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EXPERIMENTAL ARTICLES

Effect of Sodium Salicylate on the Population Dynamics of the Rhizobacterium *Pseudomonas aureofaciens* in the Wheat Rhizoplane and Adjacent Soil

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Abstract—The effect of sodium salicylate on the population dynamics of the rhizobacterium *Pseudomonas aureofaciens* BS1393 and its variant bearing the naphthalene biodegradation plasmid pBS216 was studied in the wheat rhizoplane and adjacent soil. Optimum salicylate concentration for the maintenance of the plasmid-bearing strain and for the normal growth of wheat was found to be 250 μg/g soil. When the soil was supplemented with salicylate, the population of *P. aureofaciens* BS1393(pBS216) in the wheat rhizoplane and adjacent soil was, respectively, 4- and 20-fold higher than that of the parent strain lacking the plasmid.

Key words: rhizobacterium *Pseudomonas*, biodegradation plasmids, sodium salicylate, population dynamics

The efficiency of the so-called plant growth-promoting rhizobacteria (PGPR) of the genus *Pseudomonas*, which stimulates the growth of plants and protects them from phytopathogens, can be essentially enhanced if the rhizosphere population is raised to sufficiently high levels [1]. One of the possible approaches to this problem is to use plasmid-bearing strains capable of utilizing the substrates that are inaccessible to indigenous microflora as soil inoculants. Salicylic acid, an intermediate of naphthalene catabolism in bacteria, seems to be this type of selective substrate, since its metabolism is controlled by plasmid-borne genes [2]. The use of salicylate can be especially beneficial since this compound is a natural metabolite of plants [3].

Earlier, Colbert *et al.* transformed the plant growth-promoting strain *P. putida* R20 with the conjugative plasmid NAH7 [4]. We also attempted to obtain stable transconjugants of the growth-promoting strains *P. putida* BS1380 and *P. aureofaciens* BS1393 using various naphthalene degradation plasmids, including NAH7 [5]. Plasmid NAH7 turned out to be unable to provide for the good survival of transformed rhizosphere pseudomonads cultivated on naphthalene or salicylate as the sole sources of carbon and energy. At the same time, the transconjugants of *P. putida* BS1380 and *P. aureofaciens* BS1393 bearing plasmid pBS216 were found to be very stable [5]. For further studies, we chose *P. aureofaciens* BS1393 since this strain is an antagonist to a wide range of phytopathogenic fungi and bacteria and is also the primary component of a commercial antiphytopathogenic preparation known under the trade name Pseudobacterin-2 [6].

The aim of the present work was to study the effect of exogenously added salicylate on the population dynamics of *P. aureofaciens* BS1393 and its plasmid-bearing variant BS1393(pBS216) in the wheat rhizoplane and adjacent soil.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Experiments were performed with *P. putida* BS1380 (VKM B-1743D) isolated from the rhizosphere of wild-type cereals in Armenia [7], *P. aureofaciens* BS1393 strain isolated from the barley rhizosphere, and its variant, *P. aureofaciens* BS1393(pBS216) [5] bearing plasmid pBS216, which controls naphthalene oxidation via the *ortho*-pathway of catechol cleavage [8].

Bacteria were grown in LB broth, on LB agar, and in synthetic M9 medium [9]. The ability of the strains to grow on naphthalene and salicylate as the sole sources of carbon and energy was tested using these substrates at a concentration of 1 g/l. The stability of plasmid pBS216 was determined as described earlier [5].

Soil experiments were carried out with gray forest soil [10] sieved through a 2-mm mesh screen and sterilized at 1 atm for 1 h. The soil was placed in petri dishes in 25-g portions and in seedling pans in 80-g portions. Sodium salicylate was dissolved in 10 mM sodium phosphate buffer (pH 7.0) and sterilized at 0.5 atm for 0.5 h. Bacterial cells were cultivated on LB

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Table 1. Dynamics of sodium salicylate in the soil inoculated with *P. aureofaciens* BS1393(pBS216)

Initial concentration of salicylate, μg/g soil	Cultivation time (days) and residual salicylate (μg/g soil)				
	2	4	6	8	10
1000	565	23	85	0	0
750	264	7.5	0.02	0	0
500	85	0.04	0	0	0
250	10.5	0.01	0	0	0
100	6.2	0	0	0	0

agar for 20 h at 30°C, washed from the plates with the buffer (this buffer was used in all of the subsequent procedures), washed clean of the medium with the buffer, and placed in the test tubes containing solutions of salicylate with different concentrations. Aliquots (10 ml) of the prepared cell suspensions were thoroughly mixed with 25 g of soil in petri dishes to give an initial bacterial population density of about 10^6 g^{-1} soil. The petri dishes were incubated at 16°C. Every 48 h, the soil in the petri dishes was mixed with a spatula and 1 g of soil was withdrawn to estimate bacterial population. For this purpose, the soil sample was suspended in 9 ml of the buffer in a mixer for 1 min and the soil particles were then allowed to settle for 3 min. The supernatant was serially diluted and plated on LB agar to evaluate its bacterial population (data were expressed as CFU/g soil). By the end of the 10-day experiment, the stability of plasmid pBS216 in the inoculated bacterial strain was estimated by counting the number of colonies grown on the minimal agar M9 medium containing salicylate and naphthalene as the sources of carbon and energy [5].

Experiments with plants. Bacterial cell suspensions prepared as described above were mixed with 80 g of soil contained in seedling pans to give an initial cell density of approximately 10^6 g^{-1} soil. The spring wheat (variety Kharkovskaya-6) seeds (1996 crop) were sterilized with a sodium hypochlorite solution for 1 h [11], washed twice with sterile tap water, and placed on the surface of the LB agar to germinate. The germinated seeds were placed in the soil inoculated with bacteria (two seeds per each of the 15 seedling pans used in one experiment) and incubated at 18°C for a 12-h photoperiod. Soil was supplied with water every 24 h and, in some experiments, with sodium salicylate every 72 h (salicylate was added in the form of solution in an amount of 250 μg/g soil). Samples for bacterial count were taken at 10-day intervals from the soil and the wheat rhizoplane. In the latter case, wheat roots were washed in flowing tap water and ground in 1 ml of the buffer. The resulting slurry was mixed with 9 ml of the buffer, incubated on a shaker (390 rpm) for 2 h, and then diluted with physiological saline solution. The dilutions were plated on LB agar to enumerate bacterial cells.

To estimate the bacterial population in the soil, soil samples (1 g) were taken from 7-cm depth and treated as described in the *Soil experiments* paragraph.

HPLC analysis of soil samples for salicylate was carried out as follows. Soil samples (1 g) were suspended in 9 ml of the buffer and the soil particles were allowed to settle. The supernatant was passed through a 0.45-μm-pore-size Millipore filter and applied, in 25- to 100-μl aliquots, to a (4 × 250 mm) reversed-phase C-18 Spherisorb ODS2 (particle size 5 μm) column (LKB Biotechnology) safeguarded with a (4.6 × 75 mm) Octadecyl Si-60 (particle size 20–40 μm) precolumn. Salicylate was eluted with a gradient of 0–100% B. Solvent A was 40% methanol containing 0.05 M formic acid; solvent B was 60% methanol and 40% formic acid. The column was kept at 50°C. The absorbance of the eluate was detected at 230 and 270 nm and interfaced, using a Nelson Analytical 900 Series, to an Olivetty M-24 PC. The results were processed using the Nelson Analytical software package. The concentration of salicylate in the samples was calculated by comparing the areas of salicylate peaks in the sample and reference solutions.

RESULTS AND DISCUSSION

Utilization of salicylate by *P. aureofaciens* BS1393(pBS216) in soil. To determine the optimum concentration of salicylate for the selective maintenance of the strain BS1393(pBS216) in soil and in the normal growth of plants, we tested varying concentrations of salicylate (0, 100, 250, 500, 750, and 1000 μg/g

Table 2. Population dynamics of *P. aureofaciens* BS1393 and *P. aureofaciens* BS1393(pBS216) in the soil supplemented or not supplemented with salicylate (250 μg/g soil)

Strain and amendment to soil	Cultivation time (days) and bacterial population density (CFU × 10^8/g soil)				
	2	4	6	8	10
BS1393	3.73	4.72	8.77	7.23	8.80
BS1393, salicylate	2.93	4.30	5.25	5.43	7.13
BS1393 (pBS216)	4.13	5.70	6.33	7.27	7.20
BS1393 (pBS216), salicylate	5.00	6.50	8.47	8.23	9.57

Table 3. Population dynamics of *P. aureofaciens* BS1393 and *P. aureofaciens* BS1393(pBS216) in the soil inoculated with *P. putida* BS1380 and either supplemented or not with salicylate (250 μg/g soil)

Strain and amendment to soil	Cultivation time (days) and bacterial population density (CFU × 10^8/g soil)				
	2	4	6	8	10
BS1393	0.56	2.60	2.43	1.60	2.40
BS1393, salicylate	0.59	1.70	1.67	1.47	1.70
BS1393 (pBS216)	0.50	1.40	1.87	2.27	2.40
BS1393 (pBS216), salicylate	1.77	3.37	6.47	5.10	7.87

Table 4. Population dynamics of *P. aureofaciens* BS1393 and *P. aureofaciens* BS1393(pBS216) on the wheat roots and in the soil inoculated with *P. putida* BS1380 and either supplemented or not with salicylate (250 μg/g soil every two days)

Strain and amendment to soil	Cultivation time (days) and bacterial population density (CFU × 10^8/g soil (S) or root (R))									
	10		20		30		40		50	
	S	R	S	R	S	R	S	R	S	R
BS1393	0.98	1.30	1.00	1.14	1.00	0.80	0.67	0.90	0.67	0.09
BS1393 (pBS216)	0.90	0.33	0.90	1.50	0.83	0.25	0.43	0.10	0.40	0.15
BS1393 (pBS216) salicylate	4.20	0.20	1.93	0.98	1.43	1.30	0.97	3.70	2.93	2.10

Table 5. Stability of plasmid pBS216 in *P. aureofaciens* BS1393(pBS216) cells occurring on the wheat roots and in the soil inoculated with *P. putida* BS1380 and either supplemented or not with salicylate (250 μg/g soil every two days)

Strain and amendment to soil		Cultivation time (days) and % of plasmid-containing cells				
		10	20	30	40	50
BS1393 (pBS216)	Soil	100	100	75	72	73
	Roots	100	100	75	75	65
BS1393 (pBS216) Salicylate	Soil	100	100	87	88	98
	Roots	100	100	87	90	90

soil). The HPLC analysis of salicylate in soil showed that it was efficiently utilized by the bacteria, so that its concentration in the soil considerably dropped as soon as within 2 days of cultivation (Table 1).

After 4 days of cultivation, the populations of the strain BS1393(pBS216) in the soils initially containing 250, 500, and 750 μg/g salicylate were slightly denser than in the soils supplemented with other concentrations of salicylate. After 6 days of cultivation, the populations of the strain BS1393(pBS216) were almost the same in all soils. Genetic analysis performed after 10 days of cultivation showed that, irrespective of the initial concentration of salicylate in soil, about 90% of cells retained plasmid pBS216. It should be noted that the addition of salicylate to the soil at concentrations of 500 μg/g and higher affected the growth of wheat plants: their leaves turned yellow and rolled into tubes. In view of this, salicylate was added to the soil in further experiments at a concentration of 250 μg/g soil, which does not inhibit the plants.

The population dynamics of strains BS1393 and BS1393(pBS216) in soils without salicylate and with a single addition of this substrate can be seen from the data presented in Table 2. It is evident that the population of strain BS1393(pBS216) was denser in the salicylate-containing soil, while the population of the parent strain BS1393 was greater in the soil without salicylate. This indicates that salicylate promotes the survival of soil bacteria containing the salicylate degradation genes and does not beneficially influence the bacteria that lack such genes. Based on the data of Table 1, which show that salicylate added to soil at the optimum concentration of 250 μg/g is rapidly consumed (by 96% in 2 days), it is suggested that in order to obtain denser populations of strain BS1393(pBS216) in a soil, it should be supplemented with this concentration of salicylate every two days.

In these experiments, the stability of plasmid pBS216 in *P. aureofaciens* BS1393(pBS216) cells living in soil did not depend on the presence of salicylate and comprised about 90%.

The population dynamics of strains BS1393 and BS1393(pBS216) in the soil with *P. putida* BS1380. The rhizosphere strain *P. putida* BS1380 was added to the soil in the amount of 10^6 cells/g soil. As can be seen from the comparison of the data presented in Tables 2 and 3, the addition of *P. putida* cells to soil without salicylate adversely influenced the populations of both *P. aureofaciens* strains BS1393 and BS1393(pBS216), probably due to the competition of *P. aureofaciens* and *P. putida* cells for nutrients naturally present in the soil. The addition of salicylate (250 μg/g soil) beneficially influenced the population of strain BS1393(pBS216) in the presence of *P. putida*, increasing it by almost three times (Table 3). At the same time, the addition of salicylate virtually did not influence the population of strain BS1393. This result is not surprising, since this strain is unable to utilize salicylate. This experiment clearly demonstrates the efficiency of selective carbon sources in increasing the population of alien species introduced into the soil.

In these experiments, the stability of plasmid pBS216 in *P. aureofaciens* BS1393(pBS216) cells living in soil did not depend on the presence of salicylate and comprised about 95%.

The population dynamics of strains BS1393 and BS1393(pBS216) in the wheat rhizoplane and adjacent soil with *P. putida* BS1380. These experiments were carried out similarly to those described above, except that the soil contained growing wheat seedlings. Every two days, the soil was supplied with salicylate at a concentration of 250 μg/g soil. As can be seen from the data presented in Table 4, after 10 days of cultivation, the population of strain BS1393(pBS216) in the soil supplied with salicylate was considerably higher than that of the parent strain BS1393. This result was obviously due to the fact that plasmid-bearing cells are able to utilize salicylate and thus have an advantage over the cells that lack this plasmid. In the course of further cultivation, the populations of both strains in the soil gradually decreased. However, the prevalence of strain BS1393(pBS216) remained. In the soils not supplied with salicylate, the populations of both strains were almost identical.

The enumeration of bacterial cells on wheat roots showed that, after 10-days of cultivation in the presence of salicylate, the number of BS1393 cells on the root surface was slightly greater than that of BS1393(pBS216) cells. In the course of further cultivation, however, the population of the plasmid-bearing strain on the roots was denser than that of the parent strain. When salicylate was not added, the tendency was opposite, i.e., the population of strain BS1393 on the roots was almost four times greater than that of strain BS1393(pBS216) (Table 4). In fact, growth conditions (such as the availability of carbon and nitrogen sources) in the wheat rhizoplane are more favorable than in the adjacent soil. This explains why the parent strain has an advantage over the plasmid-bearing strain in the rhizoplane when salicylate is not added.

The most important result of this experiment is that the repeated addition of salicylate to soil led to a 20 times denser population of strain BS1393(pBS216) on the wheat roots in comparison to the population of strain BS1393 (Table 4).

Salicylate beneficially influenced the stability of plasmid pBS216 in *P. aureofaciens* BS1393(pBS216) cells living in both the wheat rhizoplane and adjacent soil (Table 5). After a 30-day incubation, the plasmid was retained in 75% of the cells cultivated without salicylate and in 87% of the cells cultivated with salicylate (at this stage of cultivation, plasmid stability was the same in the wheat rhizoplane and the adjacent soil). After a 50-day incubation, however, the plasmid was more stable in the soil than on the root surface, irrespective of the presence of salicylate (Table 5). These results can be explained by the fact that various organic substances present in the root exudate [12] are readily utilized by both strains and thus diminish the selective significance of plasmid pBS216 in the cells occurring on the root surface. In the soil, which is low in readily metabolizable sources of carbon and energy, the plasmid provides for better bacterial survival, especially when salicylate is repeatedly supplied to the soil.

Thus, the data presented in this work and that available in the literature [13] show that the feeding of soil with special carbon sources can enhance the soil and rhizosphere populations of inoculated plant growth-promoting *Pseudomonas* strains capable of utilizing these carbon sources.

It is known that rhizosphere pseudomonads produce secondary metabolites that suppress the growth of soil phytopathogens only when their population densities reach a certain level [14]. In particular, there is evidence that the synthesis of phenazine antibiotics by the *P. aureofaciens* BS1393 strain is controlled by the *phzR* and *phzI* genes, which encode products highly homologous to the LuxR/LuxI regulatory proteins, whose level in cells depends on their population density [15, 16].

The data presented show that the plant-protecting efficiency of the *P. aureofaciens* BS1393 strain can be increased by obtaining its gene engineered variants capable of abundant growth in the plant rhizosphere.

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