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EXPERIMENTAL ARTICLES

Isolation and Phenotypic Characterization of Plant Growth–Promoting Rhizobacteria with High Antiphytopathogenic Activity and Root-Colonizing Ability

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Abstract—Some bacterial strains isolated from the plant rhizosphere showed high root-colonizing ability and antiphytopathogenic activity against 6 fungal species. The antifungal activity was species-specific, which could be accounted for by the fact that the isolates differed in the ability to produce lytic enzymes (chitinases, proteases, and lipases) and to secrete cyanide. The possibility of using there rhizobacteria to control phytopathogens is discussed.

Key words: rhizobacteria, phytopathogens, enzymes, colonization.

Plant growth–promoting rhizobacteria (PGPR) are universal symbionts of higher plants, which enhance the adaptive potential of their hosts through a number of mechanisms, such as the fixation of molecular nitrogen, the mobilization of recalcitrant soil nutrients, the control of phytopathogens, and the synthesis of phytohormones and vitamins [1, 2].

The antiphytopathogenic activity of PGPR is believed to be due to their ability to synthesize antifungal metabolites (phenazines, pyrrolonitrin, 2,4-diacety-lfluoroglucin, and pioluteorin), extracellular enzymes (chitinase and glucanase), and siderophores [3]. The inoculation of cultivated plants with PGPR is a promising agricultural approach, for it allows pests to be controlled without using pesticides in large amounts. The stimulation of plant growth by PGPR is closely related to their ability to colonize plant roots. However, little attention is being given to the development of efficient selection procedures for obtaining bacterial strains with high root-colonizing ability. This slows down the study of plant–bacterial symbioses and the use of PGPR in agriculture.

The aim of the present work was to develop a procedure that would allow PGPR with high root-colonizing ability to be selected, to study the possibility of using this procedure for the selection of antiphytopathogenic bacterial strains, and to determine the enzymatic activity that can efficiently suppress the growth of phytopathogenic fungi in the plant rhizosphere.

MATERIALS AND METHODS

Rhizosphere bacteria were isolated from the roots of the hexaploid wheat *Triticum aestivum* L. var. Obelisk, the wild-type diploid wheat *T. monococum* 14379 (obtained from the All-Russia Institute of Plants), the red radish *Raphanus sativus* L. var. Zarya, and the tomato *Lycopersicum esculentum* Mill. var. Karmello.

Experiments were carried out gnotobiotically. Bacterial strains with high root-colonizing ability were selected by the Berestetskii *et al.* method [4] modified by us. Axenic seedlings with roots about 1 cm long were placed in petri dishes onto the surface of 0.6% agarose (Fluka) and incubated at 24°C in the dark for 16 h, within which the root exudates could diffuse into the agarose. Then 0.5-g aliquots of soil were placed, in the form of two narrow bands, parallel to the root at a distance of 1–2 cm from it. Soil microorganisms migrated over the agarose surface toward the source of nutrients, i.e., toward the root exudates. The petri dishes were incubated at 28°C for the next 24 h, after which the soil was removed from the petri dish together with some amount of agarose, and the root was allowed to grow for 3–4 days. Bacteria were isolated from the roots using liquid or agar-solidified potato medium, which considerably augmented the recovery of bacterial strains with high root-colonizing ability.

The antifungal activity of the isolates was tested as follows: The isolates were grown at 28°C for 5 days without shaking in Erlenmeyer flasks containing LB broth [5] in an amount such that the medium thickness was 3–4 cm. A suspension (5 ml) containing 10^6 fungal conidia/ml was mixed with 250 ml of liquid Czapek agar, and the mixture was poured into petri dishes in 25 ml portions. Bacterial isolates were placed into wells made in the agar plates. Then the plates were incubated at 28°C for 2–4 days, and the antifungal activity of the isolates was estimated in terms of the diameter of the fungal growth inhibition zone around the wells.

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Table 1. The identification of rhizobacterial isolates by the ARDRA and BIOLOG methods

Strain	Gram stain	ARDRA	BIOLOG
SPB1117	+	NI	*Curtobacterium flaccumfaciens*
SPB1217	–	*Pseudomonas* sp.	*Pseudomonas chlororaphis*
SPB2137	–	*Pseudomonas* sp.	*Pseudomonas fluorescens*
SPB2142	–	*Pseudomonas* sp.	*Pseudomonas corrugata*
SPB2184	–	*Pseudomonas* sp.	*Pseudomonas corrugata*
SPB3057	–	*Pseudomonas* sp.	*Agrobacterium* sp.
SPB3062	+	NI	*Curtobacterium* sp.
SPB3185	–	*Pseudomonas* sp.	*Pseudomonas* sp.
SPB4027	+	NI	*Clavibacterium* sp.
SPB4087	–	*Pseudomonas* sp.	*Pseudomonas* sp.

Note: NI stands for "not identified."

Cyanide was detected in the form of hydrocyanic acid using indicator paper prepared by soaking the (2×2 cm) pieces of filter paper (Whatman 3 MN) in a solution of copper(II) ethylacetate (5 mg/ml) and methylene diethylamine (5 mg/ml) in chloroform. After drying, the indicator paper was placed in the inverted petri dishes with bacterial strains grown on LC agar [5]. The dishes were sealed and incubated at 28°C for 1 day, after which the indicator paper was examined for blue color (an indication of the presence of cyanide).

To assess the lipase activity of bacterial isolates, they were plated onto the surface of LC agar with 2% Tween-80, and the plates were incubated at 28°C for 5 days. The lipase activity of the isolates was proportional to the diameter of the turbid zone around the grown bacterial colonies.

Protease activity was assayed similarly, by the diameter of the transparent zone around the bacterial colonies grown on LC agar with 3% milk.

Chitinase activity was measured with chitinpentaose labeled with ^{14}C. Two microliters of [^{14}C]chitinpentaose was incubated with 10 μl of a 1-day-old bacterial culture at 28°C for 16 h. The mixture was then subjected to thin-layer chromatography on Sigma plates in an acetonitrile–water (65 : 35 v/v) mixture. The radioactivity of spots on the developed plates was determined using a Bio-Rad scanning radiometer.

The bacterial isolates were Gram stained according to the manufacturer's instructions (Sigma Diagnostics) and identified to a species level using Biolog (Hayward, United States) plates with 95 different carbon sources and analyzing (with the aid of ARDRA) the structure of the genomic locus coding for rRNA.

The colonization of the rhizoplane was studied in a gnotobiotic system [6] that included sterile quartz sand supplemented with 10% of a phosphorus–nitrogen–sulfur solution (PNS solution) containing 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and trace elements. Seeds were bacterized by incubating them with bacterial cells grown on LC agar for 12 h and washed off from it with the PNS solution (the resultant bacterial suspension had the optical density OD_{620} = 0.1, which corresponded to 10^8 cells/ml). After 15 min of incubation, the bacterized seeds were aseptically transferred to the quartz sand to a depth of 5 mm and incubated in a phytotron at 21°C for 16-h illumination periods (10000 lx) for 7 days.

Rhizobacterial cells in a mixture with the cells of *Pseudomonas fluorescens* PCL1500 (the Tn5-*LacZ* mutant of the wild-type strain WCS365 [7]) were enumerated using agar medium supplemented with 40 μg/ml X-Gal. Bacterial colonies were counted after 1–2 days of incubation at 28°C.

The motility of the bacterial isolates was determined using a semiliquid (0.3% agar) LC medium diluted twentyfold. The bacteria tested were inoculated at the center of the agar plate [6] and incubated at 28°C. The diameter of the growing bacterial colonies was measured in dynamics.

RESULTS AND DISCUSSION

We succeeded in isolating 156 bacterial strains with high root-colonizing ability from soil samples collected in the northwestern region of Russia. Forty-six strains were isolated from the roots of diploid (wild-type) wheat, fifty-eight strains were isolated from the roots of hexaploid (selected) wheat, and fifty-two strains were

Table 2. The root-colonizing ability of rhizobacteria upon the combined inoculation with *P. fluorescens* PCL1500

Plant and rhizobacterial strain	Number of cells,* 10^3 CFU/cm root		Ratio**
	rhizobacterium	PCL1500	
Wheat			
SPB1217	7.6 ± 1.8	3.8 ± 1.1	2.0
SPB2137	8.8 ± 3.9	2.9 ± 2.0	3.0
SPB2142	13.7 ± 3.1	3.3 ± 2.1	4.1
SPB2184	9.1 ± 4.0	9.3 ± 2.7	1.0
SPB3185	9.0 ± 2.8	2.4 ± 1.7	3.8
SPB4087	3.7 ± 2.2	4.2 ± 2.3	0.9
Red radish			
SPB1217	9.5 ± 2.0	1.0 ± 0.7	9.5
SPB2137	7.1 ± 1.3	1.0 ± 0.9	7.1
SPB2141	1.5 ± 0.4	2.6 ± 0.5	0.6
SPB2184	5.2 ± 1.7	5.8 ± 2.1	0.9
SPB3185	16.2 ± 2.3	5.4 ± 0.9	3.0
SPB4087	11.4 ± 2.8	15.2 ± 2.2	0.8
Tomato			
SPB1217	7.3 ± 1.3	3.3 ± 2.0	2.2
SPB2137	4.5 ± 2.0	1.0 ± 0.7	4.5
SPB2142	2.8 ± 1.2	10.9 ± 2.5	0.3
SPB2184	6.1 ± 1.8	9.0 ± 1.7	0.7
SPB3185	5.2 ± 0.9	3.7 ± 1.3	1.5
SPB4087	7.7 ± 1.8	10.8 ± 2.0	0.7

* The number of bacterial cells per 1 cm of the root tip was evaluated after 7 days of cultivation.
** The ratio of the number of rhizobacterial cells to the number of PCL1500 cells adhered to the roots (the ratio of these cells in the suspension used for inoculation was 1 : 1). The data presented are the means and the standard deviations of triplicated measurements.

isolated from red radish roots. Ten strains that showed the highest antifungal activity and growth rate were chosen for further studies. Eight of them were gram-negative, and two strains were gram-positive. Analysis with the aid of ARDRA for the structure of the genomic locus coding for rRNA and with the aid of BIOLOG for the range of utilizable carbon sources showed that the gram-negative strains were dominated by members of the genus *Pseudomonas* and that the gram-positive strains were dominated by members of the genera *Curtobacterium* and *Clavibacter* (Table 1). For strain SPB3057, the two methods of analysis gave inconsistent results.

Table 3. The motility of rhizobacteria in the semiliquid medium

Strain	Diameter of bacterial colony, mm	
	24 h	32 h
PCL1500	45.3 ± 2.1	62.5 ± 1.8
SPB1217	38.4 ± 1.0	55.4 ± 1.5
SPB2137	42.1 ± 1.9	60.7 ± 2.4
SPB2142	34.6 ± 1.7	51.9 ± 1.6
SPB2184	55.3 ± 2.2	78.6 ± 2.9
SPB3185	53.2 ± 2.4	80.1 ± 3.0
SPB4087	35.5 ± 1.5	54.6 ± 2.2

Note: The data presented are the means and the standard deviations of seven replicated measurements.

The analysis of the ability of six isolates to colonize the rhizoplane of wheat, red radish, and tomato under the conditions of a combined inoculation with the test strain *P. fluorescens* PCL1500 showed that three strains, SPB1217, SPB2137, and SPB3185, are highly competitive for the roots of all three kinds of plants (Table 2). Strains SPB2142 and SPB2184 exhibited high competitiveness only for the wheat roots, and strain SPB4087 showed low competitiveness for the roots of all of the plants tested. The motility of the strains widely varied (Table 3) and showed no correlation with the level of root-colonizing ability.

According to their activity against six phytopathogenic fungi, the bacterial isolates were divided into two groups (Table 4). The first group of broad-range antiphytopathogenic bacteria, which comprised strains SPB1217, SPB2137, SPB2142, SPB2184, SPB4027, and SPB4087, was characterized by the growth inhibition zone diameter varying within narrow limits (the variation coefficient $V = 20–78\%$). The second group of narrow-range antiphytopathogenic bacteria, which included strains SPB1117, SPB3057, SPB3062, and SPB3185, was characterized by the highly varying diameter of the growth inhibition zone (the variation coefficient $V = 99–165\%$). One of the strains, SPB2142, was found to be able to grow on the fungal mycelium.

To understand what mechanisms may be responsible for the antifungal activity of the bacterial isolates, we investigated their ability to produce hydrolytic enzymes and cyanide. As can be seen from Table 5, the narrow-range antiphytopathogenic bacteria produce the enzymes and cyanide in low amounts, if at all. At the same time, the broad-range antiphytopathogenic bacte-

Table 4. The antiphytopathogenic activity of rhizobacteria

Strain	Diameter of the growth inhibition zone, mm					
	Fusarium culmorum	*Verticillium dahliae*	*Pythium butleri*	*Bortytis cinerea*	*Rhizoctonia* sp.	*Alternaria consortiale*
SPB1117	0	0	0	0	18 ± 2	34 ± 3
SPB1217	16 ± 2	26 ± 2	25 ± 3	20 ± 2	30 ± 3	6 ± 2
SPB2137	31 ± 2	21 ± 3	20 ± 2	12 ± 1	36 ± 2	47 ± 4
SPB2142	14 ± 2	36 ± 3	38 ± 3	38 ± 3	65 ± 4	47 ± 3
SPB2184	10 ± 2	6 ± 1	6 ± 1	48 ± 4	34 ± 2	33 ± 3
SPB3057	0	36 ± 3	0	0	30 ± 2	–
SPB3062	0	20 ± 3	0	0	41 ± 2	–
SPB3185	14 ± 2	8 ± 1	5 ± 1	40 ± 4	0	20 ± 2
SPB4027	23 ± 3	20 ± 3	14 ± 2	41 ± 3	–	33 ± 2
SPB4087	25 ± 2	24 ± 2	33 ± 2	34 ± 3	–	38 ± 2

Note: "–" stands for "not determined." The data presented are the means and the standard deviations of five replicated measurements.

ria produce either all three hydrolytic enzymes and cyanide (strain SPB2142) or some of them (strains SPB1217, SPB2137, and SPB4087). The only exception was strain SPB4027, which produced none of the enzymes and no cyanide. The ability of strain SPB2142 to produce different hydrolytic enzymes may explain its capability for using the fungal mycelium as the source of nutrients.

Table 5. The production of cyanide and some hydrolytic enzymes by rhizobacteria

Strain	Cyanide	Protease	Lipase	Chitinase
SPB1117	–	–	–	–
SPB1217	+++	++	+	–
SPB2137	++	+++	–	–
SPB2142	+++	+	++	+++
SPB2184	+	–	–	–
SPB3057	–	+	+	–
SPB3062	–	–	–	–
SPB3185	+++	–	–	–
SPB4027	–	–	–	–
SPB4087	–	+	–	–

Note: "–" stands for the absence of the production of a given enzyme or cyanide, whereas "+," "++," and "+++" indicate their different production levels in seven replicated experiments.

Thus, ten of the 156 bacterial strains isolated from the plant rhizosphere showed high root-colonizing ability and antiphytopathogenic activity against 6 fungal species. The antifungal activity of the isolates was species-specific, which could be accounted for by the fact that the isolates differed in the ability to produce lytic enzymes (chitinases, proteases, and lipases) and to secrete cyanide. The root-colonizing ability of the isolates depended on the kind of plants. The bacterial isolates with both high antifungal activity and high root-colonizing ability may be of interest for the control of plant diseases. It is obvious that the mechanisms responsible for the antifungal activity of rhizobacteria need further investigations.

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