P. corrugata* indicated the production of ammonia. The role of ammonia in antagonism has been described (Howell et al., 1988). Pavlica et al. (1978) concluded that ammonia is the only gas present in sufficient concentrations in soil to inhibit soil fungi.

Petri dish-based assays carried out for the production of hydrolytic enzymes indicated that *P. corrugata* produces chitinase and lipase. When estimated quantitatively, the chitinase activity of the isolate was found to be 40.6 μmol min^{-1} mg^{-1} of protein. Production of other enzymes, amylase, cellulase, pectinase and protease, were not detected. Although the production of hydrolytic enzymes has been described for the biocontrol activity of some bacteria (Fridlender et al., 1993; Viswanathan and Samiyappan, 2001), in case of pseudomonads they have often not been described as important for biocontrol (Bagnasco et al., 1998), and such activities were not always correlated with the inhibition of fungi (de Boer et al., 1998; Sindhu and Dadarwal, 2001).

In plant disease management programmes, the use of a rapid method for screening efficient biocontrol agents is a prerequisite (Anith et al., 2003). Antagonistic activity possessed by biocontrol agents is often evaluated by measuring the inhibition zones developed in in vitro agar-based assays. There are several reports on the lack of correlation between in vitro antibiosis and biocontrol. For example, in a recent report where comparative in vitro and in vivo studies were conducted on *Bacillus subtilis* and *P. corrugata* against fungal attack causing wilting in micropropogated tea, it was observed that *B. subtilis* inhibited the fungi in vitro as well as in vivo, the isolates of *P. corrugata* inhibited the fungi only in in vivo experiments (Pandey et al., 2000). Efficient strains of *P. corrugata* have been shown to suppress damping-off in maize seedlings under glasshouse conditions (Pandey et al., 2001).

In vitro broth-based dual cultures offer a better method for evaluation of antagonistic efficiency of the biocontrol agents as the liquid medium may provide a better environment to allow the antagonistic activities from all possible interacting sites. *Pseudomonas corrugata* has been reported for its dominance in soils of colder climatic

conditions of the Himalayas and its role in plant growth promotion and biocontrol (Pandey and Palni, 1998; Pandey et al., 1998, 2000, 2001). The present study is important in understanding the possible mechanisms associated with the antagonistic bacterial species.

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