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Isolation of an Effective Nitrogen-Fixing Strain N1115 from Rice Rhizosphere by Rice Germ Lectin

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Nitrogen is an essential element for plant growth as well as is the most significant yield-limiting element in many agricultural production systems. Therefore, it is worthy to isolate nitrogen-fixation bacteria from plant rhizosphere. Our purpose is to screen effective nitrogen-fixation bacteria that show affinity to rice germ lectin from the rice root. Ashby medium absence of nitrogen source was used to isolate nitrogen-fixation bacteria from rice rhizosphere. Then, the strains were re-screened by rice germ lectin labeled with fluorescein isothiocyanate. In this study, rice germ lectin was used as a tool to screen effective and nitrogen-fixation bacteria. The physiological and biochemical characteristics of the strain N1115 were evaluated as well as the 16SrRNA gene sequence. The results showed that strain N1115 had an affinity to rice germ lectin and could fix nitrogen with the efficiency of 9.217 ± 0.148mg N/gG. Based on the analysis of 16S rRNA partial gene sequence, this strain was classified to *Bicillus megaterium*. Herein, we proposed a possible method for selecting effective PGPR to apply in research and development of biofertilizer.

Key words: Rice germ lectin; Nitrogen-fixation bacteria; 16Sr RNA; *Bicillus megaterium*.

Nitrogen is an essential element for plant growth as well as is the most significant yield-limiting element in many agricultural production systems. Although more than three quarters of the atmosphere is nitrogen gas, most of them are unavailable to be used directly, attributing to that majority organisms can not assimilate the dinitrogen molecule due to its stable form[1]. Nitrogen-fixing (N-fixing) bacteria have the ability to reduce dinitrogen to ammonium, which can be easily absorbed by plants. It is reported that N-fixing bacteria can establish symbioses with plants by forming a specialized organ for symbiosis on the host plant's roots[1]. Through this way, N-fixing bacteria can build a stable root system, and fertilize soil through nitrogen fixation, improve soil structure, and promote the virtuous cycle of the soil ecosystem.

As we know, fertilizers are essential components of modern agriculture because they provide essential chemical nutrients to plants. However, overuse of fertilizers can cause inevitable environmental issues, such as soil compaction[2]. Under this situation, biofertilizers, which can be used in improving the soil conditions, is defined as a substance which contains living microorganisms when applied to plant leaves, seeds, or soil, and colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary

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nutrients to the host plant[3]. Since the use of rhizobia as a biofertilizer is a friendly environmental alternative to mineral fertilization, inoculation of legumes is a common agricultural practice[4]. The application of biofertilizers could increase the amount of dry matter production in a root[5]. According to Veres *et al*[6], the application of biofertilizers could increase the amount of dry matter production both in a root and shoot of maize. One potential way to decrease negative environmental impacts resulting from continued use of chemical fertilizers is inoculation with plant growth-promoting rhizobacteria (PGPR)[2]. So it is important to isolate bacteria from plants rhizosphere soil to develop the potential biofertilizer and reduce environment pollution.

It is generally believed that bacteria are the main microorganisms in the plant rhizosphere, exhibiting ability in nitrogen-fixation, phosphate-releasing and potassium-releasing. Those kinds of bacteria stimulate plant producing more hormones and increasing higher ability to promote plant growth, prevent disease, increase crop yields, for that reasons those bacteria groups are known as PGPRs[7, 8]. It has been reported that PGPR (including the root surface and rhizosphere) and microorganisms will be involved in complex signal between the exchange and mutual recognition process if getting the successful colonization on plants root. Meanwhile plants could identify the microorganisms by the secreting substances, such as plant lectins and flavonoids, microbial synthesis of extracellular polysaccharides, LPS, capsular polysaccharide and root-cadherin (rhicadhesin), etc[9, 10]. As mentioned above, here we mainly focus on lectins.

Lectin, a class of sugar-binding and cell-agglutinating proteins, ubiquitous in nature, being found in all kinds of organisms[11] and lectin recognition hypothesis points out that lectin have specific binding sites both on root hair and bacterial polysaccharides, so it can be used as a bridge to link biomass and root hair. When bacteria locate in root tip, and gathered great amount, then it will start the next phase of the reaction, leading to the successful colonization in plant root. Genetic engineering also confirms that lectins play a key role in the rhizosphere adsorption and infection[12, 13]. However, the research focused on the rice germ lectin is still rare. Here, we attempt to use RGL as a possible model to explore the function between the plants and microorganisms.

The purpose of this study is to identify the role of RGL in the plants and microbes. By using the rice germ lectin labeled with fluorescein isothiocyanate (FITC), and through analysis of the physical and biochemical properties as well as 16SrRNA partial gene sequences, we isolated a nitrogen-fixing stain N1115 from rice rhizosphere soil. We proposed a possible attempt for selecting PGPR to be used as biofertilizer.

MATERIALS AND METHODS

Soil sample, rice seeds and rice germ lectin labeled with FITC

Soil sample was collected from rice root rihzosphere in Anhui Agricultural University farm and put in sterilized paper bag, stored in 4°C. Rice seeds (Guo feng No.1) were bought from Feng Le Company (http://www.fengle.com.cn/). Rice germ lectin (RGL) was extracted and labeled with FITC according to Marshall[14].

Isolation of strains from rice rhizosphere

Ten gram soil sample was weighed and blended with 100 ml sterile water, diluting to different concentration, to prepare soil solution. Rice rhizosphere soil solution was incubated on the Ashby medium with compositions of (g/l): Glucose or mannitol 10.0g, KH_2PO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.2, $CaSO_4 \cdot 2H_2O$ 0.1, $CaCO_3$ 5.0, agar 15~20, pH7.0-7.2, cultivated for 5 days at 28°C, then larger and transparent colonies were picked up to purify, and those strains was rescreened by RGL- FITC, and observed under fluorescence microscope. A FITC-specific green fluorescent could be seen through excited by blue light. One isolates N1115 was screened by this method to do the next experiments and this strain was preserved at -20°C for later study.

Phenotypic characterization of N1115

The morphology, physiological characteristics of strain N1115 were identified, including shape of colony and cell, gram staining, and the main biochemical attributes were tested, including use of glucose, activities of oxidase, catalase, amylase, H_2S production and gelation liquefaction. M.R and V.P were also evaluated.

Test of nitrogen-fixing capacity of N1115

The nitrogen-fixing ability of N1115 was tested by method used as Mal'tseva, N. M *et al*[15].

DNA Extraction, PCR amplification and analysis of 16SrRNA gene sequence

Ten mi-liter cultivated bacteria liquid was placed into a sterile microcentrifuge tube, and moved all the medium with 3500r/min centrifugation, then bacteria were precipitated by adding lysozyme, and 10% SDS and proteinase K, then sample was mixed in potassium acetate with 10000r/min centrifugation in microcentrifuge tube to collect supernatant and slowly added double volume of anhydrous ethanol, DNA was collected carefully and finally dissolved in TE buffer, preserving under the conditions at -20°C.

The complete 1.5 Kb 16SrRNA region of the isolate N1115 was amplified using primers (50 pmol/ìL) as forward primer: (5'-AGAGTTTGATCCTGGCTCAG -3') as reverse primer was: (5'- ACGGCTACCTTGTTACGACTT -3'). The total volume of PCR reaction system was 25µl. The PCR process was performed according to Park *et al*[16]. The 16SrRNA partial gene sequence of isolate N1115 was determined commercially by the Shanghai biological Engineering Technology and services Co., Ltd (http://www.sangon.com/sangonindex.aspx).

The nucleotide sequences of the 16S rDNA were subjected to BLAST analysis with the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with accession number KT964815. Sequences with high similarity scores were downloaded and a phylogenetic tree was constructed using MEGA 4.0[17].

RESULTS

In our study, one nitrogen-fixing strain N1115 was obtained by re-screening with FITC-labeled rice germ lection which was showed in Fig.1. Strain N1115 was characterized on the basis of its morphological and cultural characteristics as well as its ability to produce growth hormones, fix nitrogen and utilize different carbon sources. This strain was Gram positive with rod cell shape and was fast-growing, and single cell length was (0.5×1.6)ìm/cell. The growing colonies were circular, translucent with smooth margins. The strain N1115 could use glucose and mannitol as carbon sources, and V.P, M.P reactions were negative. Oxidase was negative while Catalase was positive. Gelatin liquefaction was positive as well as amylase (Table 1). This strain could not produce H_2S in the test. The nitrogen-fixing capacity of the strain was 9.217±0.148mg N/gG.

The total gene was successful extracted (Fig.2 A) and used as a template. 16Sr RNA gene partial sequences of strain N1115 were amplified. PCR amplification products were examined by 1.0% agarose gel electrophoresis, and it was clearly showed that an approximately l500bp nucleotide fragments was amplified (Fig.2 B). The nucleotide sequences of the 16S rDNA were subjected to BLAST analysis with the NCBI database. A phylogenetic tree was constructed using MEGA 4.0. Based on the partial 16SrRNA sequences, the strain N1115 was closely affiliated with the genus *Bacillus megaterium* (Fig.3).

DISCUSSION

The plant rhizosphere is the specific zone (2-3cm) of soil influenced by the roots, consisting of a multi-dimensional and dynamic ecological environment of microorganisms and soil system. PGPR have attracted attention because of the need to reduce the use of chemicals, especially when considering the context of sustainable agriculture and environmental protection[18]. Considering this situation, biological nitrogen fixation, which is an essential process in the nitrogen cycle, provides a major source of available nitrogen for organisms[19]. Increasing attention is currently being directed towards the contribution of beneficial microorganisms that can assimilate nitrogen from soil. In our study, we provide a possible way to use RGL as a tool to isolate nitrogen fixation bacteria from rice root.

In this study, we explore the function of RGL between PGPR and rice root. Many reports are focused on the research of plant lectins. Yegorenkova *et al*[20] have reported that *Azospirillum brasilense* might be involved in the recognition and cross-linking between wheat germ agglutinin and bacterial polysaccharide during the colonization on wheat roots. Antonyuk *et al*[21] point that wheat germ agglutinin(WGA) may promote the capacity of Brazilian *Azospirillum* in nitrogen-fixing. Taken all those progresses, we attempt to

use RGL as a mediate to isolate bacteria from rice rhizosphere.

It is important to screen beneficial bacteria from plantation soil. Several studies have shown the positive effects of endophytic bacteria inoculation in plants, e.g. sugarcane (*Saccharum* spp.), leading to increased contribution of biological nitrogen fixation, to promotion of root development, increased biomass and productivity[18]. Herein, we isolated a nitrogen fixation strain N1115 from rice root, showing

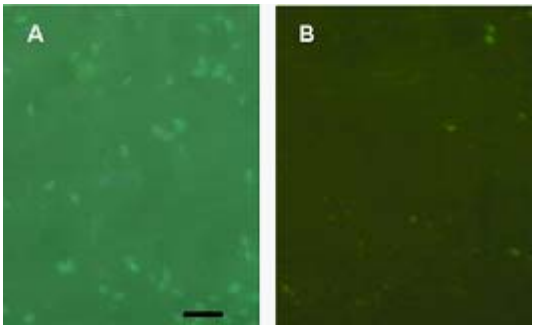

Fig.1. Fluorescent photos of isolates stained by RGL-FITC. A, Strain stained with RGL-FITC; B, Control treatment. Bar represents 5 μm.

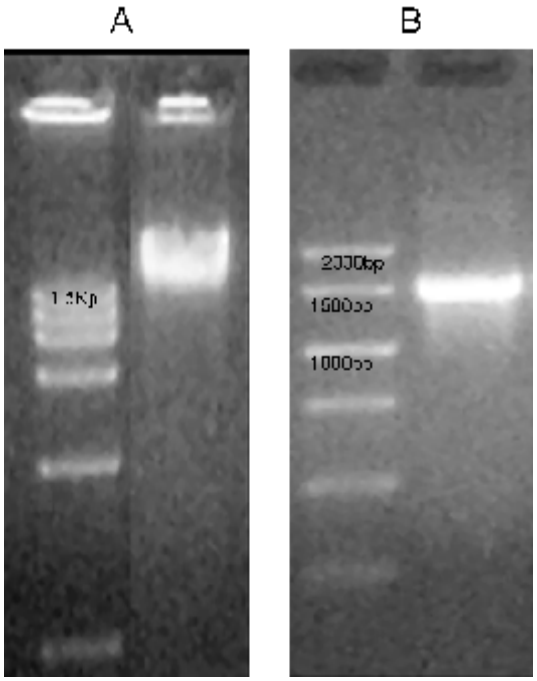

Fig. 2. Photos of agarose gel electrophoresis. A, total gene extraction of N1115, Marker maximum:1.5Kb; B, PCR amplification products (1500bp)

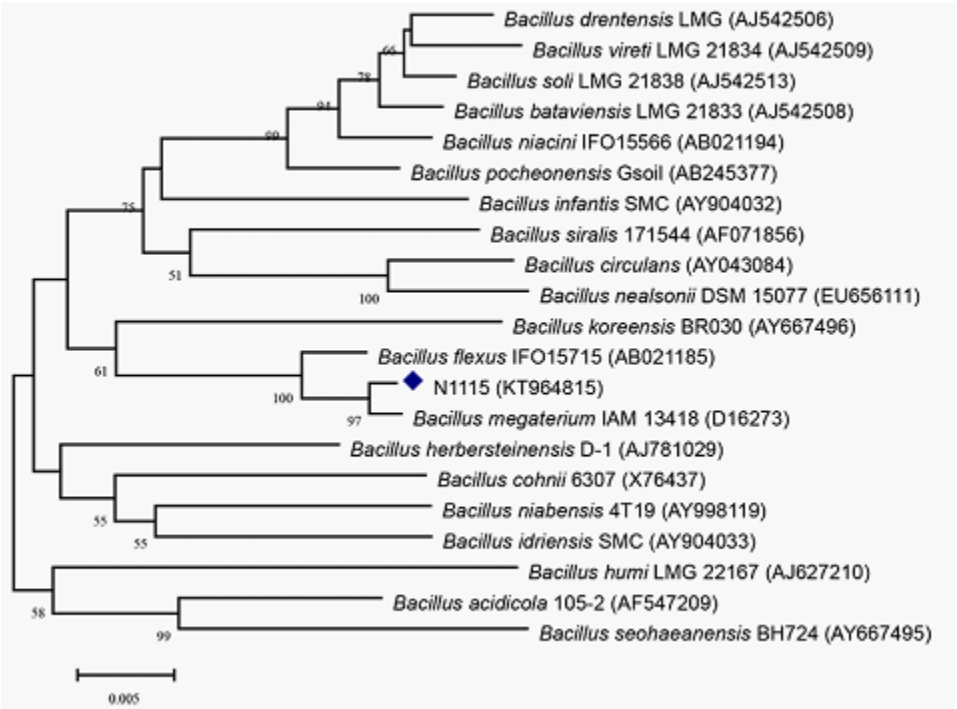

Fig. 3. Neighbor-joining phylogenetic tree based on 16S rRNA partial gene sequence Bar represents 0.005 substitutions per nucleotide position

Table 1. The physiological and biochemical characteristics of the strain N1115

Strain	Gram staining	Single Cell shape	Shape of colony			glucose utilization	oxidase	Catalase	V.P	M.P	gelatin liquefaction	amylase of H_2S	Production
			surface	edge	color								
N1115	G^+	Rod	smooth	regular	transparent	+	-	+	-	-	+	+	-

satisfactory in nitrogen-fixing capacity, of which was 9.217±0.148mg N/gG. This strain was fast-growing on the ashby medium which was lack of nitrogen resource, suggesting that strain N1115 had a strong ability to assimilate N_2 from the air.

Based on the molecular methods, we identified the 16Sr RNA partial gene sequences. According to the phylogenetic tree, strain N1115 belongs to *Bacillus megaterium*. Isolating of nitrogen fixation bacteria is still a hot research subject. Lots of bacteria have been reported in various genuses. For instance, species of *Pseudomonas*[22] and *Paenibacillus*[23] have been described as being nitrogen-fixers. Here, we obtained an isolate possessing ability to fix nitrogen in genus *Bacillus*, providing further information about nitrogen-fixing groups in the plant rhizosphere.

In summary, by using RGL as an isolating tool, one nitrogen fixation strain N1115 was obtained from rice root. According to the morphological characteristics and analysis of 16SrRNA partial gene sequence, strain N1115 was belonged to *Bacillus* genus. Herein, we proposed a possible method for selecting effective PGPR to apply in research and development of biofertilizer. Further studies are needed to test the application of strain N1115 in the field condition.

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