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Characterization of Growth-Promoting Activity of *Pseudomonas Putida* Strain MG-2.

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ABSTRACT

The present study was conducted to isolate and characterize the native plant growth-promoting bacterium from potato rhizosphere in the Republic of Tatarstan, Russia. Based on the gyrB (DNA gyrase subunit B) gene sequence analysis, bacterial isolate was identified as Pseudomonas putida MG-2. Production of indole-3-acetic acid (IAA) was dependent on tryptophan: maximum of IAA accumulation was 11.5 µg/ml on 72nd hour of growth. Furthermore, the ability to synthesize the catechol siderophores, phytate-mobilizing activity and halotolerance were revealed. The treatment of pea and rye seeds by P. putida MG-2 suspension stimulate the seedlings growth and roots biomass by up to 24-28% and 5-14%, respectively. Therefore, this bacterial isolate may be potentially beneficial as growth-promoting factor.

Keywords: Pseudomonas putida, rhizosphere, potatoes, siderophores, indole-3-acetic acid, growth-promoting factor

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INTRODUCTION

The plant rhizosphere includes a group of growth-promoting bacteria (PGPR – plant growth-promoting rhizobacteria). Bacteria that belong to genera *Pseudomonas* and *Bacillus* are the most widely reported as PGPR [1]. PGPR involved in plant growth promoting directly or indirectly. Direct stimulation of plant growth is performed by synthesizing of plant hormones, or by facilitating the nutrient enrichment of plants. Indirect stimulation is performed by the antagonistic action against plant pathogens. It occurs as a result of synthesis of antimicrobial compounds by bacteria or by induction of plant resistance [2].

PGPR could be used as a biological agent for controlling of number of the plant pathogens and stimulation of the growth and agricultural crops development. There are several *Pseudomonas* species that could be successfully used for biocontrolling of soil phytopathogens and for plant growth promotion [3-5]. The application of biological agents is important and promising strategy; because it is effective and environmentally friendly, it may be applied as an alternative to chemical pesticides [2, 6]. Therefore, searching and characterization of rhizosphere bacterial strains with a strong growth-promoting activity is important for the development of new agricultural biotechnologies.

This paper reports on the characterization of bacterial strain MG-2 isolated from the potato rhizosphere and evaluates its potential application as a biological agent.

MATERIALS AND METHODS

To isolate the bacteria from the potato rhizosphere, non-rhizospheric soil was removed from the roots by shaking. Rhizospheric soil was collected by dipping and shaking the roots in sterile water. The suspension was inoculated in LBA medium. To isolate pure cultures, the colonies of different morphologies were 3-4 times inoculated in sterile agar medium. Total 48 isolates with various colonial morphology were obtained. Among these bacteria, 23 strains were gram-negative. One strain was identified in the MaldiBioTyper (Bruker Daltonik) as the species of genus *Pseudomonas* (1.868) and used for further characterization.

Identification of the bacteria was carried out by sequencing of gyrase subunit B encoding gene. For this purpose, bacterial DNA was isolated by the method described in [7]. The PCR amplification was performed with the use of primers for gyrase B subunit gene: UP1 5'-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA and UP2r 5'-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT. The PCR analysis was conducted by method [8]. The isolate sequence was analyzed with BLAST program (htttp:/www.ncbi.nlm.nih.gov/Blast).

Siderophore production was visualized on CAS (Chrome Azurol S) agar plates [9, 10]. Siderophore production was indicated by the formation of haloes. The dinamics of catechol siderophores production in the environment was studied using a color reaction to catechols by Arnow assay [9]. Bacteria were cultured in M9 medium (g/l: KH_2PO_4 - 3, NaCl - 5, NH_4Cl -10) supplemented with 0.4% glucose, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$. For complete binding of free iron, the medium was supplemented with 2,2'-Bipyridine at final concentration of 50 µM. Cells were isolated by centrifugation at 8000g for 15 min. 50 µl 0.5M HCl, 50 µl of a 10% $NaNO_2$ and 10% Na_2MoO_4, 50 µl 1M NaOH were sequentially added to 50 µl cell-free medium. Absorbance was measured at 490 nm on a Microplate Spectrophotometer xMark^{TM}(Bio-RAD). A pink color in the test sample indicated the presence of the catechol siderophores. The siderophore concentration in the sample was determined based on the calibration curve built with the use of 2.3-dihydroxybenzoic acid (Acros Organics).

Indole-3-acetic acid (IAA) production ability was determined by colorimetric analysis [11]. Bacteria were grown in M9 medium in the presence and absence of L-tryptophan as precursor of IAA at 30 °C in a shaker incubator at 200 rpm ("INFORS HT Standard", Switzerland). Cells were isolated by centrifugation at 8000g for 15 min, and the IAA concentration was determined in the supernatant. 100 µL supernatant was added to 100 µL Salkowski reagent (1ml 0.5M $FeCl_3$, 30 ml concentrated H_2SO_4 and 50ml distilled H_2O) and allowed to react at room temperature for 20 min. IAA production was confirmed by pink color development as quantified at 540 nm.

The ability of the bacteria to hydrolyze phytate was judged by their ability to grow in a medium containing sodium phytate (0.4%) as the sole source of phosphorus and by formation of colonies around the transparent zone. Bacteria were cultured in PSM medium (2% Glucose, 0.4% Sodium phytate, 0.2% $CaCl_2$, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4$ x$7H_2O$, 0.001% $FeSO_4$ x $7H_2O$, 0.001% $MnSO_4$ x H_2O, 3% agar, pH 7.0). Sodium phytate was added to the medium after sterilization by filtration through a 0.22 µm filter [2].

To study the ability of the strain to grow in the presence of an elevated salt concentration, bacteria were cultured in LB medium containing 5.0, 7.0 and 10% NaCl. Culture absorbance was measured at 590 nm on a Microplate Spectrophotometer xMark^{TM}(Bio-RAD).

Growth-promoting activity was studied using pea and rye seeds. Pea and rye seeds in an amount of 100 in each experiment were decontaminated for 30 seconds in 70% ethanol solution, flushed 3 times with sterile water, treated for 5-7 min in 2.5% sodium hypochlorite solution, then again flushed 3 times with sterile water and dried. The seeds were further treated with bacterial suspension at concentration of 10^5 and 10^7 CFU/ml for one hour. The seeds were placed on wet filter paper in a sterile petri dish, and germinated at 25°C. After 7 days, sprouts and roots were separated, dried, and the dry weight was determined. Seeds untreated with bacterial suspension were used as control. The average dry weight of the control sprout and root were taken as 100%.

Statistical analysis was performed using the software package SPSS 12.0. Standard deviation (σ) was calculated and the results were considered significant when $\sigma \leq 10\%$.

RESULTS

Bacterial strain MG-2 isolated from the potato rhizosphere was unreliably classified as a *Pseudomonas putida* based on MALDI BioTyper instrument (the score classification of 1.868). Thus, the *gyrB* gene, which encodes the subunit B protein of DNA gyrase, was selected as an alternative phylogenetic marker. The *gyrB* gene was amplified by PCR as described above and sequenced in the Interdisciplinary centre for shared use of Kazan Federal University. Sequence similarity of *gyrB* gene in BLAST-analysis showed 97% of homology with *gyrB* genes of two *Pseudomonas* strains - *P. putida* (AN KC189956.1), and *Pseudomonas sp.* FGI182 (AN D86005.1). Based on gyrase subunit gene sequence the isolate MG-2 was identified as *P. putida*.

It was shown, that *P. putida* MG-2 is able to synthesize siderophores (Fig. 1) (changing the color from blue to orange around the colony on the CAS medium was detected). The dynamics of siderophore accumulation in the liquid culture medium was shown that catechol siderophores occur in culture medium in the exponential growth phase, and its accumulation maximum is in 24th h (the stationary growth phase). The *P. putida* MG-2 isolate produced a maximum of siderophores of 113 µM. During 48th and 72nd hours of growth the level of siderophores decreased up to 20 % and 40 %, respectively (Fig. 2).

Test with Salkowski reagent showed that *P. putida* MG-2 are able to synthesize indole-3-acetic acid (IAA) auxin when growing in M9 medium. In the absence of L-tryptophan the level of IAA in the medium was very low and did not exceed 0.7-1.7 µg/ml (Fig. 3). On 24th hour of growth, the IAA level in the medium containing 0.3 µg/ml L-tryptophan was reached 3.45 µg/ml, and with 0.5 µg/ml L-tryptophan – 8.85 µg/ml. Further culturing resulted in the increasing of IAA concentration in the medium – it reached 9.7 and 11.5 µg/ml after adding 0.3 and 0.5 µg/ml L-tryptophan, respectively. This indicates the induction of the IAA synthesis with L-tryptophan.

It was found that *P. putida* MG-2 is able to grow on medium with sodium phytate as the sole phosphorus source. Changing the medium color around bacterial colonies was indicated about the occurrence of phytate-hydrolyzing activity (Fig. 4).

P. putida MG-2 was able to grow in the culture medium with high salt concentrations. The accumulation of bacterial biomass in the medium containing 5% and 7% NaCl was about 17-24% lower than in control (LB medium) (Fig. 5). In the presence of 10% NaCl in the medium the optical density of the culture decreases to 40% compared with the control. The ability of *P. putida* MG-2 to grow in the presence of 5-7% NaCl indicates about halotolerance of this isolate.

To study the growth-promoting activity of MG-2 isolate, we used pea and rye seeds incubated prior to germination with cell suspension at a concentration of 10^5 and 10^7 CFU/ml of *P. putida* MG-2 for 1 h. As shown in the table 1, the bacterial treatment has a positive effect on an increment of pea and rye seedling biomass. Growth-promoting effect was more obvious in seedlings: the dry biomass of seedlings in both cases increased for 24-28%. Increment of roots dry biomass was 5 to 14%. We found no significant difference in the promoting activity of bacteria depending on the cell concentration used. In both cases, the effect had nearly the same range of values.

DISCUSSION

Gram-negative MG-2 bacterial isolate preliminary identified as the species of genus *Pseudomonas,* was obtained from the potato rhizosphere. Based on 97% homology of gyrase gene sequence analysis the strain MG-2 was classified as a *P. putida* MG-2. It is well-known that *Pseudomonas* species are dominant species of rhizosphere growth-stimulating bacteria [13]. Like many other species of *Pseudomonas,* strain MG-2 is able to produce siderophores [14]. It is believed that the siderophore production in soil plays an important role in the ability of various microorganisms to simulate the growth of plants [2]. However, it is still not clear whether the bacterial siderophores can satisfy the iron requirement of plants. Siderophore production adds competitive advantages to PGPR, which colonize the roots and remove other microorganisms from this ecological niche [15]. *P. putida* MG-2 is able to synthesize the catechol siderophores. Mineral medium M9 containing Fe^{2+} chelator, 2,2'-bipyridine (bipyridyl, BIP) promotes synthesis of siderophores in the exponential growth phase and with maximum values at the stationary phase (24th h). Siderophore concentration was 113 µM, which is compatible with published data. Growth-promoting bacteria *Pseudomonas aeruginosa* JAS-25 is able to produce siderophores up to 130 µM [16]. It is believed that the microbial siderophores can cause the state in plants known as induced systemic resistance (ISR) [17, 18].

Plant growth promotion is associated with the synthesis of various metabolites, and primarily with the production of indole-acetic acid [19]. The examined strain *P. putida* MG-2 is capable of tryptophan-dependent synthesis of indole-acetic acid in an amount of 9.5-11.5 µg/ml when cultured in M9 medium in the presence of 300 and 500 µg/ml of tryptophan. Another study showed that the rhizosphere-isolated strains of *Pseudomonas* could produce 11 to 13 µg/ml of IAA in the presence of 300 µg/ml of tryptophan [20]. *Pseudomonas fluorescens* MPp4 produced 15.63 µg/ml of IAA [21]. It has been shown that IAA, together with other metabolites of rhizosphere bacteria *P. fluorescens,* may be involved in biocontrol of barley disease caused by *Fusarium* [22].

It has been shown that the strain *P. putida* MG-2 has phytate-hydrolyzing activity. During the screening of the phytate-hydrolyzing bacteria, different species of genus *Pseudomonas* are often isolated from soil and rhizosphere [23-25]. It has been shown that introduction of *Pseudomonas rhodesiae* JT29 and *Flavobacterium johnsoniae* JT31 strains into the soil led to increased phytate mineralization in the soil by 16-27 times [23]. Therefore, we can conclude that the use of effective phytate-hydrolyzing bacteria allows regulating the phosphorus metabolism in agricultural soils.

Bacteria *P. putida* MG-2 can grow in the presence of 5-7% NaCl, which indicates their halotolerance and is consistent with the literature data on different isolates of *Pseudomonas*. For example, the endophytic *Pseudomonas chlororaphis* were halotolerant to 5% NaCl [26].

Treating pea and rye seeds with the cell suspension of *P. putida* MG-2 promotes increment in shoots and roots of seedlings by 24-28% and 5-14%, respectively, which indicates the growth-promoting ability of the studied bacteria. It has been shown that the strain *P. chlororaphis* Zong1 is able to stimulate the growth of shoots, roots and the formation of nodules on the roots of beans [26]. It is believed that the growth-promoting ability of the bacteria is associated with the synthesis of IAA, siderophores and the phosphate-mobilizing activity. Moreover, it has been shown that the promoting ability of the strain *P. chlororaphis* Zong1 was increasing during co-inoculation with bacteria *Mesorhizobium sp.*SQ1, indicating a synergistic interaction of rhizosphere bacteria [26].

CONCLUSION

Thus, a new strain *P. putida* isolated from the potato rhizosphere is capable of tryptophan-dependent synthesis of IAA, can produce siderophores and shows a phytate-mobilizing activity. Bacteria can grow in the presence of 5% NaCl and show a pronounced growth-promoting activity. The strain has to be further characterized in order to evaluate its use potential as biological product in agriculture.

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