CELLULOLYTIC BACTERIA CAPABLE OF NITROGEN FIXATION IN SALINE-SODIC GRASSLAND SOILS

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Abstract. Cellulolytic plate bacteria are considered to be the major drivers of litter decomposition and play critical roles in soil conditioning and nutrient cycling. With the goal to identify strategies used by soil cellulolytic bacteria to overcome nitrogen limitation characteristic of saline-sodic grasslands, cellulolytic strains were isolated from the soil of such habitats in northeast China and their abilities to fix nitrogen were evaluated. Sequencing of the 16S rRNA gene indicated that *Streptomyces* strains were the most abundant cellulolytic microorganisms in this saline-sodic soil. Most of the isolates exhibited extracellular carboxymethyl cellulase (CMCase) and xylanase activities. Importantly, all the cellulolytic isolates from grassland soils with various vegetation types demonstrated nitrogen-fixing activities ranging from 10.80 to 33.36 nmol $C_2H_4 \cdot h^{-1} \cdot ml^{-1}$. The cellulolytic isolates also exhibited indole-3-acetic (IAA) acid production abilities ranging from 2.40 to 80.69 $\mu g \cdot ml^{-1}$. These findings provided the first evidence that cellulolytic bacteria likely adapted to the low-nitrogen saline-sodic grassland soils with the acquisition of nitrogen-fixing abilities.
Keywords: *cellulolytic microorganisms, saline-sodic soils, phylogenetic analysis, nitrogen-fixing activity, IAA activity*

Introduction

Salinity inhibits plant growth due to the low osmotic potential of the soil solution, ion toxicity and ion imbalance which further reduce nutrient uptake (Rengasamy, 2006; Yan et al., 2013), as well as negatively impacts the turnover of soil organic matter by reducing the activity and quantity of microbial biomass in the soil (Tripathi et al., 2006; Yan et al., 2013). Compared to non-saline soils, microbial activity and biomass in salt-affected soils was lower; yet, they still play effective roles in soil conditioning and nutrient cycling (Yan et al., 2012; Orhan et al., 2015). Evidently, microorganisms have developed some strategies to overcome the stress conditions commonly encountered in saline-sodic soils.

The major impact of salinity on soil microorganisms has resulted in extensive studies on the mechanisms of microbial resistance and adaptation to high salinity. Another important environmental factor for soil microbial community is nitrogen-deficiency in salt-affected soils (Liu et al., 2013). Previous researches revealed that nitrogen fixation was considered as one of the most vital sources of nitrogen in nitrogen-deficient soils (Welsh et al., 1996; Reef et al., 2010). Major types of biological nitrogen fixation include symbiotic and free-living nitrogen fixation in natural ecosystems. The best known

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nitrogen fixers form symbiotic associations with plants, but are limited to select plant groups such as legumes, alders, and cycads (Poly et al., 2001). The free-living nitrogen fixing bacteria are not limited to growth with specific plant taxa, and may, therefore, play widespread roles in ecosystems (Kennedy et al., 2004; Unkovich and Baldock, 2008). These free-living nitrogen-fixing bacteria may be closely involved in the development of soil ecosystems by providing nitrogen supplies and promoting plant growth (Kennedy et al., 2004). In salt-affected habitats, the high nitrogen demand by plant and soil microorganisms may favor the growth of free-living nitrogen-fixing bacteria to convert dinitrogen gas into biologically available N (i.e. ammonia, NH_3). Specifically, some studies have observed the occurrence of nitrogen fixation in organic-matter rich and N-deficient soils (Sistla et al., 2012; Shiau et al., 2017), suggesting that microbial populations involved in soil organic matter decomposition might also have nitrogen-fixing capabilities as a strategy of adaptation to N deficiency.

Soil salinity and alkalinity are the main concerns in many grasslands, especially for the eastern and central of Eurasian steppes (from China, Mongolia, to Kazakhstan and Russia). In Northeast China, large sections of steppes have suffered from deterioration due to alkalization and salinization (Wang et al., 2003). The steppe soil is characterized by higher alkalinity (pH 8.5-11.0) and salinity (salt content > 0.5%), but low nutrient availability (Xin et al., 2012). In this saline-sodic grassland soils, plant litter cellulose, hemicellulose and lignin, were the main source of organic matter (Liu et al., 2013; Trujillo-Cabrera et al., 2013). Decomposer microorganisms are considered to be the major drivers of litter decomposition and play critical roles in soil conditioning and nutrient cycling (Liu et al., 2006; Xin et al., 2012). To break down plant litter, decomposer microorganisms require N-nutrients from the surrounding soils to maintain their activities (Schimel et al., 2003; Shiau et al., 2017). When the soil is N-deficient, it is expected that activities of soil decomposer microorganisms, plant litter decomposition, and nutrient cycling would stop, which would in turn hinder plant biomass production, unless alternative N sources are available (Schimel et al., 2003; Liu et al., 2006). Interestingly, the grassland investigated in this study, with saline-sodic soils, maintained relatively high plant biomass production (about 150-170 $g \cdot m^{-2} \cdot year^{-1}$) (Zhao et al., 2019). Thus, soil microorganisms in this grassland likely possess unique strategies to overcome N-deficiency and sustain litter decomposition and nutrient cycling. Up to now, the decomposer microorganisms in the saline-sodic soil have received increasing attention. However, very little is known about the specific mechanisms that the decomposer microorganisms employ to adapt to N-deficiency frequently encountered in saline-sodic soils in order to sustain nutrient cycling and support primary production in these environments.

For this work, we targeted cellulolytic bacteria as representatives of the soil microbial communities involved in plant litter decomposition and organic matter turnover. Soil cellulolytic bacteria were isolated from low-nitrogen saline-sodic grassland soil in Jilin, China and characterized for activities of cellulolysis and nitrogen fixation. The objective of this study was to understand the potential adaptation strategy of the cellulolytic bacteria to low-nitrogen in saline-sodic soils. We hypothesized that cellulolytic bacteria from the saline-sodic soil with nitrogen-deficiency would evolve nitrogen fixing activities within such adverse environment.

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Materials and methods

Soil samples and isolation of cellulolytic bacteria

Soil samples were collected from a saline-sodic grassland, located at the Grassland Ecological Research Station of Northeast Normal University, Jilin Province, China (44˚ 45′N, 123˚ 45′E). The climate is semi-arid continental with mean annual temperature 4-7°C and annual precipitation 280–400 mm. The soils were mixed saline and alkaline (pH 8.0–11.0). The vegetation was meadow steppe predominated by perennial *Leymus chinensis*, and other common species include grasses such as *Phragmites australis*, *Calamagrostis epigejos* and *Chloris virgata*; forbs such as *Kalimeris integrifllia*., *Suaeda glauca* and *Kochia sieversiana* (*Figure 1*).

Figure 1. *The saline-sodic grassland for collecting soil samples*

The soil samples in the saline-sodic grassland were obtained from *L. chinensis* soil, *C. virgata* soil and weeds soil (dominated by *P. australis, C. epigejos* and *S. baicalensis*) respectively. Soil samples (0–15 cm depth) were randomly collected from five sites in every types of soil, mixed together to form a single composite sample, placed in a sterile glass bottles and transferred to the laboratory in cool boxes. Each sample was separated into two parts: one was used for the isolation of cellulolytic bacteria; while the rest was air-dried to constant weight at room temperature for the determination of a suite of soil chemico-physical properties.

The cellulolytic strains were isolated on CMC-Na (carboxymethyl-cellulose, sodium salt) agar plates, which were prepared with Mandels' nutrient salts medium (Mandels, 1975) with 1% CMC-Na and 2% agar (w/v). After cultured at 30°C for 5 d, the colonies on this CMC agar medium were observed. And based on the size, color and morphology, the different colonies were picked and transferred to fresh CMC agar media plates, and then incubated at 30°C for 120 h. Pure cultures were obtained through repeated streaking on CMC-Na agar plate. After cultivation at 30ºC for 120 h, the plates were stained with 0.1% (w/v) Congo red solution for 10 min, and then washed with 0.1 M NaCl solution on each individual colony (Xu et al., 2015). A clear zone on the culture plate, indicating cellulolytic activity, was used to identify the cellulase-producing strains. The differences of isolates were performed with light microscopy and their sequences of 16S rRNA genes.

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Molecular identification of cellulolytic bacteria

The 16S rRNA genes of the isolates were amplified using a forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and a reverse primer 1492R (5'-TACCTTGTTACGATT-3') in a PCR thermal cycler (Bio-Rad, USA) under the following conditions: 94ºC for 5 min, 35 cycles of 30 sec each at 94ºC, 45 sec at 56ºC, 120 sec at 72ºC, and a final step of 10 min at 72ºC. The PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Germany). The amplified fragments were sent to Comate Bioscience Co. Ltd. (Changchun, China) for sequencing. The obtained sequences were processed following previous described protocols (Zhang et al., 2011). First, sequences with chimeric artifacts were removed by the program Chimera Check at the Ribosomal Database Project (Cole et al., 2003). The surviving 16S rRNA gene sequences were searched for homology using the BLAST program at the National Center for Biotechnology Information (NCBI), aligned with homologous sequences using Clustal X (Thompson et al., 1997), and used for the construction of phylogenetic trees by the neighbor-joining algorithm (1000 bootstrap re-samplings) with Mega-X (Tamura et al., 2007).

Enzymatic assays and IAA producing traits

Each isolate was inoculated into 100 ml of liquid CMC-Na or corn straw media (Mandels' nutrient salts solution 100 ml, 1.0% (w/v) 1-cm long corn straw 5.0 g, pH 8.5). Cultivation was carried in a rotary incubator (120 rpm) at 30°C for 5 d. Subsequently, the cells were separated from the culture medium by centrifugation at 14000 g for 20 min at 4°C. The supernatants were used as the source of the extracellular crude enzyme extract to measure the activities of CMCase (with the CMC-Na medium) and xylanase (with the corn straw medium). CMCase activity was measured by mixing 1 ml of crude enzyme extract with 1 ml of 1% (w/v) low-viscosity CMC-Na solution in 50 mM phosphate buffer (pH 8.5) and incubated at 50°C for 30 min. The reducing sugar generated by CMCase activity was determined using the dinitrosalicylic acid procedure (Miller, 1969). One unit of CMCase activity (IU) was defined as the amount of enzyme releasing 1 μmol of reducing sugars per minute under the assay conditions. Xylanase activity was assayed by the same method with birch wood xylan as the substrate (Sigma-Aldrich Co. Ltd., St. Louis, USA). One unit of xylanase activity (IU) was defined as the amount of enzyme releasing 1 μmol of reducing sugars per minute under the assay conditions.

Nitrogen fixing efficiency of isolates were estimated by using acetylene reduction assay (ARA) (Hardy et al., 1973). All the isolates were grown at 30°C for 48 h in N-free liquid medium. Following incubation, the cotton plugs were replaced with rubber stoppers (subba-seal). 10% of air inside the tubes was evacuated with syringe and replaced by the same amount of pure acetylene gas. These were further incubated at 30°C for 24 h and then reduction of acetylene to ethylene was measured with a gas chromatograph (Agilent 6890 GC, USA) using a flameionization detector. Nitrogenase activity was defined as the amount of ethylene generated (nmol $C_2H_4 \cdot ml^{-1} \cdot h^{-1}$).

The production of IAA, which was considered as a plant growth promoting compound, was determined for all isolates according to the method of Leveau and Lindow (2005). Briefly, an aliquot (1 ml) of the supernatant, obtained after centrifugation of a bacterial culture grown in LB medium (1% tryptone, 1.0% yeast extract and 1.0% NaCl) supplemented with L-tryptophan (0.5 mg/ml), was mixed with 2 ml Salkowski reagent (1 ml 0.5 M $FeCl_3$ in 50 ml 35% $HClO_4$) and 50 μl of 10-mM orthophosphoric acid. The

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reaction mixture was incubated at 25°C in the dark for 30 min. The absorbance of the pink color which developed after incubation was determined at 530 nm.

Analysis of soil chemo-physical properties

Various parameters of the soil samples were determined according to previously described methods. The soil pH and EC were determined using a soil-water ratio of 1:5. The soil organic carbon (SOC) content was measured using the Mebius method and by Walkley-Black acid digestion (Holguin and Bashan, 1996). The total nitrogen content (TN) was determined using an autoanalyzer (Foss 2100, FOSS Kjeltec1) with the Kjeldahl method following vitriol digestion. The total phosphorus (TP) content was measured colorimetrically after P extraction by Na_2CO_3 fusion (Yeomans and Bremner, 1988). Soil available nitrogen (AN) was measured using determination methods as previously described (Zhang et al., 2013).

Results

Soil chemo-physical properties

The chemo-physical properties of the sampling soil were summarized in *Table 1*. Based on the composition of 1:5 soil to water extracts, the electrical conductivity (EC) of surface soil ranged from 0.11 $ms \cdot cm^{-1}$ to 0.25 $ms \cdot cm^{-1}$, their pH ranges from 8.68 to 10.03. The *L. chinensis* soil group had the lowest pH and EC value, and the highest soil pH and EC values were observed in the weeds soil group. The analysis results of soil sample showed that the organic carbon content was 6.0–13.1 $g \cdot kg^{-1}$, total N 0.60-1.41 $g \cdot kg^{-1}$, available nitrogen 0.06–0.12 $g \cdot kg^{-1}$ and total P 4.55–6.24 $mg \cdot kg^{-1}$. The SOC and TN contents were highest in the *L. chinensis* soil and lowest in the weeds soil.

Table 1. *The chemo-physical properties of saline-sodic soil samples*

Soils	pH	Organic carbon ($g \cdot kg^{-1}$)	Total nitrogen ($g \cdot kg^{-1}$)	Available nitrogen ($g \cdot kg^{-1}$)	Total phosphorus ($mg \cdot kg^{-1}$)	Electric conductivity ($ms \cdot cm^{-1}$)
L. chinensis soil	8.68	13.1	1.41	0.09	4.55	0.11
C. virgate soil	9.47	10.9	1.18	0.12	6.24	0.19
Weeds soil	10.03	6.0	0.60	0.06	4.88	0.25

Diversity and abundance of cellulolytic bacteria

A total of 17 cellulolytic isolates were obtained in the *L. chinensis* soil, *C. virgata* soil and weeds soil. Through 16S rDNA gene phylogenetic analysis of the 17 isolates, it was confirmed that 11 strains were classified as *Streptomyces*, two strains as *Microbacteriums*, and one strain each as *Arthrobacters*, *Flavobacterium*, *Nocardioide* and *Lechevalieria* (*Figure 2*). No cellulolytic mold or yeast was isolated. In these isolates, 10 strains were obtained from *L. chinensis* soil (DS-1, DS-5, DS-6, DS-10, DS-13, DS-19, DS-24, DS-26, DS-29, DS-30), 9 strains from *C. virgata* soil (DS-2, DS-3, DS-5, DS-7, DS-10, DS-12, DS-13, DS-14, DS-30) and 4 strains from weed soil (DS-13, DS-18, DS-30, DS-34) respectively. The 16S rDNA fragment sequences of the 17 isolates showed 98–100% similarity to known corresponding strains. Strains DS-5 (*Streptomyces pratensis*), DS-10

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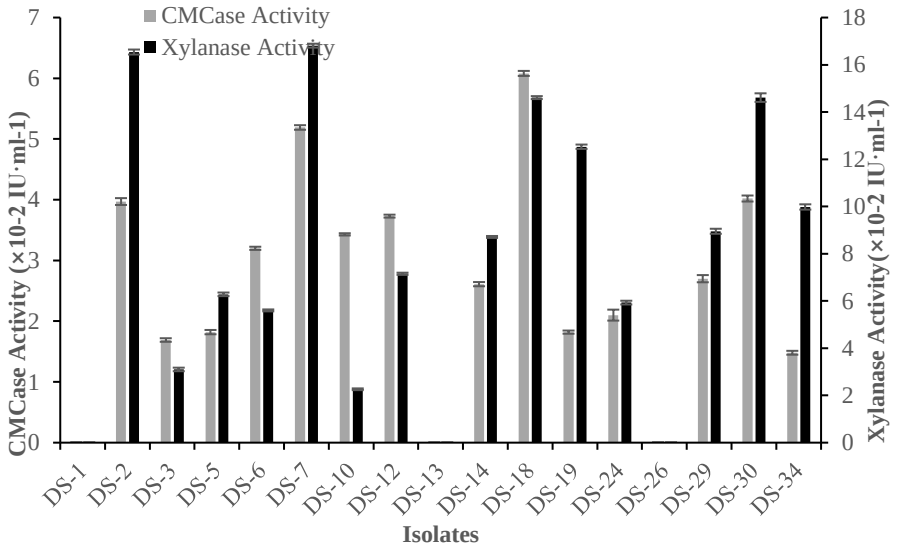
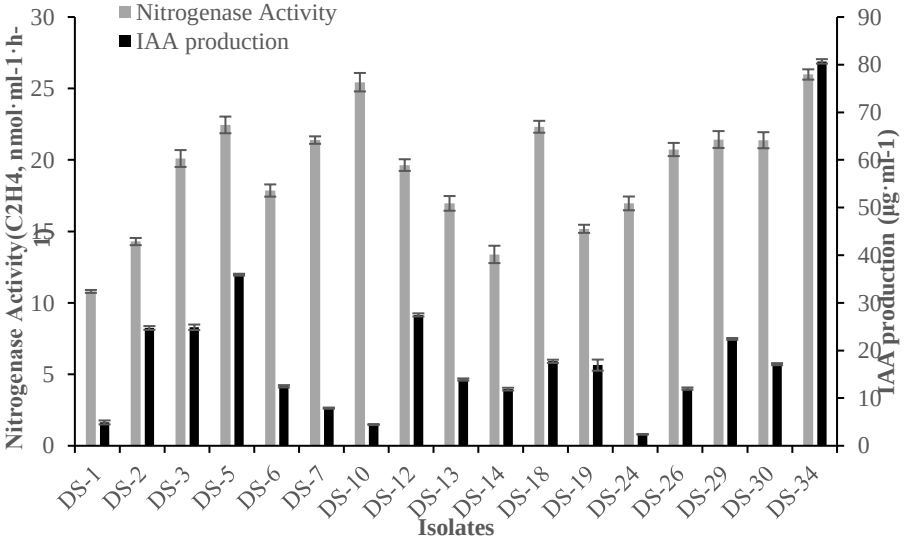
(*Arthrobacter aurescens*), DS-13 (*Streptomyces toxytricini*), DS-30 (*Streptomyces massasporeus*) were obtained from both the *L. chinensis* soil and *C. virgata* soil. Strains DS-13 and DS-30 were also obtained from weed soil.



A



B

Figure 2. *Enzymatic assays and IAA production of the cellulolytic isolates. A: The CMCase and Xylanase activity measured by the isolates. B: The Nitrogenase activity and IAA production measured by the isolates. The reported values correspond to the mean value from three independent experiments in triplicate and the error bars to one standard deviation from the mean value*

The abundance of total cultivable bacteria and cellulolytic bacteria is presented in *Figure 3*. The abundance of total bacteria ($1.44–1.78\times10^7$CFU·g^{-1}) and cellulolytic bacteria ($1.6–2.3\times10^6$CFU·g^{-1}) was similar in different vegetation's soils (*Figure 3*). Based on the morphology and 16S rDNA gene phylogenetic analysis, we found that the *Streptomyces* strains were high abundant cellulolytic microorganisms in the grassland soil

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of this study (accounting for more than 71% abundance in the *L. chinensis* soil, 71% abundance in the *C. virgata* soil and 86% abundance in the weed soil respectively). Especially, 2 *Streptomyces* strains DS-13 (*Streptomyces toxytricini*) and DS-30 (*Streptomyces iakyrus*) were obtained from the *L. chinensis* soil, *C. virgata* soil and weed soil.

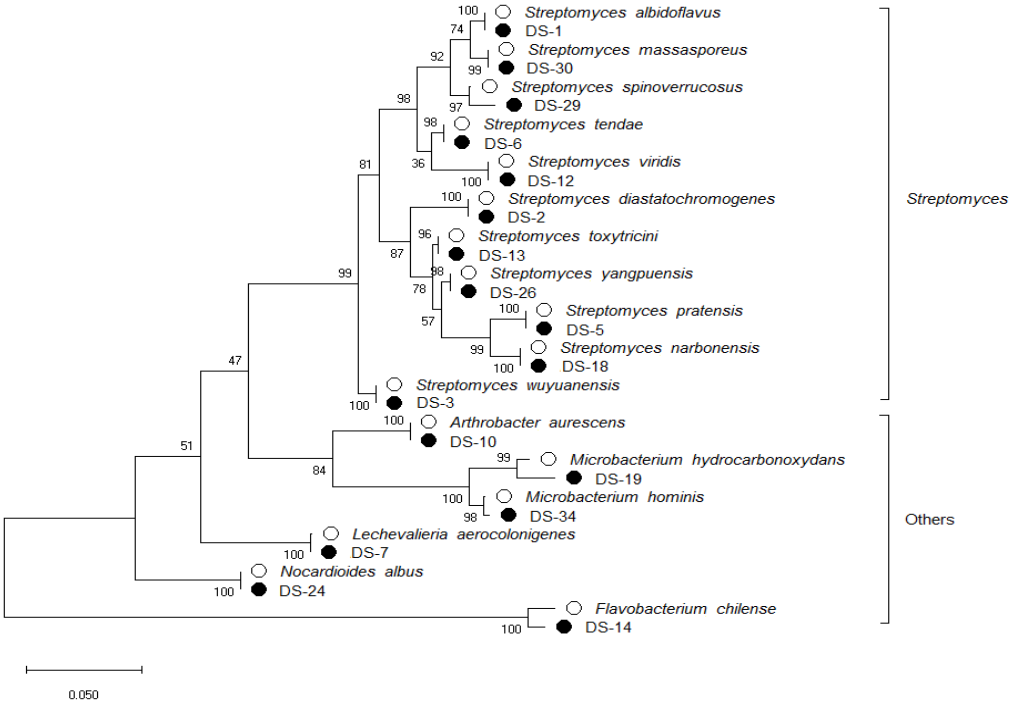

Figure 3. *Maximum Likelihood phylogenetic tree showing the relationships of partial 16S rRNA gene sequences cloned from saline-sodic grassland soil with closely related strains. The numerical values at branch nodes indicate bootstrap values per 1000 re-samplings. The scale bars indicate the number of nucleotide substitutions per sequence position. ●: isolates characterized in the study; ○: known strains closely related to the isolates identified in this study*

Enzymatic activities and IAA producing traits

After being cultured in the Mandel nutrient salts medium supplemented with CMC, all the isolates showed higher extracellular CMCase activity in the CMC media, except for the strains DS-1, DS-13 and DS-26 in which cellulolytic enzymes might distribute on the cell surface by attaching to or being inserted in the cell wall (*Figure 4*). The highest extracellular CMCase activity (6.08×10^{-2} IU·ml^{-1}) was observed for isolate DS-18 (*Streptomyces zaomyceticus*) in CMC media. Compared with CMCase activity, we found that most isolates showed considerably higher extracellular xylanase activity. When the cellulolytic isolates were inoculated on nitrogen-free CMC agar plate, all the isolates were able to develop colonies after three days. Biological nitrogenase activity of these isolates ranged from 10.80 to 25.98 nmol $C_2H_4 \cdot ml^{-1} \cdot h^{-1}$ (*Figure 4*). All the cellulolytic isolates exhibited IAA producing abilities ranging from 2.40 to 80.69 μg·ml^{-1} (*Figure 4B*). The same strains isolated from different vegetation's grassland soils had similar enzymic activities (including CMCase, xylanase and nitrogenase) and IAA producing activities.

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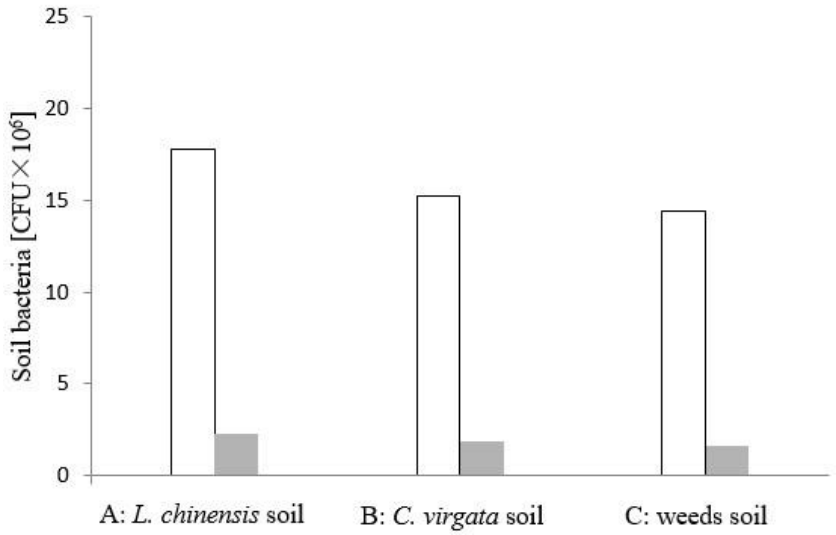

Figure 4. *Colony-forming units of culturable bacteria and cellulolytic soil bacteria in grassland soil. White bars, the abundance of total bacteria; light gray bars, the abundance of cellulolytic bacteria*

Discussion

Abundance and diversity of cellulolytic isolates

For this study, a traditional culture-dependent approach was used because the cellulolytic microbial community is very diverse phylogenetically and the cellulolytic enzymes are also very diverse (Trujillo-Cabrera et al., 2013). Our results showed that the cellulolytic isolates belonged to the Genus *Streptomyces*, *Microbacteriums*, *Arthrobacters*, *Flavobacterium*, *Nocardioide* and *Lechevalieria*, and cellulolytic *Streptomyces* strains (*Figure 2*) were the high abundant cellulolytic microorganisms in this saline-sodic grassland soil.

In nature, actionobacteria are known to occur not only in normal environments, but also in extreme environments, which are characterized by acidic/alkaline pH, salinity, low levels of available moisture, and nutrients (Zenova et al., 2011). As the represented genera of actinomycetes, the *Streptomyces* bacteria with the largest number of species and varieties are ubiquitous, are well adapted to the life in the soil where they grow by tip extension and branch through the soil searching for nutrients. *Streptomyces* bacteria can secrete large numbers of enzymes that break down insoluble organic polymers, including chitin and cellulose, into substituent sugars for binding and uptake by multiple ABC transporters (Chater et al., 2010; Thompson et al., 2010). These traits of the *Streptomyces* stains confer a competitive advantage over many other bacteria. In this study, the abundance of cellulolytic bacteria ($1.6–2.3\times10^6$ CFU·g^{-1}) was similar in different vegetation's soils (*Figure 3*), and the *Streptomyces* strains were high abundant cellulolytic microorganisms. To date, several studies on the diversity of cellulolytic bacteria isolated from different soils or environments have been performed. Ulrich et al. found that the cellulolytic community composition was dominated by *Streptomyces* (67%) in the silty sand soil and loam soil (Ulrich et al., 2008). Schlatter et al. (2009) revealed similar result that the addition of cellulose or lignin led to the high *Streptomyces* densities in a native soil. In our studied soils, plant litter cellulose, hemicellulose and lignin, were the main

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source of organic matter, and the cellulolytic bacterial community was clearly highest abundance by *Streptomyce*. Especially, 2 *Streptomyces*, strains DS-13 (*Streptomyces toxytricini*) and DS-30 (*Streptomyces iakyrus*), were obtained from the *L. chinensis* soil, *C. virgata* soil and weed soil. Our results suggested that the cellulolytic *Streptomyces* strains were likely an ecologically significant cellulolytic group, which play a vital litter decomposing role in the soil.

Nitrogen fixing characteristics of cellulolytic isolates

In saline-sodic soils, soil salinity affects the soil chemico-physical properties and soil nutrient availability. Our analysis of soil chemico-physical properties revealed that the saline-sodic soil in this study was saline-alkaline, severely oligotrophic, especially low total nitrogen (0.60–1.41 $g \cdot kg^{-1}$) and available nitrogen (0.06–0.12 $g \cdot kg^{-1}$) (*Figure 4*). Notably, when the cellulolytic isolates from these soils were inoculated on nitrogen-free CMC agar plate, all the isolates were able to develop colonies after three days. Biological nitrogenase activity of these isolates ranged from 10.80 to 25.98 nmol $C_2H_4 \cdot ml^{-1} \cdot h^{-1}$ (*Figure 4*). In soils, soil decomposer microorganisms play a vital role in soil structure maintenance, organic matter decomposition, biogeochemical cycling, and plant nutrient availability. Cellulose is the most abundant polymers in plant litter and cellulolytic microorganisms are considered to be the major drivers of litter decomposition in the saline-sodic soils (Liu et al., 2013; Trujillo-Cabrera et al., 2013). Soil nutrient availability has long been suggested as one of the most important controlling factors affecting the rate of litter decomposition, because decomposers (microorganisms) require nutrients to maintain their activities (Swift et al., 1979; Sinsabaugh et al., 1993). The results in this study showed that the saline-sodic soil was low-nitrogen soil (total N 0.60–1.41 $g \cdot kg^{-1}$) (*Table 1*). In such soil, the decomposers, i.e. cellulolytic microorganisms, are likely to develop strategies to overcome low-nitrogen and sustain cellulolytic activities.

Especially, members of the genus *Streptomyces* were higher abundant nitrogen fixing cellulolytic microorganisms in the grassland soil. Nitrogen fixing genes in actinobacteria have been presumed to have a narrow distribution, mostly restricted to the genus Frankia (Gtari et al., 2012). So far, only one nitrogen fixing heterotrophic *Streptomyces* strain from the arid soils in South Dakota Badlands is documented (Dahal et al., 2017). This is the first known report that cellulolytic *Streptomyces* strains in saline-sodic soils resisted the nitrogen-lacking environment, maintained their cellulolytic process and triggered the recycling of nutrients by the strategy of free nitrogen-fixing.

Nitrogen is the main growth-limiting nutrient for plants and microorganisms. Therefore, bacteria with N-fixation capabilities have a competitive advantage in N-deficient soils (Kennedy et al., 2004). In this study, all the cellulolytic isolates were cellulolytic nitrogen fixing bacteria, which not only decomposed cellulose in saline-sodic condition, but also exhibited nitrogen-fixation activities ranging from 10.80 to 25.98 nmol $C_2H_4 \cdot h^{-1} \cdot ml^{-1}$ (*Figure 4B*). This is most likely a typical adaptive and evolutionary paradigm that soil cellulolytic microorganisms adopted to low-nitrogen in saline-sodic environment. Previous studies also showed that the coastal saline soils can provide conducive conditions for N_2 fixation (Sengupta and Chaudhuri, 1991), and N_2 fixation can be a significant source of nitrogen in the coastal saline habitats (Welsh et al., 1996; Reef et al., 2010). In our studied soils, the cellulolytic bacteria meet their nitrogen requirements for cellulolytic processes by nitrogen fixation when growing in a cellulose-rich and low-nitrogen environment. All the isolates displayed detectable acetylene reduction activity with the highest activity reaching 25.98 nmol $C_2H_4 \cdot h^{-1} \cdot ml^{-1}$ (*Figure 4B*),

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in agreement with those of several earlier studies on nitrogen fixing bacteria, signifying their ability to grow on low-nitrogen habitats (Sengupta and Chaudhuri, 1991; Unkovich and Baldock, 2008). Meanwhile, cellulolytic bacteria are considered to be the major drivers of litter decomposition and play critical roles in soil conditioning and nutrient cycling. In this sense, the cellulolytic nitrogen fixing bacteria is "the impeller and operator" of the nutrients recycling in low-nitrogen saline-sodic ecosystems. So far, only a few reports were documented on cellulolytic nitrogen-fixing bacteria. Waterbury and coworkers showed that cellulose served as the growth substrate for a nitrogen-fixing aerobic bacterium that existed in symbiotic relation with shipworms (Waterbury et al., 1983). Leschine et al. (1988) also reported four trains of nitrogen-fixing, cellulose-fermenting bacteria from freshwater mud and soil. These studies agree with our result that the nitrogen-fixing cellulolytic bacteria could hold competitive advantages in a situation of cellulose-rich and N-deficiency.

In addition, production of IAA has been implicated in virtually all aspects of plant growth and development (Teale et al., 2006). Many microorganisms including bacteria and fungi are able to synthesize IAA (Teale et al., 2006). In this study, IAA production in cultures amended with tryptophan was detected in all isolated strains, and the amount produced was dependent upon bacterial isolates. Variation among isolates was found not only among species but also within species, such as the case of *Streptomyces* isolates. The 11 *Streptomyces* isolates showed significantly different quantitative IAA production (*Figure 4B*). In conclusion, all the isolated cellulolytic bacteria exhibited the ability to produce IAA (*Figure 4B*), meaning that synthesis of phytohormones by these cellulolytic bacteria likely played an important role for plant growth in saline-sodic soils. This IAA producing characteristic is likely a typical adaptive paradigm that soil cellulolytic microorganisms respond to oligotrophic saline-sodic environment.

Conclusions

In this study, we isolated unique cellulolytic bacteria capable of nitrogen-fixation from low-nitrogen saline-sodic soils, with higher abundant cellulolytic isolates closely related to *Streptomyces*. These cellulolytic isolates appeared to exhibit nitrogen fixing characteristic. This is the first known report that cellulolytic bacteria in low-nitrogen soil environment exhibited nitrogen-fixing activities as a potential strategy for adaptation to nitrogen limitation, providing a new insight into potential mechanisms adopted by the microbial community to sustain ecological functions in adverse soil environments. The IAA producing characteristics of all isolates meant that these cellulolytic bacteria likely played an important role for plant growth in saline-sodic grassland.

In our studied saline-sodic soils, the cellulolytic bacteria meet their nitrogen requirements for cellulolytic process by nitrogen fixation. As the vast amounts of cellulose are available in different environments (such as grassland soils, crop field soils. forest soils, and composts), the cellulolytic bacteria with nitrogen-fixing characteristics have obvious competitive advantage over other soil cellulolytic bacteria. So it is possible that the similar adapting mechanisms and specificities for cellulolytic bacteria are widely existing strategies in other cellulose-rich habitats. Furthermore, these cellulolytic nitrogen-fixing bacteria may have a potential use in improving soil fertility and promoting plant growth.

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REFERENCES

[1] Chater, K. F., Biró, S., Lee, K. J., Palmer, T., Schrempf, H. (2010): The complex extracellular biology of Streptomyces. – FEMS Microbiol Rev. 34: 171-198.
[2] Cole, J. R., Chai, B., Marsh, T. L., Farris, R. J., Wang, Q., Kulam, S. A., Chandra, S., McGarrell, D. M., Schmidt, T. M., Garrity, G. M., Tiedje, J. M. (2003): The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. – Nucleic Acids Res. 31: 442-443.
[3] Dahal, B., Nandakafle, G., Perkins, L., Brözel, V. S. (2017): Diversity of free-Living nitrogen fixing Streptomyces in soils of the badlands of South Dakota. – Microbiol Res. 195: 31-39.
[4] Gtari, M., Ghodhbane-Gtari, F., Nouioui, I., Beauchemin, N., Tisa, L. (2012): Phylogenetic perspectives of nitrogen-fixing actinobacteria. – Arch Microbiol. 194: 3-11.
[5] Hardy, R. W. F., Burns, R. C., Holsten, R. D. (1973): Applications of acetylene-ethylene assay for measurement of nitrogen fixation. – Soil. Biol. Biochem. 5: 47-81.
[6] Holguin, G., Bashan, Y. (1996): Nitrogen-fixation by Azospirillum brasilense CD is promoted when co-cultured with a mangrove rhizosphere bacterium (Staphylococcus sp.). – Soil Biol. Biochem. 28: 1651-1660.
[7] Kennedy, I. R., Choudhury, A. T. M. A., Kecskés, M. L. (2004): Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? – Soil Biol Biochem. 36(8): 1229-1244.
[8] Leveau, J. H. J., Lindow, S. E. (2005): Utilization of the plant hormone indole-3-acetic acid for growth by Pseudomonas putida strain 1290. – Appl. Environ Microbiol. 71(5): 2365-2371.
[9] Liu, P., Huang, J., Han, X., Sun, O. J., Zhou, Z. (2006): Differential responses of litter decomposition to increased soil nutrients and water between two contrasting grassland plant species of Inner Mongolia, China. – Appl. Soil Ecol. 34: 266-275.
[10] Liu, S. H., Kang, Y. H., Wan, S. Q., Jiang, S. F., Liu, S. P., Sun, J. X. (2013): Effect of drip irrigation on soil nutrients changes of saline-sodic soils in the Songnen plain. – Paddy Water Environ. 11: 603-610.
[11] Mandels, M. (1975): Microbial sources of cellulase. – Biotechnol. Bioeng. Symp. 5: 81-105.
[12] Miller, G. L. (1969): Use of dinitrosalicylic acid reagent for determination of reducing sugar. – Anal Chem. 31: 426-428.
[13] Orhan, F., Gulluce, M. (2015): Isolation and characterization of salt-tolerant bacterial strains in salt-affected soils of Erzurum, Turkey. – Geomicrobiol. J. 32: 521-529.
[14] Poly, F., Ranjard, L., Nazaret, S., Gourbière, F., Monrozier, L. J. (2001): Comparison of nifH gene pools in soils and soil microenvironments with contrasting properties. – Appl. Environ. Microb. 67: 2255-2262.
[15] Reef, R., Feller, I. C., Lovelock, C. E. (2010): Nutrition of mangroves. – Tree Physiol. 30: 1148-1160.
[16] Rengasamy, P. (2006): World salinization with emphasis on Australia. – J. Exp. Bot. 57: 1017-1023.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1902_867879

[17] Schimel, J. P., Weintraub, M. N. (2003): The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. – Soil Biol. Biochem. 35: 549-563.
[18] Schlatter, D., Fubuh, A., Xiao, K., Hernandez, D., Hobbie, S., Kinkel, L. (2009): Resource amendments influence density and competitive phenotypes of Streptomyces in soil. – Microb. Ecol. 57: 413-420.
[19] Sengupta, A., Chaudhuri, S. (1991): Ecology of heterotrophic dinitrogen fixation in the rhizosphere of mangrove plant community at the Ganges river estuary in India. – Oecologia 87: 560-564.
[20] Shiau, Y. J., Lin, M. F., Tan, C. C., Tian, G. L., Chiu, C. Y. (2017): Assessing N2 fixation in estuarine mangrove soils. – Estuar Coast Shelf S. 189: 84-89.
[21] Sinsabaugh, R. L., Antibus, R. K., Linkins, A. E., McClaugherty, C. A., Rayburn, L., Repert, D., Weiland, T. (1993): Wood decomposition: nitrogen and phosphorus dynamics in relation to extra cellular enzyme activity. – Ecology 74: 1586-1593.
[22] Sistla, S. A., Asao, S., Schimel, J. P. (2012): Detecting microbial N-limitation in tussock tundra soil: implications for arctic soil organic carbon cycling. – Soil Boil. Biochem. 55: 78-84.
[23] Swift, M. J., Heal, O. W., Anderson, J. M. (1979): Decomposition in terrestrial ecosystems. – Berkeley: University of California Press.
[24] Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007): MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. – Mol. Biol. Evol. 24: 1596-1599.
[25] Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., Higgins, D. G. (1997): The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. – Nucleic Acids Res. 25: 4876-4882.
[26] Thompson, B. J., Widdick, D. A., Hicks, M. G., Chandra, G., Sutcliffe, I. C., Palmer, T., Hutchings, M. I. (2010): Investigating lipoprotein biogenesis and function in the model Gram-positive bacterium Streptomyces coelicolor. – Mol Microbiol. 77(4): 943-956.
[27] Tripathi, S., Kumari, S., Chakraborty, A., Gupta, A., Chakrabarti, K., Byapadhyay, B. K. (2006): Microbial biomass and its activities in salt-affected coastal soils. – Biol. Fert. Soils 42: 273-277.
[28] Trujillo-Cabrera, Y., Ponce-Mendoza, A., Vásquez-Murrieta, M. S., Rivera-Orduña, F. N., Wang, E. T. (2013): Diverse cellulolytic bacteria isolated from the high humus, alkaline-saline chinampa soils. – Ann Microbiol. 63: 779-792.
[29] Ulrich, A., Klimke, G., Wirth, S. (2008): Diversity and activity of cellulose- decomposing bacteria, isolated from a sandy and a loamy soil after long-term manure application. – Microb. Ecol. 55(3): 512-522.
[30] Unkovich, M., Baldock, J. (2008): Measurement of asymbiotic N2 fixation in Australian agriculture. – Soil Biol. Biochem. 40: 2915-2921.
[31] Wang, Z. C., Li, Q. S., Li, X. J., Song, C. C., Zhang, G. X. (2003): Sustainable agriculture development in saline-alkali soil area of Songnen plain, northeast China. – Chin. Geogr. Sci. 13: 171-174.
[32] Waterbury, J. B., Calloway, C. B., Turner, R. D. (1983): A cellulolytic nitrogen-fixing bacterium cultured from the gland of deshayes in shipworms (bivalvia: teredinidae). – Science 221: 1401-1403.
[33] Welsh, D. T., Bourgues, S., de Wit, R., Herbert, R. A. (1996): Seasonal variations in nitrogen-fixation (acetylene reduction) and sulphate-reduction rates in the rhizosphere of Zostera noltii: nitrogen fixation by sulphate reducing bacteria. – Mar. Biol. 125: 619-628.
[34] Xin, W. D., Yin, X. Q., Song, B. (2012): Contribution of soil fauna to litter decomposition in Songnen sandy lands in northeastern China. – J. Arid Environ. 77: 90-95.
[35] Xu, J. X., Wang, X. F., Hu, L., Zhen, J. X., Xu, W. N., Dai, B. L., Wu, B. (2015): A novel ionic liquid-tolerant Fusarium oxysporum BN secreting ionic liquid-stable cellulase: Consolidated bioprocessing of pretreated lignocellulose containing residual ionic liquid. – Bioresour Technol. 181: 18-25.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1902_867879

[36] Yan, N., Marschner, P. (2012): Response of microbial activity and biomass to increasing salinity depends on the final salinity, not the original salinity. – Soil Biol. Biochem. 53: 50-55.
[37] Yan, N., Marschner, P. (2013): Response of soil respiration and microbial biomass to changing EC in saline soils. – Soil Biol. Biochem. 65: 322-328.
[38] Zenova, G. M., Manucharova, N. A., Zvyagintsev, D. G. (2011): Extremophilic and extremotolerant actinomycetes in different soil types. – Eurasian Soil Sci. 44: 417-436.
[39] Zhang, Y., Zamudio Cañas, E. M., Zhu, Z. E., Linville, J. L., Chen, S., He, Q. (2011): Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. – Bioresour Technol. 102: 779-785.
[40] Zhang, N. Y., Guo, R., Song, P., Guo, J. X., Gao, Y. Z. (2013): Effects of warming and nitrogen deposition on the coupling mechanism between soil nitrogen and phosphorus in Songnen Meadow Steppe, northeastern China. – Soil Biol. Biochem 65: 96-104.
[41] Zhao, Y. N., Yang, B., Li, M. X., Xiao, R. Q., Rao, K. Y., Wang, J. Q., Zhang, T., Guo, J. X. (2019): Community composition, structure and productivity in response to nitrogen and phosphorus additions in a temperate meadow. – Sci. Total. Environ. 654: 863-871.

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