Isolation of Two Cold-Tolerant PGPB Strains and Their Effects on Growth of Local Forage Grasses in Northern Tibet (Postprint)

Authors: He Min, Wang Xiupu, Li Yan, Dai Zhicong, Wang Congyan, Du Hai, Du Daolin

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Abstract

This study aimed to isolate and screen plant growth-promoting strains from the soils of degraded grasslands in the northern Qinghai-Tibet Plateau that can promote the growth of typical local forage grasses, in order to facilitate vegetation restoration in northern Tibet. By employing the dilution plating method to isolate low-temperature tolerant plant growth-promoting bacteria, and combined with pot experiment design, the effects of the isolated bacteria on the growth of local forage grasses were evaluated. This study successfully screened and obtained from Tibetan soils · α-KB mg-1 · h-1), TS27 showed strong production of $±$3.85 mg · L-1) and

Full Text

Isolation of Two Cold-Tolerant PGPB Strains from Northern Tibetan Soil and Their Effects on Local Forage Grass Growth

He Min, Wang Xiupu, Li Yan, Dai Zhicong, Wang Congyan, Du Hai, Du Daolin*

Institute of Environment and Ecology, Academy of Environmental Health and Ecological Security & School of the Environment and Safety Engineering, Jiangsu University, Zhenjiang 212013, Jiangsu, China

Abstract

This study aimed to isolate and screen cold-tolerant plant growth-promoting bacteria (PGPB) from degraded grassland soils in the northern Tibetan Plateau

to promote local vegetation restoration. Cold-tolerant PGPB strains were isolated using the serial dilution plating method, and their effects on local forage grasses were evaluated through pot experiments. Two cold-tolerant bacterial strains, TS22 and TS27, were successfully isolated from Tibetan soil and identified via 16S rRNA gene sequencing as *Brevibacterium* sp. and *Bacillus mycoides*, respectively. *In vitro* assessment of plant growth-promoting traits revealed that both strains possessed the ability to produce indole-3-acetic acid (IAA) and siderophores (SID), and exhibited ACC-deaminase activity. Strain TS22 showed high ACC-deaminase activity (264.69 nmol $\cdot$ α-KB $mg^{-1} \cdot h^{-1}$), while TS27 demonstrated strong IAA production (7.52$±3.85$mg \cdot L\hat{}${-1}$) and SID production (92%) capabilities. Pot experiments conducted at 10 °C showed that the effects of strains TS22 and TS27 on the growth of *Poa annua* and *Elymus sibiricus* varied depending on both plant and bacterial species. Strain TS22 significantly promoted *Poa annua* growth in terms of plant height, root length, aboveground dry weight, and underground dry weight, exhibiting superior growth-promoting activity compared to TS27. This study provides valuable microbial resources and a practical foundation for the application of plant-microbe technologies in vegetation restoration in the high-altitude, cold regions of northern Tibet.

Keywords: plant growth-promoting bacteria, low-temperature environment, cold-tolerant bacteria, plant growth-promoting properties, phytoremediation, grassland degradation

Introduction

In recent years, grassland vegetation in the northern Tibetan Plateau has become increasingly degraded due to overgrazing, posing a serious threat to local biodiversity, environmental health, economic development, and human quality of life. Consequently, strengthening grassland vegetation protection and restoration has garnered increasing attention (Wang et al., 2019). While various approaches—including fertilizer application, compost addition, polymer materials, and humic acid—can effectively enhance vegetation restoration efficiency, their high costs limit large-scale implementation (Weyens et al., 2009; de-Bashan et al., 2012).

PGPB are a group of bacteria that can directly or indirectly improve plant health and promote plant growth (Bashan & Holguin, 1998). These bacteria enhance plant nutrient acquisition through nitrogen fixation, production of indole-3-acetic acid (IAA), siderophores (SID), ACC-deaminase activity, and solubilization of insoluble phosphates, or they protect plants from pathogen invasion by secreting antibiotics and other substances (Bashana et al., 2000; Mayak et al., 2004; Siddikee et al., 2010).

Currently, PGPB have been widely applied in agricultural production. For example, a cold-tolerant *Pseudomonas* sp. isolated from the rhizosphere of amaranth (*Amarantus* sp.) in the Indian Himalayas, which exhibited PGP traits in-

cluding IAA and SID production and phosphate solubilization, increased wheat seedling germination rate, plant height, and root length by 19.2%, 30.0%, and 22.9%, respectively (Mishra et al., 2009). Beyond agriculture, PGPB demonstrate significant potential in ecological restoration, particularly for vegetation recovery in extreme environments. For instance, the effectiveness of PGPB (*Azospirillum*) in desert restoration with cacti has been confirmed in Mexico (Puente & Bashan, 1993; Bashan et al., 1999; Bacilio et al., 2006; Yoav et al., 2009). Cactus seeds inoculated with *Azospirillum* could grow well in crushed stone without fertilizer, while uninoculated plants withered or died (Bashan & De-Bashan, 2010a). The application of PGPB can improve plant health and growth performance in eroded soils, enhance tolerance to stresses such as drought and salinity, and facilitate in-situ vegetation regeneration in fragile habitats without additional chemical fertilizers, thereby reducing bioremediation costs. However, to date, few studies have reported the use of PGPB to promote forage grass growth and restore local vegetation in the alpine grasslands of Tibet.

Given the unique and fragile habitat conditions of northern Tibet and the influence of indigenous microorganisms, the effectiveness of exogenous PGPB inoculation is often limited. Exogenous strains face dual challenges from dramatic environmental changes and competition with native microorganisms, which restrict their survival and functional activity. Selecting indigenous PGPB as inoculants can facilitate better adaptation to local conditions and help achieve desired outcomes (Schlaeppi et al., 2016). This study isolated and screened indigenous cold-tolerant PGPB from Tibetan soil and evaluated their growth-promoting effects on two locally widespread gramineous forage grasses, *Poa annua* and *Elymus sibiricus*, under simulated low-temperature conditions representative of northern Tibet.

1.1 Soil Sample Collection

Soil samples were collected from Shenzha County, Nagqu Prefecture, Tibet (88°37 -88°38 E, 30°55 -30°56 N), where the dominant soil type is alpine steppe soil. A five-point mixed sampling method was employed at a depth of 0–10 cm, with three replicates. After removing gravel and plant residues, soil samples were placed in sterilized sealed polyethylene bags and transported to the laboratory. For each soil sample, 50 g was sent to Nanjing Convinced-Test Technology Co., Ltd. for physicochemical property analysis. The remaining soil was stored at -20 °C for subsequent bacterial isolation. Soil nutrient characteristics are presented in .

** Soil Nutrient Composition**

Parameter	Unit	Value
Total C	$g \cdot kg^{-1}$	1.96$±0.058\|\|TotalP\|g \cdot kg\^{}\{-1\}$

1.2 Isolation and Identification of Cold-Tolerant Bacteria

Soil samples (0.1 g) were suspended in 1 mL phosphate buffer solution and shaken at 250 $r \cdot min^{-1}$ and 25 °C for 30 min, followed by gradient dilution of the suspension. Aliquots (100 µL) of diluted suspension were spread on Luria-Bertani (LB) plates and incubated at low temperature (4 °C) for isolation of cold-tolerant bacteria (Reasoner & Geldreich, 1985). After 7 days, single colonies were selected and transferred to fresh LB agar for purification. Total DNA was extracted from purified isolates, and the 16S rRNA gene was amplified using universal bacterial primers 27F (5 -AGAGTTTGATCCTGGCTCAG-3) and 1492R (5 -ACGGTACCTTGTACGACTT-3). PCR products were verified and sequenced by Shanghai Sangon Biotech. Obtained sequences were compared with those in the EzBiocloud database, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA 6.06.

1.3 Analysis of PGPB Plant Growth-Promoting Properties

The growth-promoting characteristics of isolated strains were evaluated. IAA production was detected according to Glickmann & Dessaux (1995). Strains were inoculated in R2A medium supplemented with L-tryptophan and incubated for 72 h, after which cell-free supernatant was collected by centrifugation. IAA concentration in the supernatant was determined spectrophotometrically at OD_{530} nm using a colorimetric microplate method, with a standard curve for quantification.

ACC deaminase activity was quantified following Penrose & Glick (2002). Bacterial cells were cultured in DF medium without ammonium sulfate for 8 h, then harvested by centrifugation. The collected cells were resuspended in DF medium supplemented with 3.0 $mmol \cdot L^{-1}$ ACC and incubated for 24 h, after which enzyme activity was measured colorimetrically.

Siderophore production was quantified according to Cherif-Silini et al. (2016). After 72 h cultivation in King' s B medium, 500 µL of supernatant was collected by centrifugation and mixed with 500 µL Chrome Azurol S assay solution. Absorbance of the mixture was measured at 630 nm, and SID percentage was calculated using the formula:

$$SID\% = \frac{ST - Se}{ST} \times 100$$

where ST is the absorbance of the deep blue CAS solution (control) and Se is the absorbance of the sample solution that changed from blue to orange.

To assess phosphate solubilization ability, bacterial suspensions were spotted at the center of Pikovskay agar plates containing insoluble $Ca_3(PO_4)_2$ and incubated at 28 °C (Feng et al., 2005). The presence and size of clear zones around colonies were measured to evaluate phosphate solubilization capacity. All measurements were performed in triplicate.

1.4 Pot Experiment

Seeds of *Poa annua* and *Elymus sibiricus* were surface-sterilized by soaking in 75% ethanol for 1 min and 5% NaClO solution for 5 min, followed by three rinses with sterile water. Sterilized seeds were then soaked for 2 h in 10 mL of bacterial suspension at 10^8 cfu $\cdot$ mL^{-1}, with control seeds soaked in sterile phosphate buffer. Seeds were sown in culture bottles containing 180 g sterile sand and 54 mL of 0.5× modified Hoagland solution, and cultivated in a greenhouse at 25 °C. The Hoagland nutrient solution was modified according to the mineral salt composition of Tibetan soil () with the following formulation: 94.5 g $\cdot$ L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 50.6 g$\cdot$$L^{-1}$ KNO_3, 8 g$\cdot$$L^{-1}$ NH_4NO_3, 0.00276 g$\cdot$$L^{-1}$ KH_2PO_4, 49.3 g $\cdot$ L^{-1} $MgSO_4 \cdot 7H_2O$, 3.73 g $\cdot$ L^{-1} EDTA-2Na, 2.78 g $\cdot$ L^{-1} $FeSO_4 \cdot 7H_2O$, 0.83 mg $\cdot$ L^{-1} KI, 22.3 mg $\cdot$ L^{-1} $MnSO_4$, 0.25 mg $\cdot$ L^{-1} Na_2MoO_4, 0.025 mg $\cdot$ L^{-1} $CoCl_2$, 6.2 mg $\cdot$ L^{-1} H_3BO_3, 8.6 mg $\cdot$ L^{-1} $ZnSO_4$, and 0.025 mg $\cdot$ L^{-1} $CuSO_4$ (pH 6.0). After germination, seedlings were transferred to low-temperature conditions (10 °C) with a light intensity of 10,000 lx and a 14 h/10 h light/dark cycle (these conditions were based on average temperature, sunlight intensity, and photoperiod in the Tibetan Plateau during June). Each treatment consisted of five replicates. After 30 days, plants were harvested and plant height, root length, aboveground fresh weight, underground fresh weight, aboveground dry weight, and underground dry weight were measured.

1.5 Data Analysis

Statistical analysis was performed using IBM SPSS Statistics 20. Data were subjected to one-way and two-way ANOVA, with significance evaluated based on P-values. All figures were prepared using Origin software (version 2018).

2.1 Isolation and Identification of Cold-Tolerant Bacteria

Two bacterial strains, TS22 and TS27, were successfully isolated from Tibetan soil. The 16S rRNA gene sequences of the isolates were compared with the EzBiocloud database to determine their taxonomic status, and a phylogenetic tree was constructed ([Figure 1: see original paper]). Strain TS22 showed highest similarity (96%) to *Brevibacterium frigoritolerans* DSM 8801, suggesting it represents a novel species within the *Brevibacterium* genus. Strain TS27 exhibited 99% similarity to *Bacillus mycoides* DSM 2048 and was thus classified as *B. mycoides*. The 16S rRNA gene sequences of strains TS22 and TS27 have been deposited in GenBank under accession numbers MN710445 and MN710449, respectively.

2.2 Plant Growth-Promoting Properties of Isolated Strains

The plant growth-promoting properties of the two bacterial strains were assessed, including IAA production, phosphate solubilization, siderophore production, and ACC deaminase activity (). Strain TS22 exhibited extremely high ACC deaminase activity (264.69 nmol $\cdot$ α-KB $mg^{-1} \cdot h^{-1}$),

approximately 10-fold higher than strain TS27. Neither TS22 nor TS27 demonstrated phosphate solubilization ability. IAA production was relatively low for both strains, at 3$±0.31$mg \cdot L^{-1}$ and 7.52$±3.85$mg \cdot L^{-1}$, respectively. Both strains showed high siderophore production capability, with values of 89.58±0.08±$0.24%, respectively.

** Plant Growth-Promoting Characteristics of Strains TS22 and TS27**

Strain	IAA (mg · L^{-1})	SID (%)	ACC Deaminase (nmol · α-KB mg^{-1} · h^{-1})
Brevibacterium sp. TS22	3$±0.31 *Bacillus mycoides* TS27	89.58±0.0786	264.69±3.1572 ∗ 7.52±3.85	92.74±0.2426	26.35±$6.4174

Note: α-KB = α-ketobutyrate.

2.3 Effects of Isolated Strains on Growth of Local Forage Grasses

As shown in [Figure 2: see original paper] and [Figure 3: see original paper], inoculation with strains TS22 and TS27 produced different effects on the growth of the local forage grasses *Poa annua* and *Elymus sibiricus*.

For *Poa annua* ([Figure 2: see original paper]), inoculation with TS22 significantly increased plant height, root length, and both aboveground and underground dry weight compared to the uninoculated control. Inoculation with TS27 increased plant height, aboveground dry weight, and underground dry weight by 20.7%, 11.1%, 89.4%, and 74.2%, respectively, but these differences were not statistically significant.

For *Elymus sibiricus* ([Figure 3: see original paper]), neither TS22 nor TS27 inoculation showed clear growth-promoting effects; instead, slight inhibitory effects were observed. TS22 inoculation reduced aboveground and underground dry weight by 1.4% and 5.4%, respectively, while TS27 treatment significantly reduced root length by 22%.

2.4 Correlation Analysis Between Isolated Strains and Plant Growth Attributes

Two-way ANOVA results indicated that plant species significantly affected only underground fresh and dry weight, while bacterial species influenced all plant growth parameters. The interaction between plant and bacterial species affected biomass-related indices ().

** Two-Factor ANOVA Analysis of Relationships Between Plant Species (P), Bacterial Treatment (B), and Plant Growth**

Factor	Underground FW	Aboveground FW	Underground DW	Aboveground DW	Root Length	Plant Height
Plant species (P)	<0.001	ns	<0.001	ns	ns	ns
Bacterial species (B)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Plant × Bacteria (P×B)	<0.001	ns	<0.001	<0.001	ns	ns

Note: DW = Dry weight; FW = Fresh weight; ns = not significant; $P < 0.05$ indicates significant difference.

3 Discussion and Conclusion

The selection of appropriate microbial inoculants is critical for successful vegetation restoration of degraded soils using PGPB. In harsh, specialized habitats, exogenous PGPB inoculants face dual challenges from dramatic environmental changes and competition with native microorganisms, which limit their survival and functional activity. Selecting indigenous PGPB as inoculants enables better adaptation to local conditions and facilitates the achievement of desired outcomes (Schlaeppi et al., 2016).

This study targeted the unique geographical environment and cold, dry climate characteristics of northern Tibet, isolating two cold-tolerant PGPB strains—*Brevibacterium* sp. TS22 and *Bacillus mycoides* TS27—from Tibetan soil. While many *Bacillus* species have been reported to possess plant growth-promoting functions (Santoyo et al., 2012), few studies have documented such properties in *Brevibacterium* species. Recently, Wang et al. (2016) isolated endophytic *Brevibacterium* from *Kobresia capillifolia* in the Tibetan Plateau and detected multiple plant growth-promoting characteristics, though functional validation was not performed. Meena et al. (2017) isolated *Brevibacterium frigoritolerans* SMA23 from the rhizosphere of *Aloe vera*, which showed multiple PGP traits and positively affected wheat growth at 10 °C. Our evaluation of IAA production, siderophore synthesis, and ACC deaminase activity revealed that both isolated strains could produce IAA, SID, and ACC deaminase. TS22 exhibited high ACC deaminase activity (264.69 nmol $\cdot$ α-KB $mg^{-1} \cdot h^{-1}$), while TS27 showed higher IAA (7.52$±3.85$mg \cdot L^{\{-1\})andSID(92}${-1}$) in 89.47% of 38 isolated strains (Gonita-Mishra et al., 2017). As an efficient phytohormone, high IAA production can induce morphological and physiological changes in seedlings (Masciarelli et al., 2013). Siderophores play an important role in iron bioavail-

ability in plant rhizospheres (Sorty & Shaikh, 2015). Their high affinity for iron in the rhizosphere not only facilitates iron acquisition by plants but also limits iron availability to root pathogens, thereby inhibiting pathogen proliferation (O' Gara, 1992). Bacterial ACC deaminase reduces plant ethylene levels and alleviates various stresses, representing a key component of bacterial mechanisms that protect plants from flooding, drought, salinity, flower senescence, heavy metals, organic pollutants, and bacterial and fungal pathogens (Glick, 2014). The cold-tolerant strains TS22 and TS27 isolated in this study can not only adapt to the cold, high-altitude environment of the Tibetan Plateau but also possess varying degrees of plant growth-promoting potential, providing valuable microbial resources for subsequent vegetation regeneration in northern Tibet.

When TS22 and TS27 were inoculated into the rhizospheres of local forage grasses *Poa annua* and *Elymus sibiricus*, strain TS22 showed significant growth-promoting effects on *Poa annua* but no obvious effects on *Elymus sibiricus*. Strain TS27 showed minimal growth-promoting effects on both grasses. Numerous studies have demonstrated that not all bacteria with PGP traits can positively affect plant growth, as the plant-microbe interaction environment, host plant species, and bacterial strain type all influence growth-promoting efficacy (de-Bashan et al., 2012). Moreover, PGPB-mediated plant growth promotion is a complex process involving synergistic interactions among different growth-promoting traits. Bashan & de-Bashan (2010b) investigated the mechanisms of *Azospirillum brasilense*, a PGPB strain with multiple growth-promoting characteristics, and found that its mode of action is not singular; rather, these mechanisms operate simultaneously or sequentially under specific environmental conditions.

Our pot experiment results further demonstrate that plant growth responses differ depending on plant and bacterial species (). The synergistic pattern of multiple growth-promoting traits exhibited by strain TS22 may be particularly suitable for *Poa annua* growth. These findings underscore the importance of selecting appropriate PGPB for different plant species based on inoculation trial results. Further research is needed to elucidate the mechanisms underlying PGPB-host plant interactions.

In summary, this study successfully isolated and screened cold-tolerant PGPB from the unique environmental conditions of the northern Tibetan Plateau that can effectively promote local forage grass growth. The findings provide valuable microbial resources and an application foundation for plant-microbe technologies in vegetation restoration in the high-altitude, cold regions of northern Tibet.

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Note: Figure translations are in progress. See original paper for figures.