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Original Research Paper

Variations in Culturable Terrestrial Bacterial Communities and Soil Biochemical Characteristics Along an Altitude Gradient Upstream of the Shule River, Qinghai-Tibetan Plateau

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ABSTRACT

Variations in the culturable terrestrial bacterial communities and soil biochemical parameters along an altitude gradient (from 1260m to 4111m) upstream of the Shule River, Qinghai-Tibetan Plateau, were investigated. The results showed that the number of cultivable bacteria varied between 0.4×10^7 and 3.3×10^7 CFU/g, with an average of 1.6×10^7 CFU/g. 168 isolates from these soils were clustered into 34 groups by amplified ribosomal DNA restriction analysis (ARDRA). These groups are affiliated to 15 genera that belong to six taxa, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Actinobacteria, Firmicutes and Bacteroides, of which *Bacillus* and *Arthrobacter* were the dominant species at the level of the genus. The relative abundance of *Arthrobacter* increased significantly at high altitude. Correlation analysis showed that the total number of culturable bacteria in the soils decreased first and then increased below 3000m, and these trends significantly positively correlated to the soil organic C, total N and the activities of soil sucrase, and positively correlated to the activities of soil urease and catalase. However, when the altitude exceeded 3000m, cultivable bacterial number dramatically declined, although soil organic carbon, total nitrogen and enzyme activities were relatively high. While the diversity index of bacteria increased along with the increased altitude as a whole and there existed a significantly positive correlation between altitude and bacteria diversity. Together, these results illustrated that culturable bacterial numbers were mainly influenced by soil biochemical properties while bacterial diversity was mainly influenced by altitude in this region.

INTRODUCTION

Temperature is a critical factor that regulates many terrestrial biogeochemical processes, such as soil respiration (Raich & Schlesinger 1992), litter decomposition (Trofymow et al. 2002), root dynamics (Norby & Jackson 2000) and plant nutrient uptake (Reich & Oleksyn 2004). The mean global temperature has increased by 0.74±0.18°C in the past 100 years and will continue to increase by 1-3.5°C in the next 50-100 years based on the general circulation models, the effect being more apparent at higher altitudes and latitudes (IPCC 4). Higher air temperature will lead to higher soil temperature, that in turn influence biochemical processes in the soil ecosystem (Kirschbaum 2004). During the past 20 years, a series of studies have proved that soil organic matter (Kirschbaum 1995), soil nitrogen migration (Schimel & Parton 1986), soil enzyme activities (Sardans et al. 2008) and rates of soil respiration (Lloyd & Taylor 1994) will be influenced by warmer temperatures.

Soil microorganisms play an important role in maintaining global biogeochemical processes, in particular influencing cycling of the three principals of soil nutrient elements C, N and P (Beck et al. 1997). So changes in the structure of microbial communities will have a major impact on the ecosystem activities. Consequently, analysis of soil microbial community responses to global warming are of vital importance. Several artificial warming experiments have shown that the microbial activity and community structure will be affected by warmer temperatures (Zogg et al. 1997, Zhang et al. 2005, Allison & Treseder 2008, Liu et al. 2009). However, it is vital that artificial simulation of warming is complemented by the analysis of changes in natural conditions, where influences of a larger number of variables can be assessed. Soil samples derived from different altitudes pro-

vide a natural laboratory for the study of microbial communities exposed to different temperatures.

Although high-altitude, high-latitude regions are subject to extreme freezing and desiccation, soil environments in such ecosystems provide habitats for numerous microorganisms (Zhang et al. 2002, Zenoff et al. 2006, Dib et al. 2008, Martineau et al. 2010). Previous studies have shown that majority of bacterial isolates from these extreme environment belong to the Firmicutes, Alpha-Proteobacteria, Gamma-Proteobacteria and Actinobacteria (Mesbah & Wiegel 2012, Peeters et al. 2012, Shen et al. 2012, Yan et al. 2012). Correlation analysis has indicated that the altitude and soil properties are important factors influencing the number and diversity of soil microbes in these environments (Lugo et al. 2008, Ferrero et al. 2010). As an important content of soil in extreme environments, the abundance and diversity of microorganisms have received considerable attention along several altitude gradients in China, such as the Changbai Mountain area in northeast China (Lv 2011), an alpine area in the southwest (Zhang et al. 1999) and subtropical regions in the southeast (He et al. 2009, Wang et al. 2008, Wang et al. 2011). However, the biodiversity of soil microorganisms in relation to altitude requires further attention in semi-arid regions, especially in remote regions, such as in northwest China.

The upstream region of the Shule River Basin is located in the northeast margin of the Qinghai-Tibetan Plateau (QTP), China. It is considered as a 'natural water area', contributing as it does to the distribution of continental glaciers and mountain permafrost (Chen et al. 2011). Due to regional climate warming (Mu 2006), a series of ecological environment problems have become apparent, such as vegetation decline, grassland degradation, land salinization and desertification (Ding et al. 2001, Sun et al. 2011). Consequently, the area has been investigated in terms of the soil environment (Liu et al. 2012b), with surveys of vegetation in alpine grasslands (Yi et al. 2011, Chen et al. 2012, Qin et al. 2014) and soil macrofauna (Lin et al. 2011). However, there has been no analysis, to date, on soil microorganisms along an altitude gradient in this area. So in this study, we selected 12 soil samples upstream of the Shule River along an altitude gradient (from 1260 m to 4111 m) to investigate changes in the microbial community structure. We have addressed two questions: firstly, how do soil microorganisms change along the altitude gradient, and secondly, what are the main factors affecting microbial community structure in this region.

MATERIALS AND METHODS

Study sites and sample collection: The Shule River is located in the western part of the Qilian Mountains, which is in the northeast edge of the QTP, China. It is mainly controlled by westerly winds, and seldom affected by the Asian monsoon. The mean annual air temperature ranged from -4.0 to -19.4°C, and mean annual precipitation ranged from 200 to 400 mm over the period of 1960-2010. It is also considered as a natural water area contributing to the distribution of continental glaciers and mountain permafrost (Chen et al. 2011). The study area is in the upstream region of the Shule River, where alpine meadows and alpine steppes are the dominant vegetation types (Yi et al. 2011). In August 2011, 12 surface soil samples were collected at 0-100 mm depth along an altitude range from 1260 m to 4111 m above the sea level and each soil sample consisted of three replicates. According to the principle of microbiological analysis, all the samples were kept sterile during the collection. Soil samples for biochemical analyses were also collected at the same time. All samples were stored in aseptic bags and kept in ice during transport to the laboratory, then stored at -20°C.

Biochemical analyses of the soils: Soil water content was measured by weight-loss method after 24h at 100°C in a drying oven. Soil pH was measured in a 1:2.5 soil-deionized water slurry by using a digital pH meter (PT-10, Sartorius). The soil organic C and total N were determined by using an elemental analyser (Multi-N/C 2100s, Analytik Jena AG, Germany) after air-drying and grinding, to allow passage through a 80-mesh sieve. All enzymatic preparations were performed at 0-4°C. The soil enzymes urease, sucrase, catalase and phosphatase were measured. The activity of urease was measured according to a modified method described by Taylor et al. (2002) and urease activity was expressed as 1 mg ammonia-N/g dry weight (dw) soil per 24 h. Catalase activity was determined by measuring the O_2 absorbed by $KMnO_4$ after addition of H_2O_2 to the reaction mixture (Garcia-Gil et al. 1979 and Garcia-Gil et al. 2000), and the activity was expressed as 1mg N/g dw soil per 24 h. Sucrase activity was determined according to the method by Schinner & Mersi (1990) and the activity was expressed as 1 mg glucose/g dw soil per 24 h. Phosphatase activity was determined according to the method by Guan et al. (1983) and the activity was expressed as 1 mg phenol/g dw soil per 12 h.

Cultivation and total counts of bacteria: Bacterial cultivation and isolation were performed according to the method of Long et al. (2014). Beef extract-peptone medium was used to iso-late the bacteria. 2.0 g soil samples were diluted by aseptically placing them in a 50-mL flask containing 18 mL autoclaved 0.85 % NaCl solution with glass beads, and shaking at 150 rpm and 20°C for 30 min after all of the

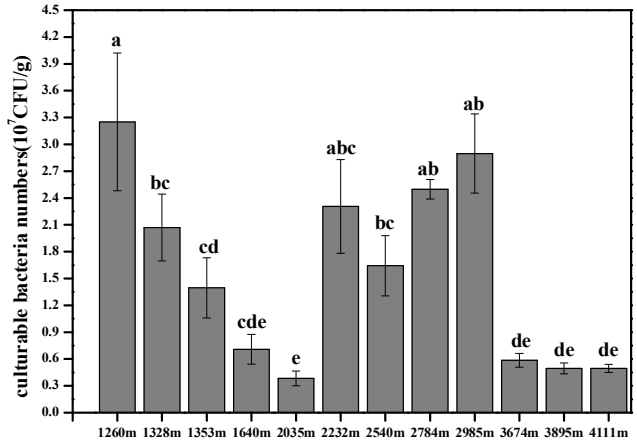

Fig.1: Population fluctuation of culturable bacteria changing with altitudes.

samples were thawed at 4°C. The suspended cells and soil particles were serially diluted with autoclaved 0.85% NaCl solution from 1 to 10^{-6} dilution. A 0.2-mL aliquot of dilution was plated with pre-chilled beef extract-peptone medium and incubated at 20°C. After a 7-day incubation period, the colony forming units (CFU) was calculated as averages of the triplicate plates. The distinct colonies on the spread plates were purified on fresh beef extract-peptone agar plates and were then immediately preserved at -70°C in liquid medium with 15 % glycerol.

Amplified ribosomal DNA restriction analysis: The bacterial DNA was extracted and purified using a TIANamp Bacteria DNA Kit (Tiangen-Biotech, China) according to the manufacturer's instructions. The 16S rRNA genes were amplified with bacteria-specific primers, 27f and 1492r (Lane 1991). The PCR procedure started with 4 min of denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 56°C and 1.5 min at 72°C, and then a final extension step of 20 min at 72°C. A 5 µL aliquot of each PCR mixture containing approximately 1.5 µg of amplified 16S rDNA was digested with 1.5 U of restriction enzymes *Hae*III and *Alu*I in a total volume of 20 µL at 37°C for 16 h. The reaction products were separated on a 2.5% agarose gel.

Sequence and phylogenetic analyses: According to the amplified ribosomal DNA restriction analysis (ARDRA) results of the isolates, one representative strain of each group was selected for 16S rRNA gene sequence determination. Two primers were utilized for sequencing reactions: 27F (5'-AGAGTTTGATCCTGGCTCAG-3',) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991). Nearly complete 16S rDNA nucleotide sequences were determined. The 16S rRNA gene sequences were aligned with representative reference sequences of the most closely related members obtained from the National Center for Biotechnology Information (NCBI) database (http://www.nvbi.nlm.nih.gov) using the multiple-alignment CLUSTALW 1.81 software package. Phylogenetic dendrograms were constructed using the neighbour-joining method, and the tree topologies were evaluated by performing bootstrap analyses of 1,000 data sets with the MEGA4.1 package, as described by Zhang et al. (2012).

Nucleotide sequence accession numbers: The 16S rRNA gene sequences of the 34 representative isolated strains have been deposited in the GenBank database under the following accession number: KC778366-KC778399.

Data analysis: The correlation coefficients (R) with their p-values were calculated according to Pearson, with the statistical program SPSS statistics, SPSS/PC software ver. 17.0 (SPSS Inc., Chicago, IL, USA). Data presented are the means of at least three independent experiments and expressed as the mean ± SE. Comparisons between the mean values were made by the least significant difference (LSD test) at $p< 0.05$.

The Shannon-Wiener diversity index was calculated according to the equation:

$$H = -\sum_{i=1}^{n} pi \ln pi \quad ...(1)$$

$$Pi = ni/N \quad ...(2)$$

where *pi* is the proportion of individuals of species *ni*, *N* is the total number of species observed in the sample.

RESULTS

Biochemical properties of the soils: The soil characteristics of 12 soil samples from upstream of the Shule River were analysed with regard to soil water content, pH, TOC, TN, urease, sucrase, catalase and phosphatase content (Table 1). The values of the soil pH in all soil samples were above 8.00. The water content of the investigated soils ranged from 1.471% to 24.191%, with differences depending on their site characteristics, with a trend of increasing water content at higher altitudes. The TOC and TN content values ranged from 0.762% to 1.683% and 0.022% to 0.131%, respectively. The activities of the soil urease, sucrase, catalase and phosphatase were relatively high at high altitude (above 2500 m). Furthermore, the values of the TOC, TN and sucrase were highest at the lowest soil sample site (1260 m) (Table 1).

The abundance of the culturable bacteria in the soils: The total number of the culturable bacteria in this area ranged from 0.4×10^7 to 3.3×10^7 CFU/g, the average value was 6×10^7 CFU/g. The total numbers of culturable bacteria in the soils decreased first and then increased below 3000 m. However, cultivable bacterial numbers dramatically declined when the altitude exceeded 3000 m. Furthermore, the total number

Table 1: Biochemical properties of soil changing with altitudes.

Altitude/m	TOC/%	TN/%	pH	Water content/%	Urease/mgN/g soil·24h)	Sucrase (mg glucose/g soil·24 h)	Catalase (μM O2/g soil·h)	Phosphatase /μg·g^{-1}· (h)$^{-1}$
1260	1.683	0.131	8.142	2.683	0.042	3.528	4.732	0.034
1328	1.639	0.106	8.191	2.366	0.021	3.271	4.637	0.021
1353	1.559	0.035	8.537	2.036	0.019	2.426	4.628	0.016
1640	0.872	0.023	8.826	1.515	0.005	0.047	4.212	0.006
2035	0.762	0.022	8.931	1.471	0.002	0.042	3.518	0.005
2232	1.142	0.045	8.634	4.625	0.035	0.779	5.235	0.040
2540	1.337	0.058	8.473	8.391	0.069	0.790	4.969	0.084
2784	1.487	0.103	8.188	15.621	0.072	3.409	5.777	0.236
2985	1.664	0.127	8.153	16.116	0.074	3.455	5.805	0.337
3674	1.414	0.073	8.761	18.223	0.072	3.158	5.912	0.174
3895	1.593	0.118	8.054	24.191	0.065	3.262	7.691	0.194
4111	1.603	0.124	8.267	10.465	0.067	3.237	5.381	0.122

Table 2: The correlation coefficients between culturable bacterial numbers and soil biochemical factors changing with altitudes.

	TOC	TN	pH	Water content	Urease	Sucrase	Catalase	Phosphatase
Cuiturable number	0.808**	0.889**	-0.889**	0.519	0.697*	0.802**	0.774*	0.54
TOC	1	0.835**	-0.859**	0.318	0.599*	0.919**	0.352	0.468
TN		1	0.468	0.420	0.656*	0.873**	0.419	0.595*
pH			1	-0.449	-0.571	-0.774**	-0.490	-0.499
Water content				1	0.522	0.383	0.984**	0.552
Urease					1	0.598*	0.455	0.819**
Sucrase						1	0.379	0.574
Catalase							1	0.457
Phosphatase								1

Table 3: Bacterial diversity index changing with altitudes.

Altitude (m)	1260	1328	1353	1640	2035	2232	2540	2784	2985	3674	3895	4111
Diversity index	0.710	0.733	1.044	0.805	0.816	1.056	0.867	1.248	1.161	0.945	1.204	1.064

of culturable bacteria at 1260 m was 8.25 times the value at 2035 m, and 6.5 times the value at 4111 m (Fig. 2). These results indicate that altitude had a significant influence on culturable bacterial numbers.

The correlations between the abundance of soil culturable bacteria and soil biochemical parameters: The total number of the culturable bacteria in the soils below 3000 m was significantly positively correlated to the soil total N, organic C and soil enzyme activity of sucrase (r=0.889, p=0.001; r=0.808, p=0.008; r=0.802, p=0.009, respectively), but significantly negatively correlated to the soil pH values (r=-0.889, p=0.002). Additionally, positive correlations between the soil bacteria numbers and soil enzyme activities of urease, catalase were investigated (r=0.697, p=0.037; r=0.774, p=0.014, respectively). However, when the altitude exceeds 3000m, correlations between the cultivable bacterial numbers and soil biochemical properties were not obvious, although soil organic carbon, total nitrogen and enzyme activities were relatively high. This indicated that the bacterial abundance increased with increasing concentrations of soil total N and organic C, and soil enzyme activities, with decreasing soil pH value when altitudes were below 3000 m. When altitudes exceeded 3000 m, the influencing factors were too complex to define.

The abundance and diversity variations of culturable bacteria related to altitude: We obtained a total of 168 isolates from soil samples. The 168 culturable bacterial isolates were clustered into 34 groups by ARDRA. Phylogenetic analysis of the dominant bands sequence revealed that the 34 groups had greater than 98 sequence similarity with another strain of the most related species in the NCBI 16S rRNA database. The 34 groups were affiliated with 15 genera that belonged to six taxa (Fig. 3). Among them, 9 genera only contained one species, they being *Limnobacter*, *Promicromonospora*, *Kocuria*, *Brevund-imonas*, *Chryseobacterium*, *Algoriphagus*, *Jeotgalibacil*, *Paenibacillus* and *Clavibacter*,

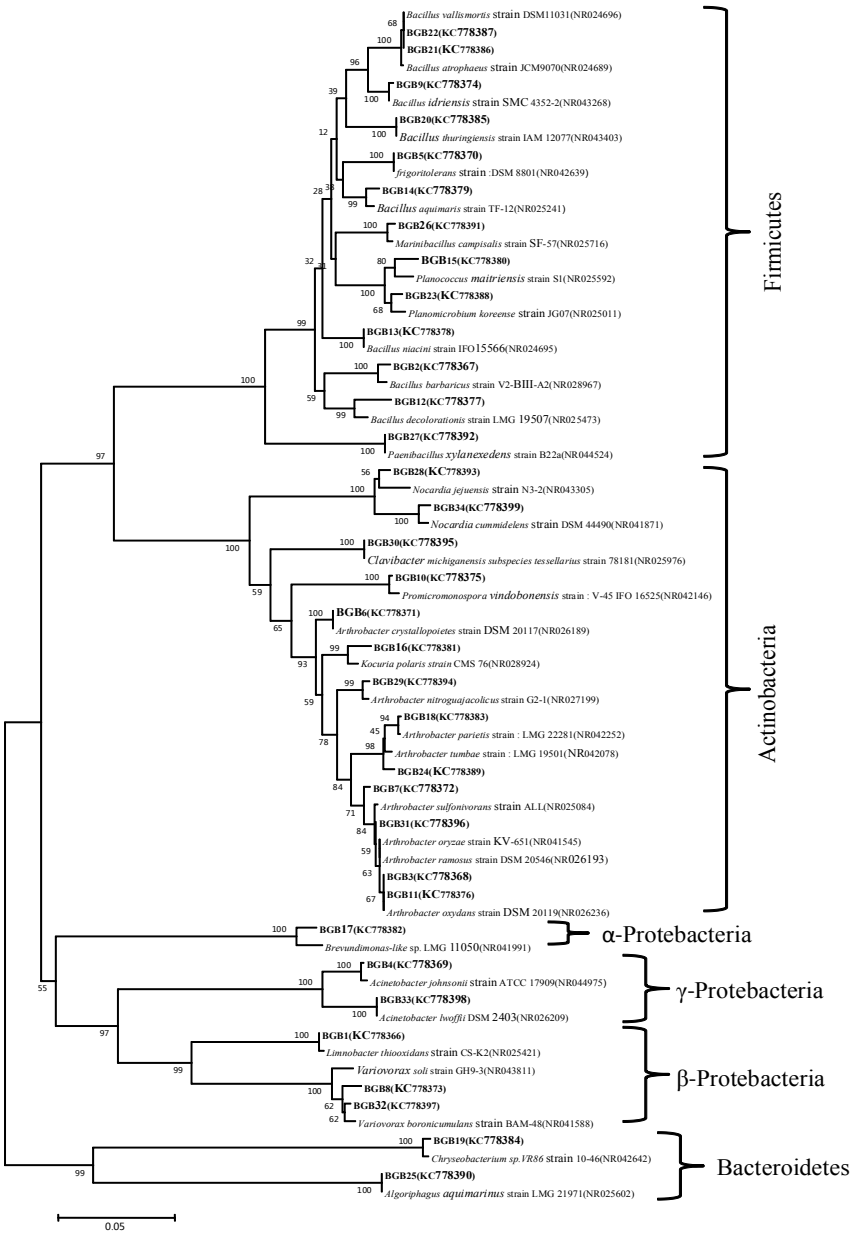

Fig. 2: Phylogenetic trees of culturable bacteria based on the 16S rDNA sequences I.

respectively. The genera *Acinetobacter*, *Planococcus* and *Nocardia* were represented by 2 species, respectively. *Bacillus* and *Arthrobacter* were the most abundant genera in this study, containing 9 and 8 species, respectively. In addition, it was interesting that the relative abundance of the genera *Arthrobacter* was higher with increasing altitude; when the altitude exceeds 3000 m, more than 50% of the genera were *Arthrobacter*, especially at 4111m where the percentage was 95.71% (Fig. 4). This was not obvious for *Bacillus*.

The values of the Shannon diversity index ranged from 0.710 to 1.246 and correlation analysis indicated that the diversity index was positively correlated with the altitude ($r=0.634$, $p=0.027$) when the altitude was below 3000m.

DISCUSSION

Altitude is considered as one of the most important factors that influences microbial numbers and diversity (Mao et al. 2013). In this study, soil culturable bacteria numbers varied between 0.4×10^7 and 3.3×10^7 CFU/g along the altitude gra-

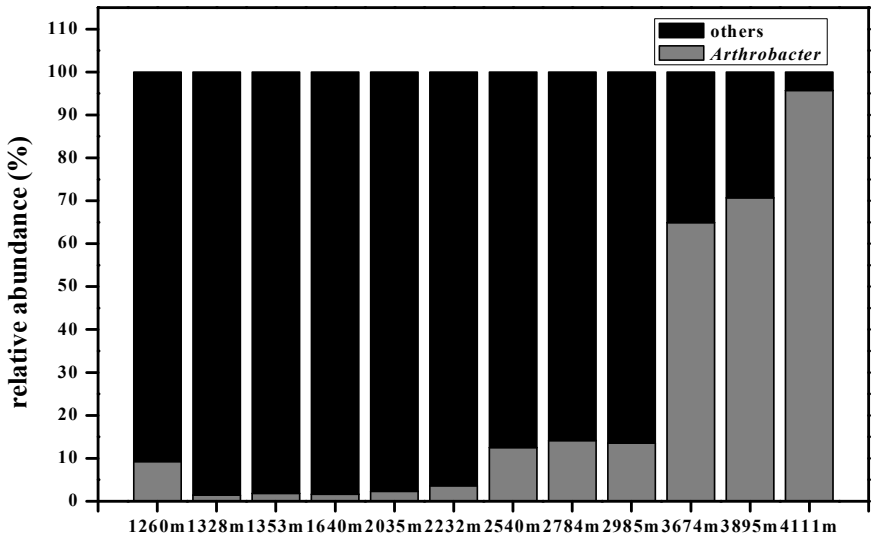

Fig. 3: Relative abundance of *Arthrobacter* changing with altitude.

dient, with an average value of 1.6×10^7 CFU/g, that was significantly lower than for bacteria isolated from the rhizosphere soil of *Splendens* from different altitudes in the same area (Yang et al. 2014), reflecting the impact of the plants on the number of bacteria. Overall, the total number of culturable bacteria in the soils decreased first and then increased below 3000 m, which was consistent with trends of *Splendens* rhizosphere bacteria (Yang et al. 2014). However, when the altitude exceeded 3000 m, cultivable bacterial numbers dramatically declined (Fig. 2). This may be due to large changes in climatic conditions at higher altitudes with, in particular, the decreased temperature likely reducing the rate of nutrient cycling and further affecting the structure and quality of organic matter, eventually leading to deterioration of living conditions for microorganisms, as suggested by others (Margesin et al. 2009). Similarly, a study in the upstream of Heihe river found that culturable bacteria varied from 4.6×10^6 to 3.7×10^7 CFU/g and showed a downward trend in the number of bacteria isolated from higher altitudes (Mao et al. 2013). Liu et al. (2010) found that the number of soil bacteria decreased with elevation in the Urumqi River area. Ma et al. (2004) investigated the number of microbes at different altitudes in different areas of Xinjiang and the results showed that the number of microorganisms at high altitudes was lower than from the low altitude areas. In north India, culturable bacterial numbers varied between 4×10^5 and 3.6×10^7 CFU/g at different altitudes and the number of bacteria also decreased significantly with altitude (Giri et al. 2007). Consistent with these studies, our data indicate that altitude has significantly negative effects on bacterial numbers, maybe due to harsh environments such as low temperature and strong UV-B radiation at higher altitudes.

The major taxa isolated in this study were Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteo-bacteria, Firmicutes, and Bacteroidetes, among which Actinobacteria and Firmicutes were the dominant taxa. The latter two taxa were also found to be dominant in *Splendens* rhizosphere soil in the same region (Yang et al. 2014). Soil samples from upstream of the Heihe river were also dominated by Actinobacteria (Mao et al. 2013). While Firmicutes were the second dominant taxa along an altitude gradient of the western Himalayan mountains (Gangwar et al. 2009). In extreme cold environments such as high altitude and high latitude regions of Antarctica, Actinobacteria was the dominant taxa too (Aislabie et al. 2006). Of the dominant bacteria identified in this study, *Arthrobacter* is widely distributed in different soils of terrestrial ecosystems, being overwhelmingly dominant in some environments due to their ability to utilize a variety of organic materials in complex environments, as well as playing a critical role in the transformation of organic matter (de Ridder-Duine et al. 2005). Their ability to grow in relatively barren soils in extreme conditions may account for the higher proportion of Arthrobacter we isolated from higher altitudes (Fig. 4). *Bacillus* is plant growth promoting rhizobacteria (Lugo et al. 2008). This genera was also dominant in the *Splendens* rhizosphere soil from the same study area (Yang et al. 2014). Ridder-Duine et al. (2005) confirmed that rhizosphere soil microbes originate from the surrounding bulk soil, since soil develops earlier than vegetation and the rhizosphere represents a relative enrichment of some soil microorganisms. This can account for why the dominant genera *Bacillus* include in both rhizosphere soil and bulk soil in this study region.

Previous studies have indicated that the soil microbial quantity is mainly affected by soil organic carbon (Zak et al. 1994), total nitrogen (Zak et al. 1990), pH (Liu et al. 2012a) and soil enzyme activities (Flechtner et al. 1998). In this study, culturable bacteria numbers were significantly positively correlated with soil organic carbon, total nitrogen and soil enzyme activities, but significantly negatively correlated with soil pH values below an altitude of 3000 m, consistent with previous studies. In the present study, we also observed a positive correlation between soil culturable bacteria numbers and urease, sucrase and catalase activities. Similar results were also found in other extreme environments, such as glacier forelands (Sigler & Zeyer 2002, Liu et al. 2012a).

In conclusion, we present for the first time data describing the soil biochemical parameters and culturable bacterial isolates obtained from upstream of the Shule river. The soil biochemical parameters and the culturable bacteria richness exhibit successional patterns along an altitude gradient that ranges from 1260 m to 4111 m. Further research is needed to identify more microorganisms related to environmental change and to determine potential drivers of bacterial community shifts along altitude gradients due to climate change. The present study, along with results of others, shows that the relationship between environmental factors and soil microorganism is complex; the main factors which influence soil microbial communities at altitude are not globally consistent.

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