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Fixation of carbon dioxide by chemoautotrophic bacteria in grassland soil under dark conditions

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ABSTRACT

Grassland is one of the most important terrestrial ecosystems for carbon (C) and nitrogen (N) cycling. However, while CO_2 fixation by phototrophic bacteria is relatively well studied, little is known about microbial CO_2 fixation without light by chemoautotrophic bacteria in grassland soils. Therefore, in this study, the isotope ^{14}C-CO_2 was used to investigate the CO_2-fixing process in grassland soils. Soil samples were collected from both fenced and adjacent continuous grazing grassland sites in Inner Mongolia and then incubated for 120 days under dark conditions. Meanwhile, the *cbbL* genes (red- and green-like) were analyzed to isolate chemoautotrophic bacteria, which are responsible for CO_2 fixation. After incubation, ^{14}C was fixed into soil organic carbon (^{14}C-SOC) and microbial biomass carbon (^{14}C-MBC) were found in both the fenced and grazing soils, and the fixation rate of ^{14}C-SOC in the fenced soils (48.55‰) was significantly higher than in the grazing soils (22.11‰). The fixation rate of ^{14}C-MBC in the fenced soils (14.05‰) was higher than in the grazing soils (7.08‰), but the difference was not significant. The red-like *cbbL* genes could be detected in all the soil samples, but the green-like *cbbL* genes could not be amplified. A greater number of identified operational taxonomic units were observed in the fenced soils compared with the grazing soils. The chemoautotrophic bacteria were mainly affiliated with Alphaproteobacteria and Actinobacteria. However, Chloroflexi was detected in only the fenced soils. The results suggested that CO_2 fixation by chemoautotrophic bacteria might be significant in carbon cycling in grassland.

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Atmospheric CO_2 fixation; *cbbL* gene; chemoautotrophic bacteria; grassland soil; RuBisCO gene

Introduction

Grassland ecosystems constitute about 40% of the global land area and play a significant role in the global terrestrial C cycle (Wang & Fang 2009). Due to the relatively high C sequestration rates (mainly associated with below-ground C pools) and extensive area, grasslands are recognized for their great potential as net sink for atmospheric CO_2 and climate change regulator (Follett & Reed 2010). Marginal changes in soil C sequestration rates in grasslands can have significant impacts on atmospheric CO_2 concentrations (Follett & Reed 2010). Globally, soils contain 1500 Pg C, which is twice the amount of atmospheric C pool (Schlesinger & Andrews 2000), with grasslands containing ~12% of the overall terrestrial soil C pool (Schlesinger 1977). However, grassland management can alter the quantity and quality of litter inputs and, subsequently, impact the amounts and stability of C stored in the soil (Dubeux et al. 2006). Conversely, adoption of improved grassland management practices such as proper grazing and soil nutrient management can also have positive impacts on soil C sequestration (Dubeux et al. 2006).

CO_2 is a greenhouse gas which is with the potential threat of global climate change. The concentration of atmospheric CO_2 dramatically increase from about 280 to more than 380 parts per million (ppm) over the last 250 years because of the consumption of fossil fuels. And it will rise up to 570 ppm in the atmosphere by the year 2100 predicted by the International Panel on Climate Change (Song 2006). Nowadays, it is an urgent need to reduce atmospheric CO_2 concentration and slow down the effects of global warming. Now many techniques (physical fixation, chemical fixation and biofixation of CO_2) are developed to alleviate atmospheric CO_2, of which the biofixation of CO_2 by microorganisms was considered an economical, effective and sanitary method. Microbial biofixation of CO_2 employs the capacity of autotrophic microorganisms, including photoautotrophs and chemoautotrophs, for CO_2 fixation (Yousuf et al. 2012), which are known to contribute

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significantly to CO_2 assimilation in aquatic environment. Photoautotrophs are capable of synthesizing their own food from inorganic substances by using light as an energy source. And the chemoautotrophs can support carbon fixation in the absence of light by using the energy from oxidizing H_2, H_2S, $S_2O_3^{2-}$,NH_4^+, NO_2 and Fe^{2+} (Santoro et al. 2013). Photoautotrophs (e.g. microalgae and cyanobacteria) can grow much faster than terrestrial plants and live in harsh conditions due to their structure. Microalgal cells absorb CO_2 to support their growth by converting solar energy into biomass, which can be converted to secondary products with high commercial value (Beer et al. 2009). In addition, microalgae are predominant carbon-fixing agents in water with efficiency being 10 times greater than that of terrestrial plants (Usui & Ikenouchi 1997). Like algae, cyanobacteria are also efficient at the carbon fixation owing to their simpler structure (Oliver et al. 2014). They are considered to play a key role among photosynthetic organisms, accounting for 20–30% of Earth's primary photosynthetic activity (Pisciotta et al. 2010).

In general, CO_2 can be fixed by five different pathways (Herter et al. 2002; Fan 2008), but the Calvin–Benson–Bassham (CBB) cycle is the major and most widely distributed pathway using autotrophic microorganisms to fix CO_2 (Yuan, Ge, Chen et al. 2012). The key enzyme of the CBB cycle is the ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) (Ellis 1979). It is a bifunctional enzyme controlling the reduction of CO_2 and the oxygenolysis of ribulose-1,5-bisphophate. RubisCO exists in four forms (I, II, III and IV), which differ in structure. And the most distributed type of RubisCO is the form I, which occurs in plants as well as in autotrophic (photoautotrophic and chemoautotrophic) bacteria (Selesi et al. 2005). The form I RubisCO is composed of eight large and eight small subunits (L8S8) (Tabita 1999). Phylogenetic studies based on these *cbbL* sequences have shown that form I RubisCO had to be subdivided into two major groups: green-like and red-like (Watson & Tabita 1997). The green-like RubisCO is divided into two types, IA and IB, and found *cbbL* sequences from plants, green algae, and Alpha-, Beta- and Gammaproteobacteria as well as from Cyanobacteria. The red-like RubisCO is also divided into two types, IC and ID, which is found in many non-green algea and Alpha-, and Betaproteobacteria (Videmšek et al. 2009).

Microbial assimilation of CO_2 is a ubiquitous process in soils. Global estimates of microbial CO_2 fixation in soils range between 0.6 and 4.9 Pg C $year^{-1}$ (Yuan, Ge, Chen et al. 2012). Nowak et al. (2015) have estimated that in the wetland soils up to 27% of soil organic matter (SOC) in the 0–10 cm layer was derived from autotrophic (photo- and chemoautotrophic) microbial fixation of CO_2. The highest CO_2 fixation by autotrophic microorganisms is found in the 0–1 cm layer and ^{14}C labeled SOC concentration in the paddy soils is higher in both 0–1 and 1–5 cm layers than those in the upland soils after incubated with continuous $^{14}CO_2$ for 110 days (Wu et al. 2014). In addition, chemoautotrophic acetogenes can assimilate CO_2 with exogenous H_2 in rice field soil incubated at 50°C (Liu & Conrad 2011). High abundance of autotrophic CO_2 fixation bacteria is shown in arid soil in northwest China and in grassland soils near natural springs with high CO_2 concentration (Videmšek et al. 2009). However, only limited knowledge is available on the CO_2 fixation by chemoautotrophic bacteria in grassland soils under the dark condition. The aims of this study were (i) to test the ability of CO_2 fixation by grassland soils; (ii) to compare the CO_2 fixation rate between the fenced and grazing grassland soils and (iii) to determine the composition of these *cbbL* types (green- and red-like) in soil bacteria isolated from both the fenced and grazing grassland soils in a semi-arid region in Inner Mongolia, China.

Materials and methods

Study sites and soil sampling

The study area is located in Baarin Right Banner of Chifeng (43°12′55″–44°27′52″ N, 118°12′09″–120°01′42″ E) in the southeastern part of Inner Mongolia. This area has a temperate, semi-arid continental monsoon climate with dry and windy winters and springs, and hot, humid summers followed by short and cool autumns. The mean annual precipitation is around 360 mm, with 60–70% of the rainfall from June to September. The annual mean temperature is around 4.9°C, with a minimum monthly mean temperature of −13°C in January and a maximum of 22.2°C in July. The annual frost-free period is approximately 125 days. From north to south it stretches 139 km, while from east to west it stretches 154 km. Elevations decrease from a high of more than 1000 m in the northwest to less than 400 m in the southeast.

All field sampling was carried out in early August 2014. A continuously grazed grassland site (GG) and an adjacent fenced grassland site (FG) with grazing exclusion of more than 20 years were selected. Five random quadrats were established at each site for soil research. One soil sample was taken from five points in each quadrat (four corners and the center of the quadrat) at depths of 0–5 cm and mixed into one sample. After carefully removing the surface organic materials and fine roots, each mixed sample was divided into two parts. One part was air-dried for analysis of soil physicochemical properties and the other was sifted through a

Table 1. Description of plant characteristics of the study sites.

Sampling site	Species number	Coverage (%)	Aboveground biomass (g m^{-2})	Shannon–Wiener Diversity Index	Dominant species
Grazing site	19	68.3 ± 7.6	156.7 ± 8.3	1.43 ± 0.27	*Carex duriuscula*; *Cleistogenes squarrosa*
Fenced site	22	91.7 ± 2.9	965.0 ± 54.1	1.16 ± 0.47	*Leymus chinensis*

2 mm sieve for microbial assays and stored at −20°C. Three aboveground plant sampling quadrats (1.0 × 1.0 m) were set up in both the grazed and fenced grassland sites. After botanical composition, plant height and canopy cover were measured, the remaining biomass in each quadrat was clipped and weighed after being dried at 80°C for 48 h. The characteristics of the plant community in this study site are summarized in Table 1.

Incubation experiments with labelled CO_2

Two sets of 25 g fresh soil of each sample was added to glass bottles (5.5 cm diameter and 11 cm height) and placed into containers (30 cm diameter and 17 cm depth). In one set as a control, the soils were fumigated under a vacuum with $CHCl_3$ for 24 h. In another set, the soil samples were just added to a bottle without any treatment. In each container, one bottle was filled with sterile distilled water to maintain the moisture level in the container, one bottle contained equal $NaH^{14}CO_3$ (specific activity 310.8 MBq $mmol^{-1}$, 2.31 MBq) and the lid was closed after HCl (1 M) was added to the $NaH^{14}CO_3$. All the containers were then incubated for 120 days at 20°C in a plant growth chamber (LH-100RD; NKS, Tokyo, Japan) under dark conditions. After incubation, each soil sample was then mixed thoroughly and divided into two portions. One portion was oven-dried at 70°C to a constant weight to determine the fixation rate of ^{14}C-SOC fixed from ^{14}C-CO_2 in the air, and the other was stored at 4°C to determine ^{14}C-MBC.

Analytical methods

Soil pH was measured using a pH meter (F-21; Horiba, Japan) at a soil-to-water ratio of 1:5 after shaking for 1 h. Electrical conductivity (EC) was measured after the pH measurement using an EC meter (CM-14P; TOA Electronics Ltd., Japan). Soil moisture was measured after being oven-dried at 105°C for 24 h. Particle size distribution was determined using the pipette method (Gee & Bauder 1986). Part of each sample was air-dried and finely ground to pass through a 0.1 mm sieve and analyzed for total N (TN) using the dry combustion method with an NC-Analyzer (Sumigraph Nc-80; Sumika Chemical Analysis Service Co., Tokyo, Japan). Soil organic carbon (C_{org}) was determined using the Walkley and Black method (Walkley 1947). The available phosphorus (Avail. P) was determined using the Bray II method (Kuo 1996), where soil samples were extracted with an extraction solution (1 M NH_4F and 0.5 M HCl) and color-developing reagent was added. The Avail. P was then determined using the absorbance measurement with a spectrophotometer at a wavelength of 710 nm (UV-142-02; Shimadzu, Kyoto, Japan). Exchangeable cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) were extracted three times with 1 M ammonium acetate at pH 7.0, and the concentration was measured using an atomic absorption spectrophotometer (AA-6800; Shimadzu, Kyoto, Japan). Microbial biomass C (C_{mic}) and N (N_{mic}) was estimated using the fumigation extraction method (Tanaka et al. 1998).

The ^{14}C-SOC was measured according to Ge et al. (2013). In brief, about 0.50 g of soil (dry soil) was added to 10 mL 0.2 M potassium dichromate and 20 mL of a mixture of concentrated H_2SO_4 and H_3PO_4 (5:1, v:v). ^{14}C-CO_2 was trapped in 25 mL NaOH (0.5 M) after digesting at 165°C for 8 min with O_2 continuously replenished. And then the mixture containing 1 mL NaOH and 5 mL Ultima Gold XR (PerkinElmer, 940 Winter Street Waltham, MA 02451, USA) was measured in an automated liquid scintillation counter (LS-6500; Beckman) for 10 min. The ^{14}C-SOC fixation rate (‰) fixed by per kilogram soil was calculated as follows:

$$^{14}\mathrm{C} - \mathrm{SOC} = [F_1(R_s - R_o)/R_pW] \times 1000,$$

where F_1 represents the factor to convert the counting volume (1 mL from 25 mL); R_s, R_o and R_p are ^{14}C-radioactivity (Bq) for a trapped solution, ^{14}C in the natural soil of the study sites and ^{14}C-CO_2 generated from $NaH^{14}CO_3$ in the container, respectively; and W, the weight (kg) of soil.

The ^{14}C-MBC was measured according to Ge et al. (2013). Moist soil samples (8 g) were fumigated for 24 h in the dark, followed by extraction with 32 mL K_2SO_4 (0.5 M). The ^{14}C-radioactivity in the extractant (1 mL) of fumigated soil sample, together with that (1 mL) extracted from equivalent unfumigated portions, was measured as above. The ^{14}C-MBC rate (‰) per kilogram soil was calculated as follows:

$$^{14}\mathrm{C} - \mathrm{MBC} = [F_2(R_f - R_{uf})/R_pW] \times 1000,$$

where F_2 represents the factor to convert the counting volume (from 1 mL to the volume of 32 mL plus soil water volume in mL); R_f and R_{uf} are ^{14}C trapped in fumigated and unfumigated soil extractant, respectively; R_p,

radioactivity of ^{14}C-CO_2 generated from $NaH^{14}CO_3$ in the container; and *W*, the weight (kg) of soil.

Microbial DNA extraction

Total DNA was extracted from 0.5 g of grassland soil using an ISOIL for Beads Beating kit (Nippon Gene Co., Ltd., Japan) according to the manufacturer's instructions. Bacterial cells in the soil sample were lysed for 45 s at 4500 rpm of the fast prep bead beating instrument (Tomy Micro Smash MS-100; Tomy Seiko Co., Ltd., Japan). The extracted pellet was dissolved in 100 µl TE and the DNA concentration determined using a spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific Inc., USA). DNA was stored at −20°C until further use.

Design of cbbL primers

New primer sets for amplification of *cbbL* (green- and red-like) sequences were designed manually. All *cbbL* nucleotide sequences, which were available from the National Center for Biotechnology Information (NCBI) sequence database, were used to establish a *cbbL* database by using CLC Sequence Viewer 7. The sequences were first translated into amino acids using Genetyx ver. 10, and the deduced amino acid sequences were then aligned using CLC Sequence Viewer 7. Amino acid alignments were performed manually and nucleotide sequences were aligned accordingly. Based on these data, we designed two primer sets specific for the selected *cbbL* sequences of the red-like and green-like groups. The primers *cbbL*RA and *cbbL*RB, used to amplify the red-like *cbbL* gene, were designed from multiple sequence alignment data for the *cbbL* genes of *Ralstonia eutropha* H16, *Ralstonia eutropha megaplasmid* pHG1, *Sinorhizobium meliloti* WSM419, *Rhodobacter sphaeroides* HR and *Rhodopseudomonas palustris* CGA009. The primers *cbbL*GA and *cbbL*GB, used to amplify the green-like *cbbL* genes, were designed from multiple sequence alignment data for the *cbbL* genes of *Nitrobacter vulgaris* T3, *Acidithiobacillus ferrooxidans* ATCC 23270, *Hydrogenophaga pseudoflava* DSM1083, *Thiobacillus denitrificans* ATCC 25259 and *Nitrospira* sp. strain TCH716. The primers designed and used for this study are listed in Table 2.

Table 2. Primers used for amplification of *cbbL* gene.

Primer	Sequence (5′ to 3′)	Positions	Amplified region
*cbbL*RA	AARGAYGAYGARAAYATHAA	609–627	*cbbL* red-like gene
*cbbL*RB	ATNGTNCCNCCNCCRAAYTC	1206–1224	
*cbbL*GA	GAYTTYACNAARGAYGAYGA	573–591	*cbbL* green-like gene
*cbbL*GB	TCYTCYTTDATYTCYTTCCA	1365–1383	

Note: Y = C or T; S = G or C; R = A or G; H = A or C or T.

PCR amplification, cloning and sequencing

The previously designed primers of the *cbbL* (green- and red-like) genes were used for amplification. In brief, each individual PCR mixture contained approximately 150 ng soil DNA, 2 µl 10×Ex Taq buffer, 1 U Ex Taq polymerase (TaKaRa, Japan), 200 µM deoxynucleoside triphosphates, 2.5 µM of each *cbbL* primer and made up to 20 µl with sterilized H_2O. The thermos-cycle conditions were as follows: 4 min of initial denaturation at 95°C, followed by 40 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 50°C for the red-like and 52°C for the green-like *cbbL* primers and 1 min of elongation at 72°C. The reaction was completed by a final extension for 10 min at 72°C. PCR products were checked in 0.8% (wt/vol) agarose gels (PeqLab Biotechnology GmbH, Erlangen, Germany) using horizontal gel electrophoresis at 75 V for 45 min. DNA was observed using UV excitation after staining with ethidium bromide.

PCR products were purified using a Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) and ligated into p3 T Vector (MoBiTec, Germany), and the resulting ligation products were used to transform *Escherichia coli* DH12S (Invitrogen, Japan) competent cells. The inserted products were amplified with primers (*M13-47* and *RV-M*) and were sequenced directly from white colonies grown in LB supplemented with ampicillin and X-gal. The sequences were compared with known *cbbL* gene sequences from the GenBank (NCBI) database using BLAST. Sequence data have been submitted to the DDBJ database under accession numbers LC195753 to LC195828.

Statistical analysis

Data were processed using Excel 2013 for the means and the standard errors. The Student's *t*-test was used to compare the difference in soil physicochemical characteristics between the fenced and grazing sites. Multiple comparisons of significant differences were made using one-way analysis of variance followed by a Tukey test ($P < .05$). All analyses were performed using SPSS 19.0 for Windows XP.

Results

Soil properties changes

According to the results, grazing significantly decreased soil moisture and EC compared with the fenced grassland soil ($P < .01$) (Table 3). The soil was slightly acidic (6.84–6.97), and the differences in soil pH between the fenced and grazing sites were not significant ($P > .05$). There was a significant difference in soil C_{org} and TN

Table 3. Soil physicochemical characteristics of study sites in 0–5 cm layer.

Soil characteristic	GG	FG
Soil moisture (%)	6.94 ± 1.24	11.18 ± 1.13**
Soil pH (H_2O)	6.97 ± 0.14	6.84 ± 0.07
EC (mS m^{-1})	4.76 ± 0.87	9.02 ± 1.05**
C_{org} (g kg^{-1})	18.81 ± 1.84	26.39 ± 2.60**
TN (g kg^{-1})	2.00 ± 0.10	3.10 ± 0.41**
Soil C/N	11.18 ± 0.13	11.09 ± 0.26
Sand (%)	62.05 ± 3.59	56.87 ± 6.02
Silt (%)	14.43 ± 1.71	16.68 ± 2.11
Clay (%)	23.52 ± 2.03	26.45 ± 3.96
Exchangeable Ca^{2+} ($cmol_c$ kg^{-1})	11.03 ± 1.35	19.81 ± 1.83**
Exchangeable Mg^{2+} ($cmol_c$ kg^{-1})	2.70 ± 0.44	2.41 ± 0.07
Exchangeable K^+ ($cmol_c$ kg^{-1})	0.77 ± 0.06	1.04 ± 0.14**
Exchangeable Na^+ ($cmol_c$ kg^{-1})	0.21 ± 0.02	0.20 ± 0.03
Avail. P (mg kg^{-1})	32.34 ± 7.38	39.21 ± 6.34
C_{mic} (mg kg^{-1})	704.99 ± 127.20	1218.12 ± 118.22**
N_{mic} (mg kg^{-1})	96.6 ± 21.53	159.4 ± 34.57**

Note: Average value and standard deviation; $n = 5$.
GG: grazing grassland site; FG: fenced grassland site; EC: electrical conductivity; C_{org}: organic carbon; TN: total nitrogen; soil C/N: soil carbon to nitrogen; Ca: calcium; Mg: Magnesuim; K: potassium; Na: sodium; Avail. P: available phosphorus; C_{mic}: microbial biomass carbon; N_{mic}: microbial biomass nitrogen.
*Significant difference between grazing and fenced grassland ($P < .05$).
**Significant difference between grazing and fenced grassland ($P < .01$).

between the fenced and grazing sites ($P < .01$). The results revealed that the mean C_{org} and TN significantly increased from 18.81 g kg^{-1} in the grazing site to 26.39 g kg^{-1} in the fenced site and from 2.00 g kg^{-1} in the grazing site to 3.10 g kg^{-1} in the fenced site, respectively ($P < .01$). However, there was no difference in the C-to-N ratio between the fenced and grazing sites ($P > .05$). The soil silt and clay content decreased from 16.68% and 26.45% in the fenced site to 14.43% and 23.52% in the grazing site, while the soil sand content increased from 56.87% in the fenced site to 62.05% in the grazing site. There were no significant differences between the mean value of exchangeable Mg^{2+} and Na^+ for the fenced and adjacent grazing grassland ($P > .05$), but the mean value of exchangeable Ca^{2+} and K^+ decreased significantly from 19.81 $cmol_c$ kg^{-1} in the fenced site to 11.03 $cmol_c$ kg^{-1} in the grazing site and 1.04 $cmol_c$ kg^{-1} in the fenced site to 0.77 $cmol_c$ kg^{-1} in the grazing site, respectively ($P < .01$). The results showed that the mean value of Avail.P decreased from 39.21 mg kg^{-1} in the fenced site to 32.34 mg kg^{-1} in the grazing site. And the mean value of C_{mic} and N_{mic} significantly decreased from 1218.12 and 159.37 mg kg^{-1} in the fenced site to 704.99 and 96.63 mg kg^{-1} in the grazing site, respectively ($P < .01$).

^{14}C-CO_2 fixation rate in the fenced and grazing soils

After 120 days of incubation under dark conditions, ^{14}C in the soils was detected in all the samples (Figure 1). The highest ^{14}C-SOC fixation rate appeared in the

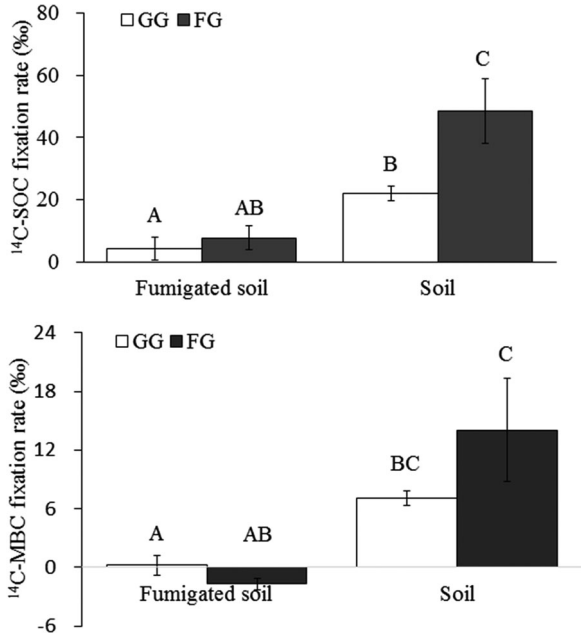

Figure 1. The fixation rate of ^{14}C-SOC and ^{14}C-MBC in soils after incubation in darkness for 120 d.

Note: $n = 3$. GG and FG represent grazing grassland site and fenced grassland site, respectively.

fenced grassland soils (48.55‰), which was significantly higher than in the grazing grassland soils (22.11‰) ($P < .05$). After fumigation, the fixation rates in the soils decreased to 7.68‰ and 4.18‰ in the fenced and grazing grassland soils, respectively, and the difference was not significant. However, these rates were all significantly lower than in the unfumigated soils.

As with the ^{14}C-SOC fixation ratio, the highest ^{14}C-MBC fixation rate also appeared in the fenced grassland soils (14.05‰), which was higher than in the grazing grassland soils (7.08‰). The ^{14}C-MBC fixation ratios in the fumigated grazing and fenced grassland soils were 0.24‰ and −1.66‰, respectively. The ^{14}C-MBC fixation rates in the unfumigated soils were significantly higher than in the fumigated soils.

Phylogenetic analysis of the cbbL *gene clones from soils*

The red-like types of RubisCO *cbbL* genes were detected in both the grazing and fenced grassland soil samples, but the green-like *cbbL* genes were not detected with the used primers. 30 and 46 red-like *cbbL* operational taxonomic units (OTUs) were retrieved from the grazing and fenced soil samples, respectively (Figure 2). The unique phylotypes were distributed into four phyla – Proteobacteria (46.1%), Actinobacteria (38.1%), Chloroflexi (7.9%) and Cyanobacteria (7.9%) – with two phyla dominating (Figure 2). Proteobacteria was dominated by the

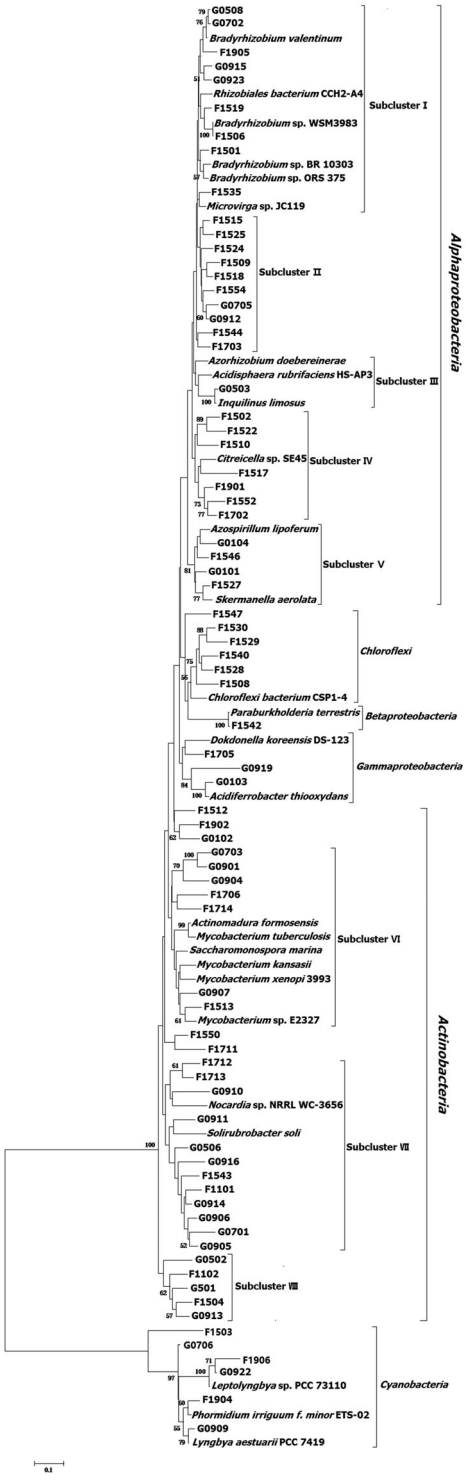

Figure 2. Neighbor-joining phylogenetic tree of red-like *cbbL* genes in the grazing and fenced grassland soils.

Notes: Bootstrap values are known as percentage of 1000 bootstrap replicates. The bar indicates 10% estimated sequence divergence. Bootstrap values higher than 50% are shown at the branch points. G and F represent gene isolated from grazing and fenced site.

subgroup Alphaproteobacteria (88.5%), followed by Gammaproteobacteria (8.6%) and Betaproteobacteria (2.9%).

These sequences in the Alphaproteobacteria were tentatively grouped into five subclusters. Subcluster I was grouped with *Bradyrhizobium valentinum* (accession number WP_057851213), *Rhizobiales bacterium* CCH2-A4 (WP_068078378), *Bradyrhizobium* sp. WSM3983 (WP_027529094), *Bradyrhizobium* sp. BR 10303 (WP_066503379), *Bradyrhizobium* sp. ORS 375 (WP_009031241) and *Microvirga* sp. JC119 (WP_046868905). Close resemblance (100% similarity) of one phylotype was observed with *Bradyrhizobium* sp. WSM3983. Subcluster III was grouped with *Inquilinus limosus* (WP_034847255), *Azorhizobium doebereinerae* (WP_051356539) and *Acidisphaera rubrifaciens* HS-AP3 (GAN77260). Subcluster IV was grouped with *Citreicella* sp. SE45 (WP_008884071). Clones in this cluster were all from the fenced grassland soil samples. Subcluster V was grouped with *Azospirillum lipoferum* (WP_014188404) and *Skermanella aerolata* (WP_044434920). Both Betaproteobacteria and Gammaproteobacteria were grouped into one subcluster, which was affliated with Betaproteobacteria *Paraburkholderia terrestris* (SAL14411) with 99% resemblance and Gammaproteobacteria *Acidiferrobacter thiooxydans* (WP_065968746) and *Dokdonella koreensis* DS-123 (ANB18496), respectively. The clones of Proteobacteria in the fenced grassland soil samples accounted for 68.6% of the total, whereas only 31.4% corresponded to the grazing grassland soil samples.

The sequences in the Actinobacteria were tentatively grouped into three subclusters. Subcluster VI was grouped with *Actinomadura formosensis* (WP_067800405), *Mycobacterium tuberculosis* (CNE30029), *Saccharomonospora marina* (WP_009153612), *Mycobacterium kansasii* (WP_063467152), *Mycobacterium xenopi* 3993 (EUA52020) and *Mycobacterium* sp. E2327 (WP_068106460). Subcluster VII was grouped with *Nocardia* sp. NRRL WC-3656 (WP_030514398) and *Solirubrobacter soli* (WP_028064187). The clones from the fenced grassland soil samples accounted for 44.8% of the total, whereas 55.2% corresponded to the grazing grassland soil samples.

There was one subcluster in Chloroflexi, which was grouped with Chloroflexi *bacterium* CSP1-4 (KRT63449). The clones were all from the fenced grassland soil samples. There was one subcluster in Cyanobacteria, which was grouped with *Leptolyngbya* sp. PCC 73110 (BAE80672), *Phormidium irriguum f. minor* ETS-02 (CBL80832) and *Lyngbya aestuarii* PCC 7419 (BAE80673).

Discussion

Soil physicochemical properties changes

We found significantly higher soil moisture, C_{org}, EC, TN, Ca^{2+}, K^{+}, C_{mic} and N_{mic} in the fenced grassland soils

compared with the grazing grassland soils (Table 3). Fencing significantly increased soil moisture in the present study (Table 3), which was consistent with previous studies (Deng et al. 2014). In this study, fences enhanced aboveground biomass and coverage (Table 1) that could decrease soil evaporation. Moreover, due to continuous grazing and trampling by cattle, the ground surface at the grazing site became bare and could easily become hotter than the covered ground, which caused a decrease in soil moisture and an increase in soil erosion risk (Wang et al. 2015). There was no difference in soil pH between the fenced and grazing areas ($P > .05$) and was constant at about 7.0 (Table 3). Similar results were reported about grazing intensity that did not affect soil pH in a Mediterranean rangeland (Akhzari et al. 2015) and in the *Stipa grandis* and *Stipa bungeana* steppe in northern China (Xie & Wittig 2004). The mean value of EC in the fenced grassland soils was remarkably higher than in the grazing grassland in terms of its effect on soil texture (Table 3). Soil clay content decreased after long-term grazing (Table 3). Soil leaching decreased while soil clay content increased, which can reduce natural soil drainage and conserve water. These lead to the accumulation of salts and minerals in the surface soil, which cause EC to increase.

Our results showed that soil C_{org} and TN significantly increased in the 0–5 cm layer after long-term fencing (Table 3). A similar increase in soil C_{org} and TN following fencing was reported in other arid and semi-arid rangelands (Steffens et al. 2008). In grassland, soil C_{org} is determined by carbon input from plant productivity, litter decomposition, root turnover and animal excreta, and carbon output through soil respiration, soil erosion and leaching (Cui et al. 2005; Wen et al. 2013; Zuo et al. 2015). Aboveground litter accumulates on the soil surface after fencing and vegetation grows better and develops a better root system compared with grazing plots and is conducive to SOC formation and accumulation (Su et al. 2004). However, there were no significant differences in soil C/N ratios between the grazing site and fenced site, indicating the rate of change for C and N after grazing exclusion is the same.

Particle size distribution showed more silt and clay and less sand in the soils of the fenced site compared with the soils of the grazing site (Table 3), but the difference was not significant. Grazing and trampling leads to a decrease in ground cover, enlarging patches of bare ground (Ludwig & Tongway 1995) and leaving the land surface directly exposed to strong wind erosion, which causes loss of fine soil particles and degradation of soil structure (Gomes et al. 2003).

Grazing exclusion increased the concentration of Avail. P, but the difference was not significant (Table 3). This result may be due to the runoff from soil erosion (Vadas et al. 2015), especially in the grazing site where the soils lacked protection from vegetation because of grazing. In addition, livestock grazing can cause energy and nutrient loss from the ecosystem (Miao et al. 2015). The decrease in nutrient feedback from the litter might be related to the decrease in Avail. P in the grazing site. The mean concentrations of Ca^{2+} and K^{+} were significantly higher in the fenced site than in the grazing site, but the difference in mean concentrations of Mg^{2+} and Na^{+} was not significant. All four of the major cations are subject to loss by leaching (Phillips & Burton 2005), but among the exchangeable cations, Ca^{2+} is usually dominant, often amounting to 60–85% of the total in non-acid soil (Domagała-Świątkiewicz & Sady 2011), and when Ca^{2+} is the dominant cation in the soil, it may be the highest amount leached (Whitehead 2000), which was consistent with this study. In addition, potassium, magnesium and calcium appear to compete with each other in the uptake by plants (Evangelou et al. 1994), and because sodium is not essential to plant biochemistry, plants exclude it when taking up water and other cations, which can explain the constant Na^{+} concentration in the fenced and grazing sites.

In the present study, C_{mic} and N_{mic} were found to significantly decrease in the grazing site compared with the fenced site, which indicated that continuous grazing was deleterious to microbial growth. This finding was similar to those of many other studies (Northup et al. 1999; Wang et al. 2008). Soil organic carbon is the major source of energy for soil microorganism growth. In the fenced site, higher organic matter input from plant litter and root exudates may have enhanced the rate of C_{mic} production in the soil (Bird et al. 2002; Ge et al. 2011).

The differences in ^{14}C-CO_2 fixation rates between the fenced and grazing sites

Labelled ^{14}C was detected in the SOC in both the fenced and grazing grassland soils after 120 days' incubation under dark conditions (Figure 1). This result was in agreement with previous studies in which CO_2 fixation was found in two artificial soils after incubation in the dark for 14 days (Šantrůčková et al. 2005), in agricultural soil in northeast Georgia (Shimmel 1987) and in synthetic soil after incubation up to 91 days in the dark (Miltner et al. 2005). However, this finding was inconsistent with the study conducted by Ge et al. (2013), who used ^{14}C to incubate subtropical upland and paddy soils for 110 days, but no ^{14}C was fixed in the soils incubated in continuous darkness.

This result indicates that fixed ^{14}C was mainly derived from chemoautotrophic processes and chemoautotrophic

microorganisms that sequester atmospheric CO_2 in the grassland soils. Our results also showed that ^{14}C-SOC and ^{14}C-MBC fixation rates were significantly higher in the fenced soils than in the grazing soils (Figure 1). In addition, the ^{14}C-SOC and ^{14}C-MBC fixation rates in the fumigated soil samples significantly decreased compared with those in the unfumigated soil samples in both the fenced and grazing sites. A previous study reported that the red-like *cbbL* genes were found only in small clay and silt fractions and not in coarse particle fractions (Selesi et al. 2007). Moreover, it was found that there is a close link between bacterial cell numbers and smaller silt and clay fractions (Van Gestel et al. 1996; Kandeler et al. 2000). In this study, clay and silt content in the fenced site was around 12.0% higher than in the grazing site (Table 3). In addition, both the amount of the red-like *cbbL* genes and RubisCO activity had a significant positive relationship with SOC content (Yuan, Ge, Wu et al. 2012; Yuan et al. 2013). In our study, the mean amount of C_{org} in the fenced soils increased by 28.7% compared with the grazing soils (Table 3). These findings can explain the high fixation rate in the fenced site.

The effect of grazing on the red-like cbbL *genes*

Grassland is widespread, accounting for 40% of the land area in China and one-third of the world terrestrial area, and is one of the most important ecosystems for C and N cycling (Kang 2012). Until now, most previous studies on the distribution and quantification of *cbbL* gene-containing bacteria have focused on aquatic systems (Yuan, Ge, Wu et al. 2012) or terrestrial agricultural bulk soil (Selesi et al. 2005, 2007), with few studies on grassland soil. The most common pathway of CO_2 fixation used by most photoautotrophic and chemoautotrophic bacteria is via the Calvin cycle with the key enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the large subunit of which is encoded by the *cbbL* gene. In this study, no green-like bacteria was found in the fenced and grazing sites, which was consistent with previous studies (Miltner et al. 2005; Videmšek et al. 2009; Yousuf et al. 2012). Miltner et al. (2005) concluded that the growth of obligate lithotrophs carrying the green-like *cbbL* genes was suppressed by the input of readily available carbon sources, and the absence of the green-like *cbbL* genes could be attributed to primer specificity bias in the rhizospheric soil of groundnut (Yousuf et al. 2012).

In contrast to the green-like *cbbL* genes, the red-like *cbbL* genes were detected in both the fenced and grazing grassland sites. Our results indicate that the composition of microbial communities carrying red-like *cbbL* genes was affected by continuous grazing (Figure 2). Compared with the 30 OTUs detected in the grazing site, there were 46 OTUs detected in the fenced site. In particular, in the cluster Alphaproteobacteria, 22 OTUs were identified from the fenced site compared with 9 OTUs identified from the grazing site. The cluster Alphaproteobacteria contained five subclusters, mainly *Bradyrhizobium valentinum*, *Microvirga* sp. and *Azospirillum lipoferum* bacteria, which are known to promote plant growth and fix nitrogen and CO_2 (Videmšek et al. 2009). In addition, a high diversity of red-like *cbbL* sequences was found in grassland soils close to natural carbon dioxide springs, but there was no difference in the number of OTUs between low and high CO_2 concentration (Videmšek et al. 2009). The amount of the *cbbL* genes was found to increase by applying straw and chemical fertilizers (Yuan, Ge, Wu et al. 2012), and Selesi et al. (2007) found a positive relationship between the amount of the red-like *cbbL* genes and SOC content. The study by Tang et al. (2015) revealed the amount of *cbbL* and 16S rRNA genes were lowest in the soils under *Cleistogenes chinensis* where SOC and pH were lowest. The number of OTUs showed no difference between the fenced and grazing sites in the cluster Actinobacteria, mainly with *Mycobacterium* sp. and *Solirubrobacter soli* (Figure 2). Also, phototrophic Cyanobacteria was detected in both the fenced and grazing soils. However, phototrophic Chloroflexi was found in only the fenced soils. Previous studies showed that the microbial autotrophic community is significantly affected by edaphic factors (Selesi et al. 2005; Tolli & King 2005; Nigro & King 2007; Videmšek et al. 2009; Yuan, Ge, Wu et al. 2012). The absence of Chloroflexi in the grazing soils indicated continuous grazing shaping the fixation bacterial communities in the grassland soils.

Disclosure statement

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