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***Article***

# Isolation and functional characterization of root nodule-associated bacteria from the rare legume *Chesniella macrantha* in Mongolia

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## Abstract

A total of 18 bacterial strains were isolated from the root nodules of *Chesniella macrantha* Cheng f. ex H.C.Fu, a very rare, subendemic, and relict plant species in Mongolia. Based on 16S rRNA gene sequence analysis, the strains were assigned to four genera: *Priestia* (10 strains), *Rhizobium* (6 strains), *Peribacillus* (1 strain), and *Neobacillus* (1 strain). Ten strains exhibited 99.65–100% similarity to *Priestia aryabhattai* B8W22$^T$ or *Priestia megaterium* NBRC 15308$^T$, while strains Gr2-1, Gr2-2, Gr2-3-1, Gr3-1, Gr3-2-1, and UN2-3 showed 99.78–100% similarity to *Rhizobium mongolense* subsp. *mongolense* USDA 1844$^T$. Two strains, Gr2-4-1 and UN1-2, were most closely related to *Peribacillus simplex* NBRC 15720$^T$ (99.93–100%) and *Neobacillus drentensis* LMG 21831$^T$ (99.71%), respectively. Eight strains produced indole-3-acetic acid (IAA) in LB medium supplemented with 5 mM L-tryptophan, and three exhibited phosphate solubilization activity. Six *Priestia* strains, Gr2-3, Gr2-4-2, Gr3-2, Gr3-3, Gr3-3-1, and UN1-3, showed high IAA production, whereas *Priestia* strains Gr2-3, Gr3-3, and UN2-5 solubilized phosphate on Pikovskaya's agar, with a solubilization index (SI) of 2.16 ± 0.02 cm. Among all isolates, *Rhizobium* strain Gr3-2-1 was the most effective in promoting both root elongation and branching in *C. macrantha* plantlets, indicating its potential use in propagation and conservation of this endangered Mongolian legume.

**Keywords:** *Chesniella macrantha Cheng f. ex H.C.Fu; rhizobia; 16S rRNA gene; indole acetic acid; phosphate solubilization;*

## Introduction

*Chesniella macrantha* (Cheng f. ex H.C.Fu) is a subendemic perennial legume native to the arid regions of Mongolia and extending into northwestern China. The species was formerly known as *Chesneya macrantha* [1]. According to *The World Flora Online*, *Spongiocarpella grubovii* (N.Ulziykh.) Yakovlev has also been recorded as a synonym of this species [2]. Legumes (Fabaceae) form one of the most ecologically and evolutionary significant plant families in arid and semi-arid ecosystems, largely due to their ability to establish mutualistic symbioses with nitrogen-fixing rhizobia housed within specialized root nodules [3]. In recent years, due to climate change and significantly reduced precipitation, the plant's flowering and seed development have drastically declined. Moreover, the mature seeds exhibit poor germination capacity, resulting in a sharp decrease in both its distribution and population. Thus, propagation of *C. macrantha* through biotechnological method is important to ensure its conservation and restoration.

Endophytic bacteria are microorganisms that inhabit the internal tissues of plants without causing disease, often establishing beneficial relationships with their hosts. They are increasingly recognized as key contributors to plant health by promoting growth, enhancing stress tolerance, and suppressing pathogens [4,5]. Their functional diversity includes nitrogen fixation, phosphate solubilization, and production of phytohormones such as indole-3-acetic acid (IAA) [6,7]. Among them, bacteria that inhabit nodule of legume plants are of special interest because of their ability to convert atmospheric nitrogen into ammonia, a usable form of nitrogen for the host plant [8].

Some endophytes are known to function as plant growth-promoting (PGP) bacteria, playing an important role in enhancing plant development under challenging environmental conditions [9].These properties make them valuable both for sustainable agriculture and the conservation of threatened plant species.

Recently, we developed an *in vitro* propagation protocol for this species for the first time. As a result, *in vitro* propagated shoots treated with 300 mg/L IBA solution successfully rooted at a rate of 92% under *ex vitro* conditions [10]. In this study we aimed to explore root nodule-associated bacteria of *C. macrantha* and their plant growth-promoting properties, such as phosphate solubilization, and IAA production, as well as their effect on the plantlets of *C. macrantha*. These findings are expected to identify promising bacterial candidates for use in the propagation and conservation of this species, either as an alternative to, or in combination with, the above-mentioned propagation method.

**Materials and methods**

*Trapping of Nodules*

Nodulation was induced using the "plant trap" method under greenhouse conditions as described by Vincent [11], with minor modifications. Soil samples were placed in 3-kg capacity plastic pots that had been surface-sterilized with 70% ethanol for 5 seconds. Subsequently, two-month-old *in vitro*–grown shoots were transplanted and cultivated under greenhouse conditions. After seven months, pink and healthy nodules were collected and transferred to sterile Petri dishes using forceps sterilized by dipping in ethanol and flaming.

*Isolation of Bacteria from Root Nodule*

Collected nodules were surface sterilized with 70% ethanol for 60 seconds, and transferred to 3% (v/v) solution of sodium hypochlorite (NaOCl) for 3-4 minutes. The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse the sterilizing chemicals. Then nodules were transferred into sterile Petri dishes and crushed with alcohol-flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl) inside a laminar air flow hood. Then 0.1ml (loopful) of the suspension was streaked on a plate containing Yeast Extract Mannitol Agar (YEMA) [11] and incubated at 28 ± 2°C for 3-5 days. The pure cultures were preserved in glycerol suspensions (20%, V/V) at -80°C in the Mongolian National Culture Collection of Microorganisms (MNCCM), Institute of Biology, Mongolian Academy of Sciences.

*Identification of the Isolates*

Molecular identification was carried out using bacterial isolates grown on YEMA at 28°C for 2-7 days. Total genomic DNA was extracted using the Bacterial DNA Extraction Kit (ZanaaSpex) according to the manufacturer's instructions.

The 16S ribosomal RNA (rRNA) gene was amplified using the pair of primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCC-3') as described previously [12]. The amplification was performed in the GeneAmp® PCR System 9700 (Thermo Fisher Scientific Inc., USA) according to the following conditions: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 15 sec, and extension at 72°C for 1 min; and a final extension step at 72°C for 5 min. The PCR products were visualized by electrophoresis on 1% agarose gel and subsequently purified using a PCR Purification Kit (ZanaaSpex), and sent to Macrogen, Korea, for commercial sequencing. The sequences were analyzed by BLAST similarity search at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) and EzBioCloud (https://www.ezbiocloud.net) based on their identity values [13].

The obtained sequences were submitted to GenBank, and the accession numbers are LC895371-LC895388.

*Phosphate Solubilization Assay*

Screening of bacterial isolates for the ability to solubilize phosphate was carried out on Pikovskaya's (PKV) agar medium as described previously [14]. Agar plugs (6 mm), cut from a 2-day-old culture of endophytic bacteria, were placed on the respective plates in triplicate and incubated at 28°C. The clear zones formed around the colonies were measured after the incubation period, 1-5 days after inoculation, for the efficiency of solubilization of phosphorus.

*IAA Production Assay*

The production of IAA by endophytic bacterial isolates was assessed using a colorimetric method. The bacterial strains were cultured in 5 mL of LB medium supplemented with 5 mM L-tryptophan and incubated at 28°C for 5 days. Following incubation, the cultures were centrifuged at 5000 rpm for 10 min, and 1 mL of the resulting supernatant was combined with 2 mL of Salkowski reagent (prepared from 1 mL of 0.5 M $FeCl_3$ and 50 mL of 35% perchloric acid). The mixture was then incubated in the dark at room temperature for 20 min. A control was prepared by mixing LB medium containing 5 mM L-tryptophan with Salkowski reagent. The appearance of a pink coloration indicated IAA production, with the intensity of pink to red color categorized as low, medium, or high [15]. The absorbance of the reaction was measured at 530 nm using a UV–VIS spectrophotometer, and the IAA concentration was determined from a standard curve constructed with known concentrations of pure IAA [14].

*Test of Bacterial Effect on Plantlet Development*

Cell suspensions of all 18 strains were tested to evaluate their effects on plantlet growth and development in *ex vitro*. The assay was conducted using 2–3 cm *in vitro*-grown shoots of *C. macrantha*. Shoots were treated with 100 µL of each bacterial suspension for 20 minutes, and one shoot was planted per pot with sterilized soil, sealed with food-grade plastic film. The experiment was conducted in 30 replicates for each treatment. The positive control consisted of explants treated with 300 mg/L indole-3-butyric acid (IBA) for 20 minutes before transfer to soil. The negative control involved direct transfer to soil without any hormone treatment. Additionally, YEMA medium without bacteria was used as a medium control. The plantlets were cultivated in a tissue culture room under a photoperiod of 16 hours light and 8 hours dark, with a light intensity of 3000–3500 lux, and a temperature range of 25–30°C.

## Results

### *Isolation and Identification of Root Nodule Bacteria*

After 7 months of greenhouse incubation in the plant-trap assay, healthy pink nodules measuring 3–8 mm in diameter were observed on *C. macrantha* roots. A total of 18 bacterial strains were successfully isolated from these nodules (Figure 1). Six isolates were Gram-negative, forming mucoid colonies indicative of substantial exopolysaccharide (EPS) production, consistent with the typical characteristics of rhizobia and other members of the family *Rhizobiaceae* [16], while the remaining isolates were Gram-positive.

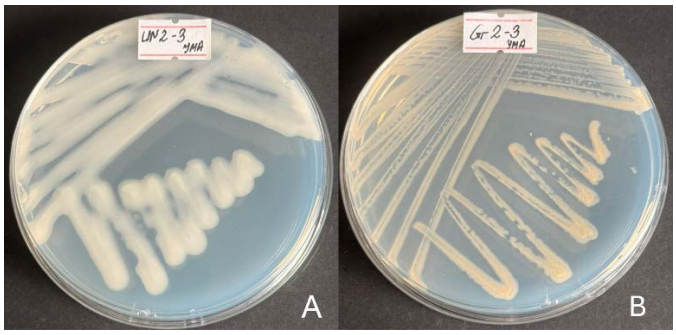


***Figure 1***. Isolated *Rhizobium* (A) and *Priestia* (B) strains.

Identification based on 16S rRNA gene sequences revealed that the strains belonged to four bacterial genera: *Priestia* (10 strains), *Rhizobium* (6 strains), *Peribacillus* (1 strain), and *Neobacillus* (1 strain). Strains Gr2-1, Gr2-2, Gr2-3-1, Gr3-1, Gr3-2-1, and UN2-3 were assigned to the rhizobial genus of *Rhizobium* and were closely related to *R. mongolense* subsp. *mongolense* USDA 1844$^T$ with sequence similarities of 99.78–100%. Nine strains, Gr2-3, Gr3-2, Gr3-3, Gr3-3-1, UN1-1, UN1-3, UN2-1, UN2-2, and UN2-5 showed 99.65–100% similarity to *P. aryabhattai* B8W22$^T$, and Gr2-4-2 showed 99.72% similarity to *P. megaterium* NBRC 15308$^T$. Two strains, Gr2-4-1 and UN1-2, were most closely related to *P. simplex* NBRC 15720$^T$ (99.93–100%) and *N. drentensis* LMG 21831$^T$ (99.71%), respectively (Table 1).

**Table 1.**

*Chesniella macrantha* root nodule bacterial strains identified by 16S rRNA gene sequence comparison with the BLAST match with the EzBioCloud database.

| No | Strain | Obtained GenBank accession No. | Type species with most homologous sequence (accession No) | Similarity % |
|---|---|---|---|---|
| 1 | Gr2-1 | LC895371 | *Rhizobium mongolense* subsp. *mongolense* USDA 1844$^T$ | 100 |
| 2 | Gr2-2 | LC895372 | *Rhizobium mongolense* subsp. *mongolense* USDA 1844$^T$ | 100 |
| 3 | Gr2-3 | LC895373 | *Priestia aryabhattai* B8W22$^T$ | 100 |
| 4 | Gr2-3-1 | LC895374 | *Rhizobium mongolense* subsp. *mongolense* USDA 1844$^T$ | 100 |
| 5 | Gr2-4-1 | LC895375 | *Peribacillus simplex* NBRC 15720$^T$ | 100 |
| 6 | Gr2-4-2 | LC895376 | *Priestia megaterium* NBRC 15308$^T$ | 99.72 |
| 7 | Gr3-1 | LC895377 | *Rhizobium mongolense* subsp. *mongolense* USDA 1844$^T$ | 100 |
| 8 | Gr3-2 | LC895378 | *Priestia aryabhattai* B8W22$^T$ | 100 |
| 9 | Gr3-2-1 | LC895379 | *Rhizobium mongolense* subsp. *mongolense* USDA 1844$^T$ | 100 |
| 10 | Gr3-3 | LC895380 | *Priestia aryabhattai* B8W22$^T$ | 99.72 |
| 11 | Gr3-3-1 | LC895381 | *Priestia aryabhattai* B8W22$^T$ | 99.65 |
| 12 | UN1-1 | LC895382 | *Priestia aryabhattai* B8W22$^T$ | 99.93 |
| 13 | UN1-2 | LC895383 | *Neobacillus drentensis* LMG 21831$^T$ | 99.71 |
| 14 | UN1-3 | LC895384 | *Priestia aryabhattai* B8W22$^T$ | 100 |
| 15 | UN2-1 | LC895385 | *Priestia aryabhattai* B8W22$^T$ | 100 |
| 16 | UN2-2 | LC895386 | *Priestia aryabhattai* B8W22$^T$ | 99.86 |
| 17 | UN2-3 | LC895387 | *Rhizobium mongolense* subsp. *mongolense* USDA 1844$^T$ | 99.78 |
| 18 | UN2-5 | LC895388 | *Priestia aryabhattai* B8W22$^T$ | 99.93 |

***IAA Production and Phosphate Solubilization Assay***

The strains were further studied for their plant growth-promoting traits, including IAA production and phosphate solubilization. Nine strains exhibited positive results for one or more traits. The production of IAA was evaluated after 24, 48, and 72 hours of incubation in LB medium supplemented with 5 mM L-tryptophan. IAA synthesis was detected in eight strains, among which six *Priestia* strains, Gr2-3, Gr3-2, UN1-1, UN1-3, UN2-2, and UN2-5, exhibited particularly high levels of production. In all strains, IAA concentration increased progressively with incubation time, reaching the maximum at 72 hours. Strain UN1-1 exhibited the highest IAA production, reaching 335.6 µg/mL at 72 hours. (Figure 2). 88

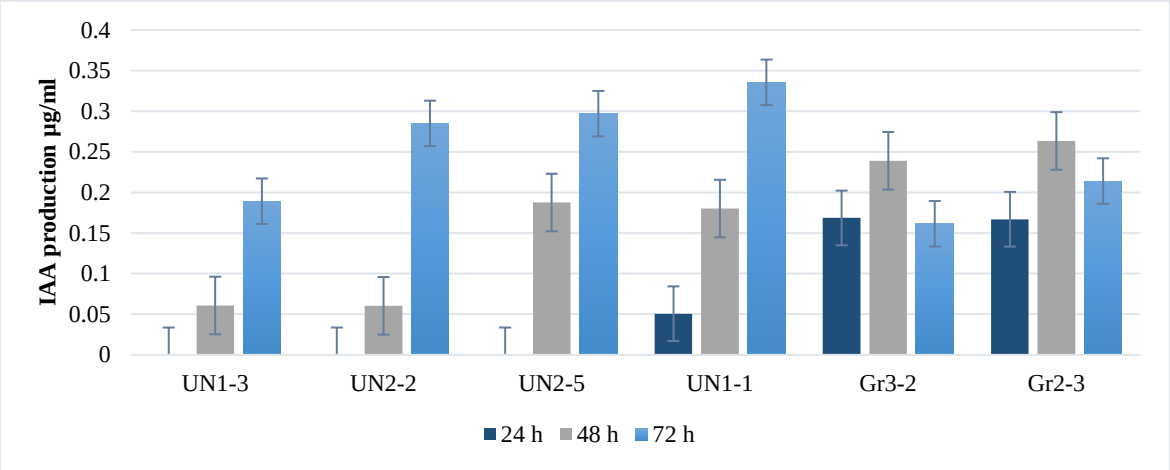


**Figure 2.** Quantitative production of IAA by endophytic *Priestia* strains

Three *Priestia* strains, Gr2-3, Gr3-3, and UN2-5, demonstrated phosphate solubilization activity on solid Pikovskaya (PVK) medium with a solubilization index (SI) of 2.1±0.02, 2.3±0.02, and 2.5±0.02, respectively (Figure 3).

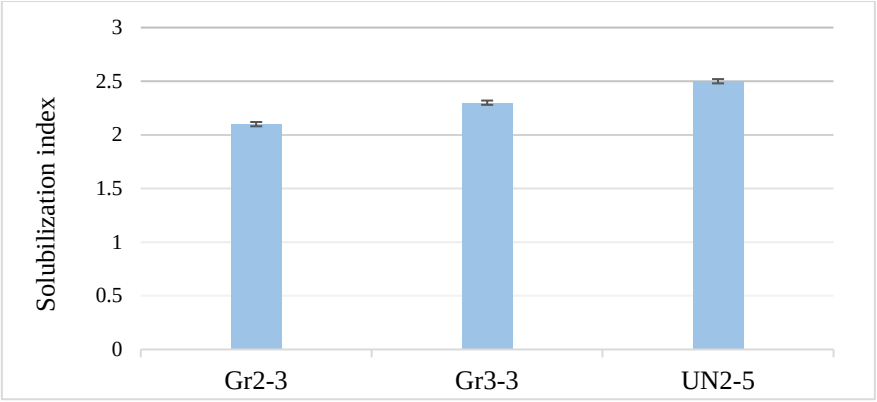


**Figure 3.** Phosphate solubilization by endophytic *Priestia* strains

***Effect of Bacterial Inoculants on Plantlet Development***

After 60 days, plantlet roots treated with Gr3-2-1 and UN2-3 suspensions reached 1.28 ± 0.5 cm and 1.42 ± 0.8 cm, respectively, both significantly longer than other suspensions and the indole butyric acid (IBA)-treated control (0.54 ± 0.5 cm) (Table 2). Branching of roots was also assessed by counting the number of lateral roots per shoot. The number of lateral roots induced by the two treatments was 6.0 ± 2.9 and 2.6 ± 1.0, respectively, compared to 2.2 ± 1.98 in the IBA-treated control (Table 2).

**Table 2.**

Effect of *Rhizobium* strains Gr3-2-1 and UN2-3 on primary root elongation and lateral root initiation in *Chesniella macrantha* plantlets in comparison with the IBA-treated control.

| No | Strains and treated control | Root Length (cm) | Lateral Roots (cm) |
|---|---|---|---|
| 1 | Gr-3-2-1 | 1.28 ± 0.5 | 6.0 ± 2.9 |
| 2 | UN2-3 | 1.42 ± 0.8 | 2.6 ± 1.0 |
| 3 | IBA Control | 0.54 ± 0.5 | 2.2 ± 1.98 |

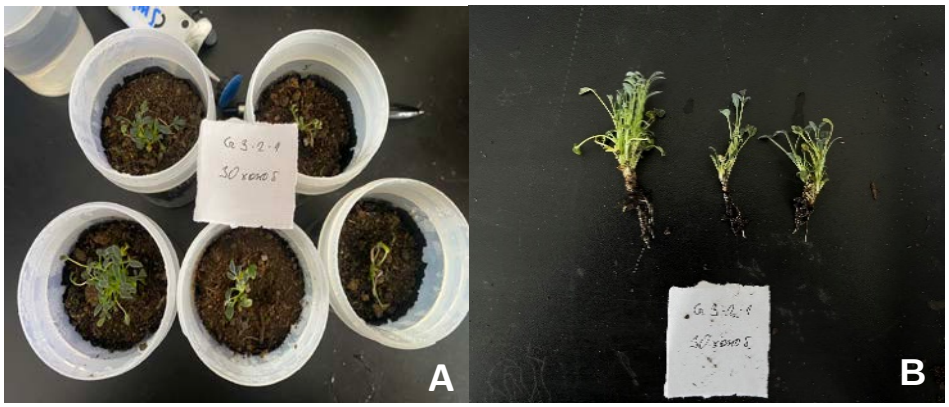


**Figure 4.** (A) *Chesniella macrantha* plantlets treated with cell suspension and transferred to soil. (B) Root formation in *Chesniella macrantha* plantlets.

## Discussion

The present study revealed that *C. macrantha* root nodules harbor diverse bacterial communities dominated by *Rhizobium* and *Priestia* species. While *Rhizobium* is a well-known nitrogen fixer in leguminous nodules, the consistent presence of *Priestia* suggests a potential endophytic or co-symbiotic role, contributing to phytohormone production and nutrient solubilization.

Several *Priestia* strains exhibited strong auxin and phosphate-solubilizing activities, implying indirect enhancement of plant growth through nutrient mobilization and hormonal stimulation. Similar findings have been reported in other legumes, where endophytic bacteria with PGP traits contributed to improved plant vigor [17].

The presence of mucoid colonies among some isolates indicates substantial production of exopolysaccharides (EPS). EPS production is a crucial physiological trait that enables bacterial attachment to root surfaces, protects cells against desiccation and environmental stress, and contributes to the establishment of effective symbiotic interactions with host plants [18]. Similar findings have been reported in other rhizobial studies, where EPS-producing strains exhibited enhanced nodulation efficiency and tolerance to adverse conditions such as drought and salinity [19]. Therefore, the observed mucoid phenotype suggests that these isolates may possess adaptive advantages for survival and symbiosis in harsh soil environments.

The *ex-vitro* assays demonstrated that *Rhizobium* strains Gr3-2-1 and UN2-3 were more effective in enhancing root elongation and lateral root formation. This result aligns with the established role of *Rhizobium* in nitrogen fixation and symbiotic signaling processes that directly influence root development [20]. Taken together, these findings suggest complementary but distinct functions of the two genera: *Priestia* may indirectly promote plant growth through hormone production and nutrient mobilization, while *Rhizobium* confers more immediate developmental benefits, likely mediated by nitrogen fixation and host–microbe signaling pathways [21]. Such functional differentiation highlights the potential value of combining strains with diverse PGP traits to optimize the propagation and conservation of *C. macrantha.*

## Conclusion

Our study provides the first insight into the cultivable bacterial diversity associated with root nodules induced by the plant trap method of the rare legume of Mongolia, *Chesniella macrantha* Cheng f. ex H.C.Fu. Among the 18 strains, *Priestia* and *Rhizobium* were the most abundant and functionally significant. Experiments investigating the combined effects of *Priestia* and *Rhizobium* strains on plantlet growth and development are currently underway to identify the most effective strains and combinations for application in the propagation of *C. macrantha.*

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

**Auhtors' contribution**

M.S. contributed to investigation and data analysis and wrote the original draft. M.N. contributed to methodology, investigation, and data analysis. M.T. and S.M. conducted plantlet experiments and analyzed the data. B.Ba. and B.Bo. performed DNA isolation and PCR analyses. A.K. and O. Y. contributed to study design and project administration. E.J. conceptualized and designed the study; contributed to methodology, project administration, and data analysis; and validated, reviewed, and edited the manuscript. All authors read and approved the final manuscript.

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