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EXPERIMENTAL ARTICLES

Role of Bacterial Adhesin RAPA1 in Formation of Efficient Symbiosis of *Rhizobium leguminosarum* with Bean Plants

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Abstract—Bacterial adhesins are the proteins responsible for attachment of plant growth-promoting rhizobacteria (PGPR) to plant roots, are involved in formation of stable associative symbiosis. In the present work, enhanced expression of the adhesin gene *rapA1* in *Rhizobium leguminosarum* PVu5 was shown to improve the efficiency of root nodulation on bean roots inoculated with the modified strain. The gene *rapA1* was cloned into the pJN105Turbo plasmid, this construct was used for transformation of *R. leguminosarum* PVu5, bean plants were inoculated by this transgenic strain, and efficiency of root nodule formation was determined. In the plants treated with *rapA1*-transgenic rhizobia, the number of root nodules was on average two times higher than in the plants inoculated with the original strain. Aggregation of *R. leguminosarum* was achieved when the gene *rapA1* expression was enhanced either in rhizobia or in the co-cultured modified strain *E. coli* pJN105TurboRapA1.

Keywords: nodule bacteria, RAPA1, agglutination, nodulation

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The bacteria capable of rhizosphere colonization and plant growth acceleration by different mechanisms are called Plant Growth-Promoting Rhizobacteria (PGPR). These properties are typical of microorganisms from different species, including *Rhizobium, Azoarcus, Azospirillum, Azotobacter, Arthrobacter, Bacillus, Clostridium, Enterobacter, Gluconoacetobacter, Pseudomonas*, and *Serratia* (Somers et al., 2004). PGPR are directly involved in improvement of plant nutrition by supplementing such major nutrient elements as nitrogen, phosphorus, iron and/or secreting the substances with antibacterial and antifungal activities, which enhance plant resistance to pathogens (Maksimov et al., 2011).

Root nodule bacteria of the genus *Rhizobium*, which form nitrogen-fixing symbiosis with legumes, can also act as PGPR and form symbiotic associations with the most important agricultural crops such as rice, wheat, barley, etc. (Chabot et al., 1996; Mongiardini et al., 2008). At the initial stages of establishment of symbiosis, root nodule bacteria are attached to the root fibrils of legumes (Lodeiro and Favelukes, 1999); they can also be attached to the surfaces of epidermal cells of nonsymbiotrophic plant species (Reddy et al., 1997; Fujishige et al., 2006), as well as to bacterial, fungal, and animal cells (Ausmees et al., 2001; Seneviratne et al., 2003; Horiuchi et al., 2005) and some inert surfaces such as soil, various particles, or glass (Fujishige et al., 2006). Thus, the nonspecific adsorption of rhizobia at different surfaces allows them to colonize the rhizosphere, to form biofilms on plant roots (Ramey et al., 2004), and to successfully compete with other species of microorganisms.

The conditions when *R. leguminosarum* rhizobia are adsorbed at the root fibers have been studied in detail in the works of Smith et al. (1989). A Ca^{2+}-binding adhesion protein (rhicadhesin) was found, which mediates the attachment of rhizobia to plant root fibrils under alkaline conditions. It was shown that rhicadhesin-mediated adsorption was not specific for the legume–rhizobia symbiosis (Smith et al., 1989).

The RAPA1 protein isolated from *R. leguminosarum* and belonging to the RAP protein family is similar to rhicadhesin, since it can bind Ca^{2+} and is involved in bacterial adsorption at the surface of plant root fibrils; however, it has some distinctive biochemical properties (Ausmees et al., 2001). Several proteins of this family are known: RAPA, RAPB, and RAPC. The RAPA protein has two isoforms: RAPA1 and RAPA2. Overexpression of RAPA1 was shown to increase the number of rhizobia adhered to the root fibrils of legumes and to cause enhanced nodulation (Mongiardini et al., 2008, 2009).

The role of RAP proteins in the legume–rhizobia symbiosis has not yet been elucidated. Hence, it seems to be interesting to determine whether the RAPA1 protein can be a mediator of rhizobial adsorption at plant roots and influence on nodulation in the *R. leguminosarum*–kidney bean nitrogen-fixing system.

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The goal of the present work was to obtain rhizobial strains with enhanced expression of the *rapA1* gene, to study their agglutinating properties, and to determine the efficiency of nodulation by these strains on kidney bean roots.

MATERIALS AND METHODS

Research subjects and materials. The cloning experiments were carried out using the plasmid vectors pJN105, pTurboGFP-B, pTurboRFP-B, pAL-TA, and the strain *E. coli* XL1-Blue. The experiments on plant inoculation were carried out using the rhizobium *R. leguminosarum* PVu5 from the collection of bacterial strains of the Institute of Biochemistry and Genetics of the Ufa Scientific Center, Russian Academy of Sciences, originally isolated from kidney bean root nodules. The kidney bean (*Phaseolus vulgaris*) of the Eureka variety was used as a host plant. Bacterial agglutination was visualized using the strains of *R. leguminosarum* and *E. coli* labeled with the fluorescent RFP protein (Baymiev et al., 2011).

DNA manipulations. The isolation of high-molecular bacterial DNA and plasmid DNA, the analysis of recombinant clones, the cloning of amplified DNA into plasmid vectors, the cleavage of DNA by restriction endonucleases and ligation, the preparation of competent cells and their transformation by plasmid DNA, and the electrophoresis of DNA fragments and their elution from low-melting agarose were carried out according to Sambrook et al. (Sambrook, 1989). The polymerase chain reaction (PCR) was performed using standard DNA amplification kits (DNA Technology, Russia) in a Tercyc MS2 amplifier (DNA Technology, Russia). The DNA nucleotide sequences DNA were determined with an ABI PRISM 310 sequencer (Applied Biosystems, United States) using Big Dye Terminator v. 3.0 sequencing kits. The nucleotide sequences were analyzed with the Lasergene software package (DNASTAR, Inc., United States).

Binary cocultures of bacteria. Bacterial agglutination was visualized by the method of binary coculture. Bacteria were transformed by the plasmids pJNTurboRFP (*Rhizobium*) and pTurboRFP-B (*E. coli*) to obtain the microorganisms emitting red fluorescence. Then the fluorescently labeled and recombinant bacterial strains were grown in 100 mL of YM medium (mannitol, 1%; yeast extract, 0.04%; NaCl, 0.01%; $MgSO_4$, 0.01%; $K_2HPO_4 \cdot 3H_2O$, 0.05%) for rhizobia and LB medium (bacto tryptone, 1%; yeast extract, 0.5%; NaCl, 1%) for *E. coli* at 28°C and 37°C, respectively, up to OD_{660} 0.75.

The two bacterial cocultures were inoculated (1 mL each) into flasks with the medium (50 mL) and incubated together overnight on an orbital shaker at 100–150 rpm and at 28°C (*R. leguminosarum* or *R. leguminosarum* + *E. coli*) and 37°C (*E. coli*). Then the fluorescently labeled bacteria were examined under an Axio Imager M1 fluorescence microscope (Carl Zeiss, Germany). RFP was detected using a no. 15 set of light filters (excitation BP 546/12, emission LP 590).

Analysis of root nodule formation on plants. Kidney bean seeds were sterilized for 2 min in 70% alcohol and then for 15 min in 15% sodium hypochlorite solution with the addition of a few drops of Tween-20. The seeds were germinated on filter paper moistened with sterile water in petri dishes for 2 days and then inoculated with rhizobia carrying the pJN105TurboRapA1 plasmid and planted into sterile universal soil TERRA VITA (Agrokhimzem, Russia). The seeds not treated with the bacteria and the seeds infected with the same rhizobial strain but without the plasmid pJN105TurboRapA1 were planted as controls. The nodules on plant roots were counted after 1-month cultivation of the plants.

Determination of nitrogenase activity of the bacteria. Nitrogenase activity was determined by the method based on acetylene reduction to ethylene (Umarov, 1989). Plant roots with the nodules were placed into 200-mL flasks, and acetylene was added to a concentration of 10% (by volume). After incubation in the dark for 1–1.5 h, 1-mL gas samples were taken with a syringe three times. The acetylene and ethylene content was measured by the time of each gas output using a Gas Chromatograph GC-2014 (Shimadzu, Japan) equipped with a plasma ionization detector. The acetylene and ethylene concentrations were calculated by the calibration curve (Mineev, 2001).

RESULTS AND DISCUSSION

The *rapA1* gene cloning. The vector construction carrying the rhizobial *rapA1* gene under the regulation of a strong constitutive promoter, which is expressed in bacteria, was obtained by selecting and synthesizing the oligonucleotide primers rapF (5'-atggctgttcacgcaaccgacgat-3') and rapR (5'-gcggcgggcgttgtttttgattg-3') for the *rapA1* gene sequence registered in the GenBank under the accession no. AF265222. These primers were used to amplify the encoding part of the *rapA1* gene from the DNA of the *R. leguminosarum* strain isolated from kidney bean root nodules. Then the amplified DNA was cloned into the intermediate vector pAL-TA. The *GFP* gene was cut out of the plasmid pJN105TurboGFP (Baymiyev, 2011) using the *Bam*HI and *Hin*dIII restriction endonucleases, and the *rapA1* gene was recloned at its position under the control of the strong constitutive phage T5 promoter (Fig. 1). After that this construction was used to transform *R. leguminosarum* PVu5 and *E. coli* XL1-Blue. The resultant bacterial strains carried the plasmid pJN105TurboRapA1, as was confirmed by PCR analysis.

Bacterial agglutination in the binary coculture system. Under natural conditions, the RAPA1 protein, being an extracellular Ca^{2+}-binding protein, recognizes polysaccharides on bacterial surface and pro-

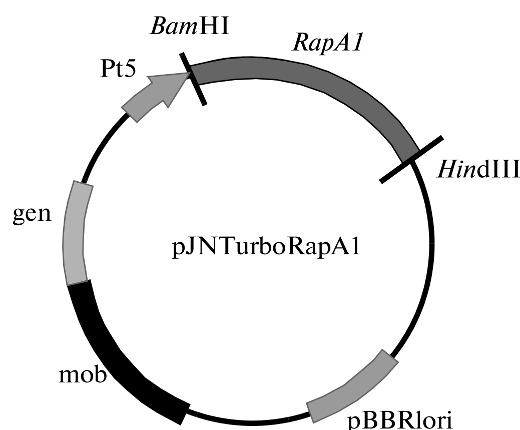

Fig. 1. Schematic representation of the vector pJN105TurboRapA1.

motes rhizobial agglutination via cell poles (Ausmees et al., 2001). Therefore, it would be sufficient for the binding of two bacterial cells if one of them produced RAPA1 and the other had a specific polysaccharide on its surface. Hence, to visualize the degree of bacterial aggregation without overloading the producer strain RAPA1 with the marker genes, it was cocultured with the strain *R. leguminosarum* producing a fluorescent protein.

The efficiency of the *rapA1* gene function in the created constructions in the homo- and heterologous systems of *R. leguminosarum* and *E. coli* XL1-Blue was tested in cross agglutination experiments, the results of which are shown in the table and on Fig. 2.

Visualization of the cells in a fluorescence microscope revealed that agglutination of both *E. coli* and *R. leguminosarum* cells occurred mainly in the presence of *rapA1*-transgenic bacteria (Figs. 2a, 2d, and 2f). At the same time, in the experiment where the *rapA1*-transgenic *E. coli* strain was cultivated with the fluorescent *E. coli* strain, agglutination was associated rather with the rapid cell division of *E. coli* and, accordingly, the higher production of its biomass than with the specific binding of the RAPA1 protein to cell surface (Fig. 2d). This fact also accounts for weak agglutination in the experiment with the initial strain of *E. coli* cultivated together with the fluorescent strain of *R. leguminosarum* (Fig. 2e). Formation of large cell conglomerates observed in the experimental variant, where the *rapA1*-transgenic *E. coli* strain was cultivated with the fluorescent strain of *R. leguminosarum*, was due to higher production of the RAPA1 protein in *E. coli* cells (Fig. 2f). The subsequent specific binding with rhizobial cells in this case resulted in formation of large conglomerates, which was less pronounced in the experimental variant, where the fluorescent strain of *R. leguminosarum* was cultivated with the *rapA1*-transformed strain of *R. leguminosarum* characterized by the lower production of biomass and, accordingly, of the RAPA1 proteins than the *E. coli* strains (Fig. 2b).

Undoubtedly, it seems interesting that the production of RAPA1 in the cocultured microbial strain was sufficient for the binding and aggregation of rhizobia. Importantly, these may be both root nodule bacteria and other bacteria, e.g., like in this case, *E. coli*. This result is a prerequisite for development of binary biopreparations on the basis of root nodule bacteria which, in addition to the basic rhizobial strain, will contain the RAPA1-producing strain contributing to efficient nodulation.

Our results are in agreement with the work of Ausmees et al. (Ausmees et al., 2001), where the treatment of the bacterium *R. leguminosarum* R200 with the RAPA1 protein resulted in autoagglutination; however, interbacterial interactions were not studied in this work. These authors were also the first to suggest that RAPA1 was similar to soybean lectin and trifolin A,

The scheme and results of the experiments on bacterial cross agglutination

Cocultured strains of microorganisms	*R. leguminosarum* PVu5, transformed by the plasmid pJNTurboRFP	*E. coli* XL1-Blue, transformed by the plasmid pTurboRFP
R. leguminosarum PVu5 (as a control)	–	–
R. leguminosarum PVu5 transformed by the plasmid pJN105TurboRapA1	++	–
E. coli XL1-Blue	+/–	–
E. coli, transformed by the plasmid pJN105TurboRapA1	+++	+/–

Designation for agglutination intensity: "–," no agglutination; "+," weak agglutination; "++," average agglutination; "+++," strong agglutination.

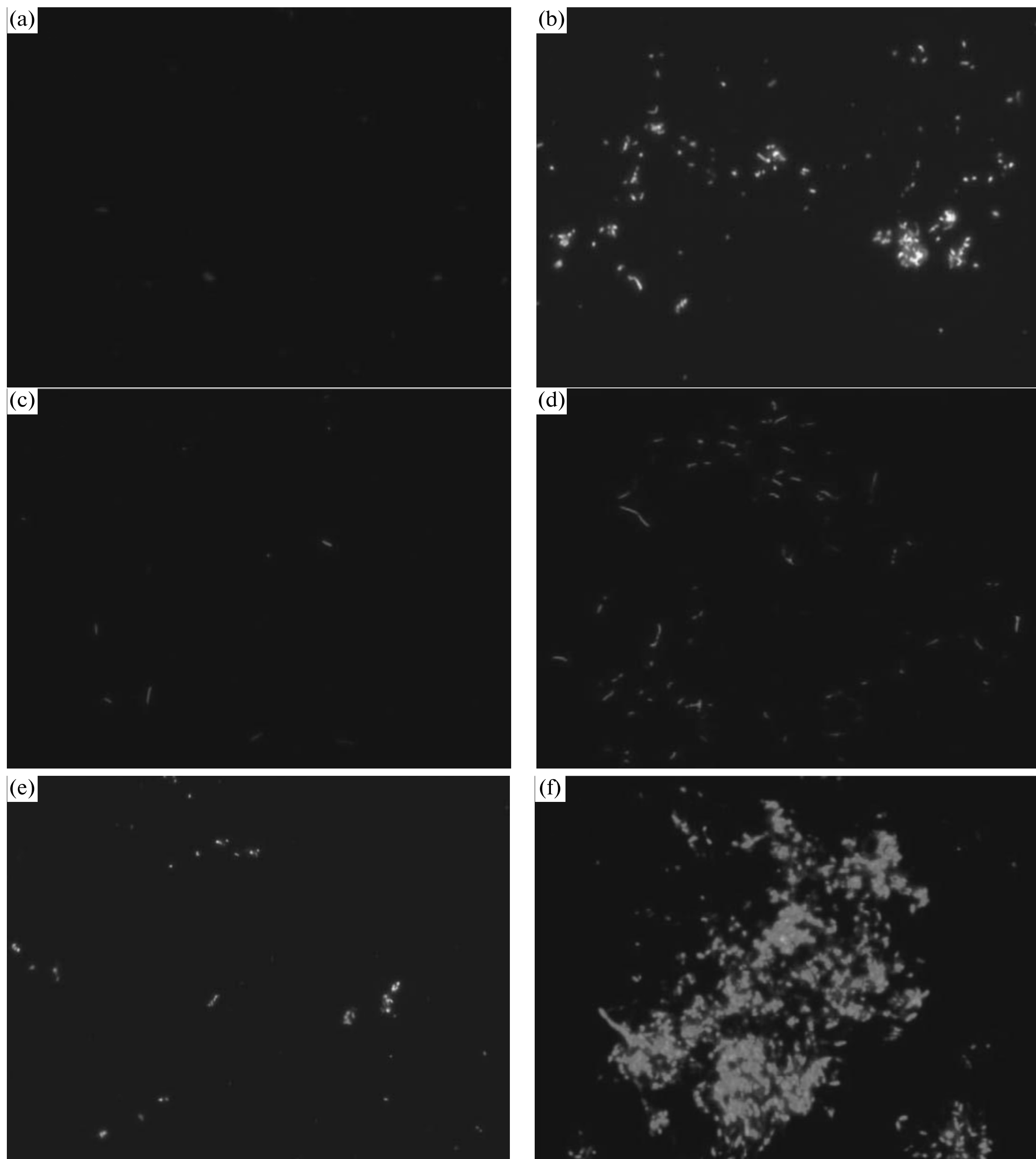

Fig. 2. Visualization of agglutination: the control, the original strain of *R. leguminosarum* + *R. leguminosarum* pJN105TurboRFP (a); *R. leguminosarum* pJN105TurboRapA1 + *R. leguminosarum* pJN105TurboRFP (b); the control, the original strain of *E. coli* + *E. coli* pTurboRFP (c); *E. coli* pJN105TurboRapA1 + *E. coli* pTurboRFP (d); the control, *R. leguminosarum* pJN105TurboRFP + the original strain of *E. coli* (e); and *R. leguminosarum* pJN105TurboRFP + *E. coli* pJN105TurboRapA1 (f).

recognizing not only bacterial but also plant polysaccharides, and playing an important role in establishment of nitrogen-fixing symbiosis (Ausmees et al., 2001).

It is considered than adhesion and specific recognition during the establishment of nitrogen-fixing symbiosis depend on the interaction between the Nod factors (lipochitooligosaccharides) produced by rhizobia and the LysM-receptor kinases of the host plant (Oldroyd and Downie, 2008). However, one cannot state that this system is the only mechanism for the recognition of symbiotic partners and bacterial adsorption at plant surfaces. It is probably the main link in a complex process involving also bacterial RAPA1 proteins.

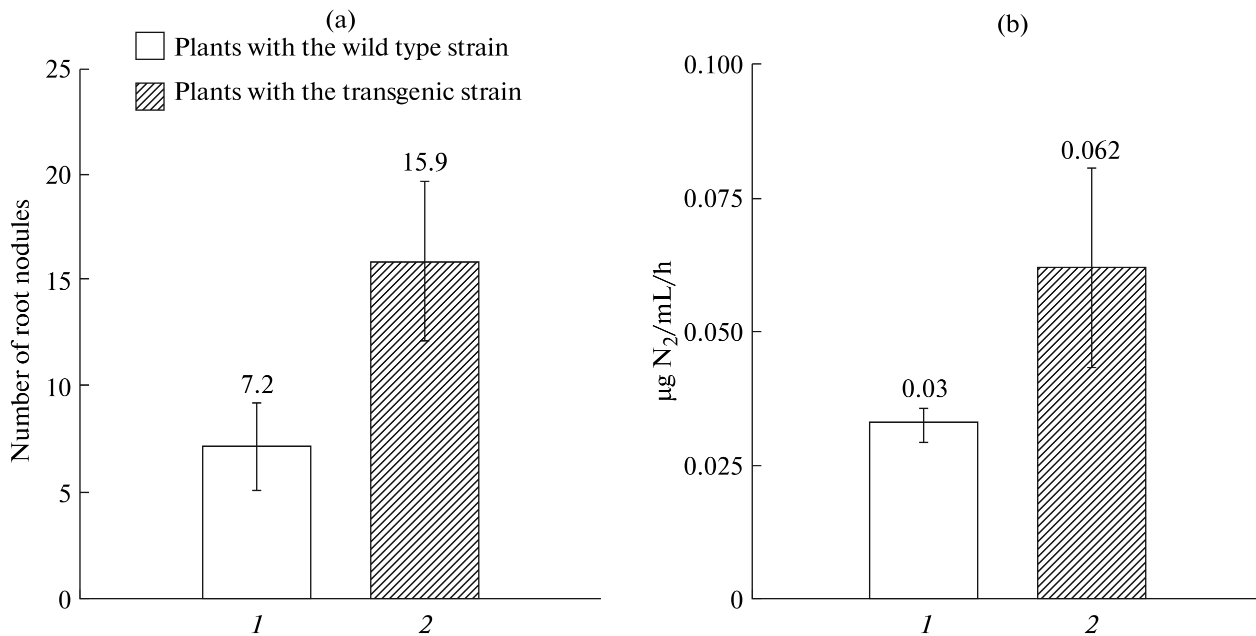

Fig. 3. Comparison of the number of root nodules (a) and the values of nitrogenase activity (b) on kidney bean roots treated with wild type (*1*) and modified (*2*) rhizobial strains.

Moreover, the specific interactions between rhizobia and plants involve plant lectins. For example, lectins such as PSL from *Pisum sativum* (Diaz et al., 1989), Le1 from *Glycine max* (van Rhijn et al., 1998), DB46 and GS52 from *Dolichos biflorus* and *Glycine soja*, respectively (Day et al., 2000; Etzler et al., 1999), were shown to participate in specific recognition of rhizobia in a plant–host system. Adhesion with the RAPA1 protein is probably one of the mechanisms of adsorption and of root colonization by rhizobia which, in case of legumes, is directly associated with subsequent nodulation.

Nodulation efficiency. The influence of constitutive expression of the *rapA1* gene on the efficiency of nodulation was tested in the experiment on inoculation of kidney bean plants with the initial and *rapA1*-knockout rhizobial strains of *R. leguminosarum*. Visual assessment of root nodule development a month after the beginning of the experiment showed the absence of root nodules in uninoculated control kidney bean plants. In all other experimental variants, root nodules were formed but their number in the kidney bean plants treated with the *rapA1*-transgenic rhizobia was approximately twofold higher (15 ± 3) than in the control plants inoculated with the initial rhizobial strain (7 ± 2) (Fig. 3a).

Our data show that the rhizobia with overexpression of gene *rapA1* contribute to the increase in the number of nodules formed on the roots of experimental plants, which may be indicative of the RAPA1 binding to polysaccharide components of the root cell wall. The previous experiments with clover and soybean confirmed the positive effect of the gene on plants. However, the formed root nodules were not enumerated (Bhuvaneswari et al., 1980; Mongiardini et al., 2008.)

Previously, the effect of the RAPA1 protein on attachment of *R. leguminosarum* to clover roots was already studied in other works (Mongiardini et al., 2008). It was shown that the adsorption capacity increased two- to fivefold. Adsorption of this strain at alfalfa and soybean roots was also studied; although these plants are not its natural symbionts, the cells of *R. leguminosarum* were attached to plant roots and the overexpression of *rapA1* increased the level of bacterial adsorption (Mongiardini et al., 2008). Thus, it may be concluded that the RAPA1 protein has a higher specificity, rather than host affinity only to plants of the family of legumes. Other rhizobial adhesins described in some works can also nonspecifically recognize the root surface of legumes (Lodeiro and Favelukes, 1999). They include agglutinins such as the α-L-fucose-binding lectin from *Rhizobium lupine* (Wisniewski et al., 1994) and the BJ38 protein from *Bradyrhizobium japonicum* (Ho et al., 1990). The existence of such nonspecific adhesins enhances the adaptive possibilities of rhizobia and provides for their more successful competition with other soil bacteria (Bogino et al., 2013).

The analysis of acetylene output by the measurement of the nitrogenase activity of root nodules method showed an increase in total nitrogen-fixing activity of the kidney bean root system, most likely due to the greater number of nodules on plant roots. Com-

parison of nitrogenase activity values confirmed the nearly twofold enhancement of nitrogen fixation: 0.032 μg N_2/mL/h (the plants infected by the initial strain) and 0.062 μg N_2/mL/h (the plants infected by the recombinant strain) (Fig. 3b). These results confirm the possibility of increasing the yield of legumes due to the crop/seed treatment with the strains with enhanced adsorption activity.

Thus, in this work we have studied the RAPA1 protein, which not only contributes to bacterial agglutination but also participates in interbacterial interactions and in establishment of nitrogen-fixing symbiosis. It was shown that the constitutive expression of *rapA1* both in rhizobia and in the cocultured strain contributes to bacterial aggregation and has a positive effect on both nodulation and nitrogen fixation efficiency in general. Undoubtedly, the *rapA1* gene used as a transgene can be useful for the establishment of associative interactions between rhizobia and nonlegume plants (Vershinina et al., 2012), where this protein, similar to the legume lectins, may be a good tool for modification of symbiotic interactions.

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