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ARTIFICIAL ACTIVATION OF *NIF* GENE EXPRESSION IN NODULE BACTERIA *EX PLANTA*

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❀ **Background.** Rhizobia are the most effective nitrogen-fixing organisms that can fix nitrogen only in symbiosis with leguminous plants. The general transcriptional activator of nitrogen fixation genes in diazotrophic bacteria is NifA. In this work, the possibility of modifying the regulation of nitrogen fixation in the nodule bacteria *Mesorhizobium*, *Ensifer* and *Rhizobium* was studied by introducing an additional copy of the *nifA* gene into the bacterial genomes during the regulation of induced bacterial promoters. **Materials and methods.** A series of expression genetic constructs with *nifA* genes of nodule bacteria strains under the control of an inducible promoter Pm were created. The resulting constructs were transformed into strains of nodule bacteria. The obtained recombinant strains were investigated for the appearance of their nitrogen-fixing activity in the free-living state. **Results.** It was shown that the expression of *nifA* in recombinant cells of all three genera of bacteria leads to the appearance of insignificant nitrogenase activity. At the same time, the level of nitrogenase activity does not have a correlation with the level of expression of the introduced *nifA* gene, which, most likely, is a consequence of the multilevel regulation of nitrogen fixation. **Conclusion.** The possibility of artificial activation of nitrogenase activity in nodule bacteria in the free-living state by introducing the NifA regulatory protein gene into bacteria was shown.

❀ **Keywords:** nitrogen fixation; nodule bacteria; *nifA*; recombinant bacteria; plasmid vector; promoter.

ИСКУССТВЕННАЯ АКТИВАЦИЯ ЭКСПРЕССИИ *NIF*-ГЕНОВ У КЛУБЕНЬКОВЫХ БАКТЕРИЙ *EX PLANTA*

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❀ Исследована возможность модификации регуляции азотфиксации у клубеньковых бактерий *Mesorhizobium*, *Ensifer* и *Rhizobium* путем привнесения в их геном дополнительной копии гена *nifA* под регуляцией бактериальных индуцируемых промоторов. Показано, что экспрессия *nifA* в рекомбинантных клетках всех трех родов бактерий приводит к появлению незначительной нитрогеназной активности. При этом уровень нитрогеназной активности не имеет явной корреляции с уровнем экспрессии привнесенного гена *nifA*, что, скорее всего, является следствием многоуровневости регуляции азотфиксации. Наиболее интересны результаты, полученные с рекомбинантным штаммом *Ensifer* sp. Mlu10PmNifA, который продемонстрировал нитрогеназную активность при свободноживущем состоянии, в несколько раз превышающую таковую у контрольного свободноживущего азотфиксирующего штамма *Pseudomonas* sp. K749. При этом штаммом была утеряна способность к клубенькообразованию, а также у рекомбинантных бактерий наряду с нитрогеназной выявлена нитраредуктазная активность.

❀ **Ключевые слова:** азотфиксация; клубеньковые бактерии; *nifA*; рекомбинантные бактерии; плазмидный вектор; промотор.

INTRODUCTION

The absorption of molecular nitrogen from the air, known as nitrogen fixation, is one of the most important biological processes and is essential for much of the life on Earth. The most effective nitrogen fixers are nodule bacteria (rhizobia), which live in symbiosis only with legumes (with very few exceptions). These bacteria are characterized by high genome plasticity, recombination activity, and active involvement in the process of horizontal gene transfer. The symbiotic *nif*, *fix*, and *nod* are the most actively involved in gene transfer. The main method by which nodular bacteria undergo horizontal gene transfer is conjugation [1]. As this process is frequently interrupted, a cell is not always able to obtain all genes required for the

full expression of nitrogen fixation, although theoretically cells may obtain the missing genes in the next round of conjugation they undergo. As this process is not limited to only one species of bacteria and can occur among bacteria of different taxons, the combination of *nif* from different bacteria can occur, which is an element of the combinative evolutionary process [2–4]. The genes of the bovine proteins of nitrogenase are arranged in a single operon and are primarily inherited together; this is not the case for the genes of complexing proteins of nitrogen fixation, which form individual operons in many nitrogen-fixing bacteria. This is especially typical for nodular bacteria, which demonstrate dissociation of *nif* in different operons. Because of this, these microorganisms serve as ideal models for examining the combinative evolution of nitrogen fixation in bacteria. *nifA* is one of the least associated with genes coding for bovine proteins; however, it is an integral part of the nitrogenase complex of rhizobia; it encodes the regulatory protein NifA, the protein on which the activation of entire nitrogenase complex depends. As an enhancer element, it activates expression of all genes involved in nitrogen fixation, and is the final link in the signal cascade of activation of the genes of the nitrogenase complex.

nifA of symbiotic free-living nitrogen fixers is kept in a repressed state in case of oxygen abundance. This results in the subsequent repression of gene expression of the nitrogenase complex [5] and in the production of nitrogenase proteins; therefore, nodular bacteria *ex planta* do not display nitrogen fixation activity. However, currently there are studies focused on activating the gene of the nitrogenase complex of the free-living nitrogen fixers by means of the modification of the expression of *nifA* regulation [6]. It is known that constitutive expression of this gene in non-symbiotic nitrogen fixers results in an increase of nitrogen fixation intensity [7] and causes nitrogenase activity when nitrogen compounds are abundant in the environment [8]. In this study, we assessed the possibility of inducting the nitrogenase activity of nodular bacteria in the free-living state using artificial activation of *nifA* at the detectable level. This will allow the use of *nifA* of nodular bacteria as a suitable marker for studying the functionality of heterologous combinations of *nif*. This is because this gene is also an integral part of the nitrogen fixation apparatus of all rhizobia, and does not have a rigid association with the genes of bovine proteins of nitrogenase. Thus, based on the occurrence of nitrogenase activity of nodular bacteria *ex planta* using the artificial activation of *nifA*, judgments can be made about the functionality of this gene in heterologous systems of *nif*.

Besides this, the possibility of activation of nitrogen fixation of rhizobia in a saprophytic state can have practical value. It has been demonstrated that some strains of rhizobia can serve as plant growth-promoting *Rhizobacteria* when grown in association with plants [9, 10]. Giving these cells the ability to fix nitrogen *ex planta* could significantly improve their growth-promoting properties.

METHODS

The strains of bacteria from the collection of the IBG of UFIC RAS were used: "Symbiont" *Rhizobium* sp. VSy9, *Ensofer* sp.Mlu10, and *Mesorhizobium* sp. LZh7 had been extracted from nodules of the wild-growing legumes of the South Urals attributed to tribes Fabeae, Trifolieae, and Loteae: wood vetch (*Vicia sylvatica* L.), black medic (*Medicago lupulina* L.), and bird's foot (*Lotus zhegulensis* Klokov) accordingly and *Escherichia coli* XL1Blue for genetic manipulations.

Nodular bacteria were cultivated on JM medium (1 % mannitol, 0.05 % K_2HPO_4, 0.02 % $MgSO_4$, 0.01 % NaCl, and 0.1 % yeast extract) at 28 °C. *E. coli* were cultivated on LB medium (1 % bacto-tryptone, 0.5 % yeast extract, and 0.5 % NaCl) at 37 °C. Ampicillin (100 mg/mL) was used as a selective antibiotic during transformation. The expression of *nifA* in plasmid *pJB658* was activated using *m*toluene acid (2 mM).

Total DNA was extracted from bacteria by lysing cells in 1 % Triton X100 and 1 % suspension Chelex100. For this purpose, a small amount of bacterial mass was placed in 1.5-ml flasks with 100 μL of 1 % Triton X100 and 1 % suspension Chelex100 and incubated after suspending at 95 °C for 10 min. Cellular debris were deposited by centrifuging at 12000 g for 3 min. Supernatant liquid was used as matrix for PCR. Plasmid DNA was extracted using the kit diaGene ("Diem," Russia).

To express plasmid-borne *nifA*, the broad-host-range plasmid *pJB658* with replicon *RK2* was selected [11, 12]. The sequence of the target gene was identified using PCR along with primers nifAviciaNdeIF 5′tttgtacatatgattaaaccagaggcgcggctccata-3′ and nifAviciaBamHIR 5′ttacggggatcctggcgatcgcggtcactccttcttcaca-3′ in which restriction sites were introduced: Nde1 was in the first primer, and BamH1 was in the back primer. Amplified product included *nifA* with the size of 1560 bp. The availability of restriction sites at the ends of obtained PCR products facilitated directed cloning of the target sequence and allowed access to the reading frame (Fig. 1).

Cell transformation with vectors was performed with electroporation using MicroPulser (BioRad, USA) based on the program and protocol for agrobacteria transformation in a 0.1 cm electroporation measuring cell. Electrocompetent cells were prepared according to the technique described by Lin [13].

The visual observation of fluorescent-labeled bacteria was performed using a fluorescent microscope (Axio Imager M1; Carl Zeiss, Germany). The No. 10 set of color filters was used for GFP detection (BP excitation band, 450–490 nm; BP emission, 515–565 nm).

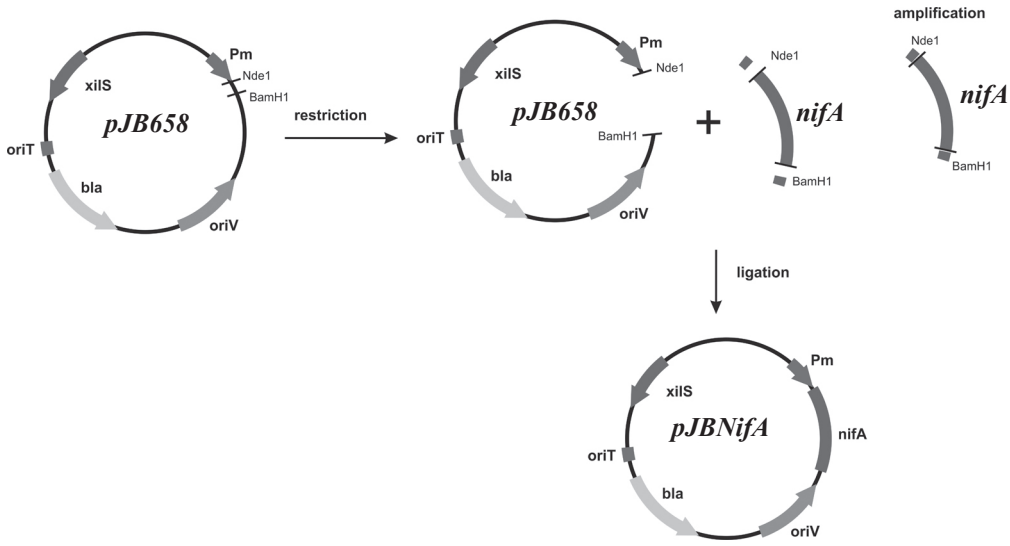

Fig. 1. The strategy of cloning *nifA* genes into plasmid *pJB658*

To assess the expression of *nif* using RT-PCR, RNA was extracted using FastRNA Pro Blue Kit (USA). The first DNA chain complementary to mRNA was obtained by the ferment of MMLV revertase (reverse transcriptase obtained from mouse leukemia virus) ("Eurogen," Russia). The reaction was performed at 42 °C for 2 h. The reaction mixture contained 1–5 μg of RNA, 20 μM of random hexamer primer (Fermentas, USA), normal strength buffer for revertase (50 mM trisHCl with pH 8.3, 10 mM $MnCl_2$, 10 mM DTT, and 0.5 mM of spermidine), 500 μM of each deoxinucleotide triphosphate, and 5 units of MMLV revertase. After incubation, the reaction mixture was inactivated by heating at up to 70 °C for 10 min. Obtained samples were split into aliquots, and the primers used for RT-PCR were as follows: NifHF (5′acgctagccgcccttgtgga-3′) and NifHR (5′gagttcgcgatcggtttgacg-3′) for the amplification of *nifH* fragment with the size 513 bps and NifAF (5′cggcagcgcgacggtgacatt-3′) and NifAR (5′caagaggagcgccgaagaaca-3′) for the amplification of *nifA* fragment with the size 246 bps.

Proteins from the nodular bacteria cell culture were extracted using an aforementioned method [14]. Proteins for PVDFmembrane (BIORAD, USA) for Western blot analysis were transferred using previously described method [15] and instrument MiniTransBlot® Module 170-3924EDU BioRad (USA) at 4 °C for 1 h at 100 V and 18–22 mA. To detect NifH, polyclonal chicken antibodies were used, which were obtained against bovine NifH (Agrisera, Sweden). PVDFmembrane was treated with the solution of antibodies, which was diluted 1000 times in TBSbuffer (20 mM Tris base, 150 mM NaCl), with 1 % of powdered skim milk and 0.1 % Twin-20 and was incubated for 10–16 h at 4 °C. After this, the solution of primary antibodies was drained, and the membrane was washed out in TBSbuffer with 0.1 % Twin-20.

The detection of primary antibodies associated with antigen was performed using secondary monoclonal rabbit antibodies to polyclonal chicken antibodies marked with horseradish peroxidase HRP (Agrisera, Sweden). Serum was diluted in 0.2 mL of water; the obtained solution (2 μL) was further diluted in 10 mL of TBSbuffer with 0.01 % of Twin-20. The membrane was incubated in the solution of secondary antibodies for 1.5–2 h at room temperature. The detection of peroxidase on the membrane was performed using chromogene DAB (Cell Marque Corporation, USA).

To determine nitrogenase activity, microorganisms were cultivated for 1–2 days on liquid nitrogen-free nutrient medium, with selective antibiotics and inducers in required concentrations, in an orbital shaker ES20 at 160 rpm and an optimal growth temperature of 28 °C until they reached a CFU of $1–2 \times 10^8$. Following this, 1 mL of culture liquid was obtained from each sample and placed in sterile glass bottles of 15 mL. Ten bottles of sand were required for controlling non-specific ethylene genesis; 5 grams of sterile sand was used as a carrier and was placed in each bottle before placing the liquid culture. After the culture liquid was placed, the sand humidity amounted to 60 % of full water capacity. Ten bottles with samples in three replicas were used for each recombinant and reference strain (30 samples per strain). Bottles closed with cotton wool plugs were incubated for 24 h at 28 °C; then, the bottles were closed with rubber plugs, and acetylene was injected into each up to the concentration of 10 % volume. After 1–1.5 h of incubation, 1 ml of gas samples were taken with syringe. Acetylene and ethylene contents were deter-

mined using gas chromatography (Shimadzu Gas Chromatograph GC-2014, Japan) with a flame-ionization detector.

The content of exchangeable ammonium in soil was determined using photometric method (GOST 2648985) in five replicates. The filtrates of soil extracts either not inoculated or inoculated with recombinant and wild strains of rhizobia were used for analysis. Two cm^2 of filtrates and reference solution of 0.25 mg/cm^3 NH_4Cl were placed in 100-mL glass flasks. Following this, 40 cm^3 of the colored solution of 5.7 % sodium salicylate, 1.7 % potassium-sodium tartrate, 2.7 % sodium hydroxide, 0.04 % sodium nitroprusside, and 0.2 % Trilon B was added to the flasks; finally, 2 cm^3 of 0.125 % solution of NaOCl was added. The solutions were thoroughly mixed and photometrically scanned relative to the reference solution at a wavelength of 655 nm after 1 h on the photoelectric photometer KFK 301 in glass measuring cells with a transparent layer thickness of 1 cm. Every filtrate was measured in three replicates. The graduated diagram of dependence of the readings on ammonia concentration in the samples was plotted; the results expressed in ppm and rounded to the first decimal point.

The mass fraction of nitrogen in the nitrates in conversion to the dry soil (X_1) in ppm was calculated using the formula: $X_1 = X \cdot K_1 \cdot K_2$, where X is the mass fraction of nitrates nitrogen, mln.$^{-1}$; K_1 and K_2 are factors considering the mass fraction of moisture in soil and the increase of the extracting solution interacting with the analyzed sample by means of the moisture content in soil.

Nitrate content was determined using an ionometric method (GOST 2695186). This method is based on nitrate extraction using aluminum potassium sulfate solution (1 % K_2SO_4) with a ratio of soil sample weight to solution of 1 : 2.5. Further determination of nitrates in the extraction was performed using an ion-selective electrode. The filtrates of extractions from untreated soil and from soil treated with recombinant strains and with wild strains of nodular bacteria were used for analysis. Twenty grams of soil samples were placed in cone glass flasks; further, 50 cm^3 of 1 % K_2SO_4 solution was added, and the sample was mixed for 3 min. Nitrate content was calculated using the obtained suspensions, which were agitated before measurement. A nitrate ion-selective electrode was thoroughly rinsed by holding in distilled water for 10 min. Following this, the electrode pair was placed in the suspension, and readings were taken minimum 1 min after the termination of the reading drift.

Based on the determination of EDS (in microvolts) in the reference solution, the graduated diagram of dependence of the pH meter readings on nitrate concentration was plotted. Results were expressed in ppm and rounded to the first decimal point.

RESULTS AND DISCUSSION

Currently, many different signal cascades are known to activate nitrogen fixation in bacteria. The last stage of the transcriptional regulation of many nitrogen fixation bacteria, including the groups of nodular bacteria, is the activation of the expression of the genes of the nitrogenase complex by the regulator protein NifA [16].

Here, we formed a series of genetically engineered structures with *nifA* of nodular bacteria under control of inducible promoter *Pm* [17, 18], which was expressed using the broad-host-range plasmid *pJB658* with replicon T2: *pJB658nifAvicea*, *pJB658nifAMesorh*, and *pJB658nifASinorh*, with genes *nifA Rhizobium* sp.VSy9, *Ensifer* sp. MLu10, and *Mesorhizobium* sp. LZh7 accordingly. To ensure the functionality of the structures in plasmid *pJB658*, a similar structure was constructed in parallel in rhizobia strains using a reporter *TurboGFP* in place of the target gene. When this structure was transformed into *Rhizobium* sp. (VSy9), *Ensifer* sp. (MLu10), and *Mesorhizobium* sp. (LZh7), green-colored bacterial colonies grew on media with methylbenzoic acid, demonstrating the functionality of the *Pm* promoter. The structures with *nifA* were then transformed into the aforementioned strains of nodular bacteria. Recombinant strains were formed on the basis of the donors of the target gene. In fact, that strain's own copy of *nifA* was added to bacterial cells regulated with an inducible promoter. Obtained strains were analyzed for nitrogen fixation activity in the free-living state using the acetylenic method.

When analyzing recombinant strain *Rhizobium* sp.VSy9 with structure *pJB658nifAvicea* (strain VSy9PmNifA) without induction, the occurrence of nitrogenase activity was detected. Interestingly, at low induction of NifA, a reduction in nitrogen fixation activity was observed (Fig. 2).

Moreover, recombinant strain *Mesorhizobium* sp. LZh7 with the structure *pJB658nifAMesorh* (strain LZh7PmNifA) demonstrated nitrogenase activity outside of plants. The minimum induction of *nifA* did not remarkably change the level of acetylene reduction. it is known that *Mesorhizobium* sp. contains two copies of the gene for the regulatory protein NifA, namely, *nifA1* and *nifA2* [19–21]. As *nifA2* is involved in the regulation of nitrogen fixation genes, and *nifA1* does not affect nitrogen fixation activity [22], only homologous *nifA2* was used in this study. The ability of recombinant strain *Mesorhizobium* sp. LZh7nifA to convert acetylene to ethylene in the free-living state confirms that the *nifA2* is involved in the activation of the nitrogenase complex.

An unexpected result was obtained during the analysis of recombinant strain *Ensifer* sp. MLu10 when transformed with structure *pJB658nifASinorh* (recombinant strain Mlu10PmNifA); the ability to recover acetylene was

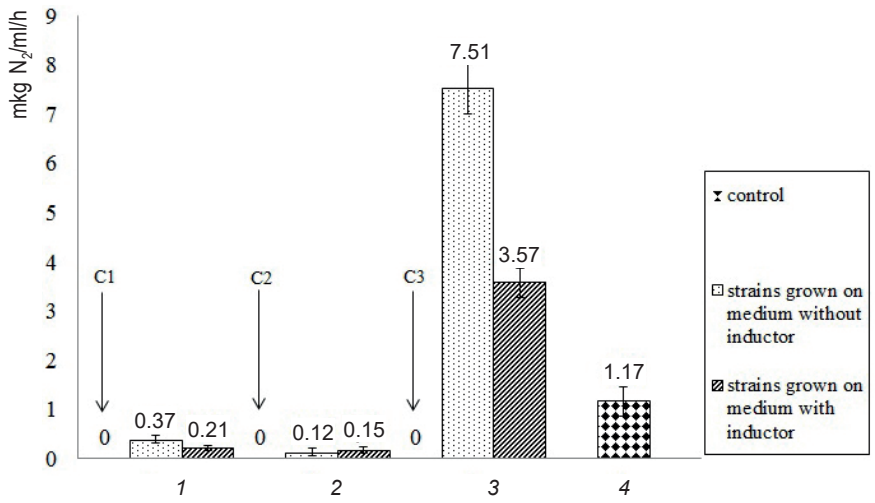

Fig. 2. Analysis of the nitrogenase activity of bacteria. *1* — *Rhizobium* sp.VSy9PmNifA; *2* — *Mesorhizobium* sp. LZh7PmNifA; *3* — *Ensifer* sp. Mlu10PmNifA; *4* — control (*Pseudomonas* sp. K749 — free-living nitrogen fixer). K_1, K_2, K_3 — wild variants of strains of nodule bacteria

detected in these microorganisms, which exceeded the ability of the reference strain *Pseudomonas* sp. K749 by >6 times, whereas weak induction (the same as that in the case of *Rhizobium* sp.VSy9PmNifA) resulted in the reduction of nitrogenase activity. It is likely that a minimal amount of regulator protein is sufficient for the induction of nitrogen fixation activity, whereas an increased concentration of the same does not directly affect nitrogenase activity; likely, at this point, other limiting factors start to affect the process [23–25].

To evaluate this, RT-PCR analysis of the expression of *nifA* was conducted along with the analysis of one of the genes (*nifH*) of the bovine nitrogenase proteins in the recombinant strains. The induction of promoter of the target *nifA* resulted in the activation of its transcription (demonstrated on phoregram in the form of occurrence of appropriate PCR products, Fig. 3); this was not observed in the non-stimulated samples. It is assumed that this is because when the inducer is absent, an insignificant amount of mRNA of the target gene is produced, and therefore, it is not possible to detect it.

When analyzing expression of *nifH*, transcription was observed in the recombinant strains during the induction of the expression of *nifA*; transcription was also observed in the strains without induction (see Fig. 3).

Thus, the total level of expressed mRNA of *nifH* depends on NifA protein, but does not directly depend on the expression of *nifA*, once again confirming the complicated multilevel mechanism of the induction of nitrogen fixation activity in rhizobia.

The strains were examined for the availability of NifH protein using dot-blot analysis, because Western blot anal-

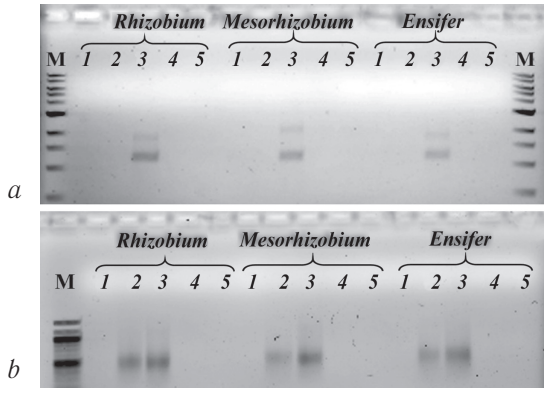

Fig. 3. RT-PCR analysis of the transcriptional activity of the *nifA* (*a*) and *nifH* (б) genes in cells of wild and recombinant rhizobia strains of *Rhizobium* sp. VSy9, *Mesorhizobium* sp. LZh7, *Ensifer* sp. Mlu10. *1* — wild strains of bacteria; *2* — recombinant bacteria strains without induction; *3* — recombinant bacterial strains with induction; *4* — PCR of a total recombinant bacteria nucleic acid preparation after DNAse treatment; *5* — negative control

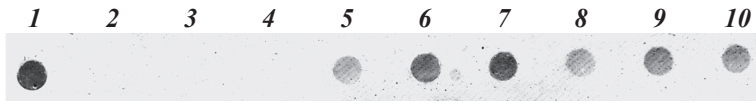

Рис. 4. Dot-blot analysis of NifH protein production by recombinant strains of nodule bacteria grown on a nutrient medium without and with the addition of an inducer of *m*-toluic acid (0.5 mM). *1* — positive control of strain *Pseudomonas* sp. K749; *2* — non-recombinant strain of bacteria *Rhizobium* sp. VSy9; *3* — Non-recombinant bacteria strain *Ensifer* sp. Mlu10; *4* — non-recombinant strain of bacteria *Mesorhizobium* sp. LZh7; *5* — recombinant strain of bacteria *Rhizobium* sp. VSy9PmNifA, cultivated on medium without inductor; *6* — recombinant strain of bacteria *Ensifer* sp. Mlu10PmNifA, cultivated on medium without inductor; *7* — recombinant strain of bacteria *Mesorhizobium* sp. LZh7PmNifA, cultivated on the medium without inductor; *8* — recombinant strain of bacteria *Rhizobium* sp. VSy9PmNifA, cultivated on medium with inductor; *9* — recombinant strain of bacteria *Ensifer* sp. Mlu10PmNifA, cultivated on medium with inducer; *10* — recombinant strain of bacteria *Mesorhizobium* sp. LZh7PmNifA, cultivated on medium with inductor

ysis with splitting proteins in polyacrylamide gel for analyzing the total protein of recombinant strains detected only one target band. It was demonstrated that NifH is absent in wild strains in the free-living state. In all recombinant strains, NifH is present during the induction of foreign *nifA* and without it (Fig. 4).

Thus, the artificial activation of *nifA* in all sampled strains of bacteria, attributed to three main species of rhizobia, results in detectable nitrogenase activity *ex planta*. The occurrence of nitrogenase activity without the induction of target gene is probably caused by the so-called "promoting" of promoter *Pm*, during which genes are weakly expressed under the control of this promoter. Owing to this, the insignificant production of the target protein that takes place is capable enough to invoke the induction of nitrogenase activity.

The interaction of plants and growth-promoting microorganisms is a complicated process, which involves products of multiple genes. Any changes in this mechanism can affect the plant–microbe interaction. We examined how the change of nitrogen fixation regulation in recombinant rhizobia affects their ability to form nodules in comparison with their wild-type strains.

It was found that the introduction of additional copy of *nifA* in the cells under the control of bacterial promoters exhibited an insignificant effect on their ability to form nodules. No difference was seen in comparison with the wild-type strains (data shown). The exception to this was a recombinant strain *Ensifer* sp. Mlu10PmNifA, which demonstrated the highest nitrogenase activity and whose inoculation did not result in the formation of nodules. Probably, this strain exhibited more significant changes in the expression of the gene due to recombination; this altered the genes that were involved in the formation of symbiosis with the host plant.

Because strain *Ensifer* sp. Mlu10PmNifA demonstrated high nitrogen fixation activity, we examined the change of the amount of nitrogen compounds in the soil after the introduction of these bacteria. Thirty days after the soil inoculation, the content of ammonia-based nitrogen significantly increased, while the amount of nitrate-based nitrogen significantly decreased (Table 1). This could indicate that bacteria of this strain are able to switch to nitrate respiration, blocking oxygen delivery. This particular fact likely allows them to exhibit high nitrogen fixation activity. Such a possibility was already demonstrated for *E. meliloti* 1021 during initial incubation in the conditions of low oxygen concentration (2 %) [26]. The denitrification power of nodular bacteria plays a key role in preventing the early formation of nodules [27, 28]. The loss of ability to form nodules by recombinant strains and the appearance of nitrate respiration can be interrelated.

Here, it was demonstrated that nitrogen fixation activity outside of plants can be induced in nodular bacteria by modifying the regulation of the expression of *nifA*. The degree of this activity does not have direct dependence on the level of expression of the regulatory protein. The nitrogenase activity of *nifA*-recombinant strains is easily detectable, although it is significantly lower than that of the free-living nitrogen fixers. In some cases, recombinant strains can manifest super nitrogen fixation properties, which are considerably superior to those of the free-living nitrogen fixers.

Table 1

Analysis of the composition and amount of nitrogen compounds in the soil

Sample	NH_4^+, mg/kg	NO_3^+, mg/kg
Untreated soil	30.86 ± 3.2	1050 ± 11
Inoculation with the wild type strain *Ensifer* sp. Mlu10	31.15 ± 2.7	1050 ± 13
Inoculation with a recombinant strain *Ensifer* sp. Mlu10PmNifA	83.6 ± 1.6	355 ± 7

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