Diversity of Culturable Bacteria Isolated from Highland Barley Cultivation Soil in Qamdo, Tibet Autonomous Region

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Abstract

The soil bacterial communities have been widely investigated. However, there has been little study of the bacteria in Qinghai-Tibet Plateau, especially about the culturable bacteria in highland barley cultivation soil. Here, a total of 830 individual strains were obtained at 4°C and 25°C from a highland barley cultivation soil in Qamdo, Tibet Autonomous Region, using fifteen kinds of media. Seventy-seven species were obtained, which belonged to 42 genera and four phyla; the predominant phylum was Actinobacteria (68.82%), followed by Proteobacteria (15.59%), Firmicutes (14.29%), and Bacteroidetes (1.30%). The predominant genus was Streptomyces (22.08%, 17 species), followed by Bacillus (6.49%, five species), Micromonospora (5.19%, four species), Microbacterium (5.19%, four species), and Kribbella (3.90%, three species). The most diverse isolates belonged to a high G+C Gram-positive group; in particular, the Streptomyces genus is a dominant genus in the high G+C Gram-positive group. There were 62 species and 33 genera bacteria isolated at 25°C (80.52%), 23 species, and 18 genera bacteria isolated at 4°C (29.87%). Meanwhile, only eight species and six genera bacteria could be isolated at 25°C and 4°C. Of the 77 species, six isolates related to six genera might be novel taxa. The results showed abundant bacterial species diversity in the soil sample from the Qamdo, Tibet Autonomous Region.

Keywords: Qinghai-Tibet Plateau, Streptomyces, 16S rRNA, novel taxa, high-altitude area

Introduction

Bacteria constitute a major proportion of biodiversity in soil ecosystems; they are the main driving force for the conversion and circulation of carbon, nitrogen, and phosphorus, and also the prominent participants in biochemical processes of soil organic matter decomposition and humus formation (Fulthorpe et al. 2008; Řeháková et al. 2015; Malard et al. 2019). Bacterial assemblies are essential components of soils in arid ecosystems, especially in remote high-elevation mountains (Margesin et al. 2009; Yuan et al. 2014). While global surveys of microbial diversity and functional activity have already been conducted (Bodelier 2011; Deldago-Baquerizo et al. 2018), the number of Qinghai-Tibet Plateau samples is restricted, and, therefore bacterial data is still lacking in this area, especially in the most high-altitude area (Zhang et al. 2016).

Highland barley (Hordeum vulgare L.) is the fourth most consumed grain worldwide, only ranked after rice, wheat, and maize (Shen et al. 2016; Deng et al. 2020). Highland barley is a hulless barley cultivar and used as the main staple food for the Tibetan people widely grown in Qinghai-Tibet Plateau in China (He et al. 2019; Zhang et al. 2019). Extreme environments such as cold and hypoxia in Tibet have promoted the unique ecological environment and soil bacterial composition (Zhang et al. 2007; 2010a). However, the extreme environments also have led to the decline of soil bacterial activity and the impoverishment of soil for growing highland barley (Yu et al. 2009; Zhao et al. 2014). The research of soil bacteria in the highland barley planting field has important significance for highland barley yield increase, pest control, and soil quality improvement (Bailly and Weisskopf 2012). At present, there were few studies on bacteria in the soil of the highland
barley-planting field (Liu et al. 2019). Significantly, the culturable bacteria isolated from highland barley cultivation soil have not been reported systematically.

The Qamdo region’s temperature is between 20°C and 28°C from June to September, a significant growth period for highland barley. While the temperature is below 10°C from November to March, no crops were planted on the land during this period. So the culturable bacteria were isolated from a high-altitude highland barley cultivation soil collected in Qamdo using 15 media at 4°C and 25°C to simulate the temperature conditions over these two periods in this study. The composition of bacterial communities was characterized based on the 16S rRNA gene (Furlong et al. 2002; Li et al. 2019). Our aims were: (1) to reveal the diversity of culturable bacteria isolated from highland barley cultivation soil in the high-altitude area; and (2) to study the effect of different culture temperatures on the species of culturable bacteria in highland barley cultivation soil.

Experimental

Materials and Methods

Study site and samples collection. The sampling site was located in the Zhu Village, Banbar County, Qamdo, Tibet Autonomous Region (30°55′48.9″N, 94°58′13.4″E, Altitude: 4,011 m); the sampling site is the typical high-altitude patches farmland in Qamdo, which is about one-third of Qamdo’s farmland. The sample site belongs to the plateau temperate subhumid climate type, the air temperature range is –40–29°C, the annual average air temperature is –1°C, and the yearly frozen period is from September to April. The soil type was sandy loam, and the pH value is 7.6. The previous frozen period is from September to April; the soil type belongs to the plateau temperate subhumid climate type, the air temperature range is –40–29°C, the annual average air temperature is –1°C, and the yearly frozen period is from September to April. The soil type was sandy loam, and the pH value is 7.6. The previous frozen period is from September to April, and the samples were collected in April 2018. Once retrieved, the soil sample was immediately stored at 4°C, and bacteria were isolated in the laboratory in Lhasa in May and June 2018.

Isolation and maintenance of bacteria. The bacteria in highland barley cultivation soil sample were isolated using X1, R, L1, ISP2, GW1, and DSM372 media to isolate Gram-negative bacteria. Two sets of plates were incubated at 4°C and 25°C, respectively; the bacterial strains were obtained across 3–60 days. The pure culture isolates were preserved in glycerol suspensions (20%, v/v) at –80°C for further research.

PCR amplification and sequencing of the 16S rRNA gene. According to the manufacturer’s protocol, the genomic DNA of bacteria was extracted using a bacterial genomic DNA FastPrep Extraction Kit (TIANGEN DP302). Polymerase chain reaction (PCR) amplification of the partial 16S rRNA gene was performed using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GTCCTACCTTGTTACGACTT-3′), PCR was performed using the extracted highly purified genomic DNA as a template under the following conditions: 95°C for 10 min, followed by 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s for 30 cycles with a final 10 min extension at 72°C. The PCR products were detected by agarose gel electrophoresis and then sent to GENEWIZ.Inc for the 16S rRNA gene sequencing. The phylogenetic status of the species was determined by a reaction of 700–750 bp (V1-V4) using the universal primers 27F, if the similarity was less than 98.65% (Kim et al. 2014), then the phylogenetic status of the species was further analyzed by nearly full-length 16S rRNA gene (1,300–1,400 bp).

Phylogenetic analysis. Similarity searches of the 16S rRNA gene sequences were performed in the NCBI and EzBiocloud database for BLAST; then the 16S rRNA gene sequences with the highest homology were obtained for phylogenetic analysis. The sequence alignments were performed using Clustal X, the phylogenetic trees were constructed from evolutionary distances using the neighbor-joining method with a bootstrap of 1,000 repetitions, and the phylogenetic analysis was conducted using the MEGA 7 software (Kumar et al. 2016b).

Nucleotide sequence accession numbers. The full and partial 16S rRNA gene sequences of the strains were submitted to the NCBI GenBank database under the accession numbers (MT611248-MT611324).

Results

The isolated strains. Bacterial populations were successfully isolated from the highland barley cultivation soil sample using fifteen kinds of media, a total of 830 individual strains were obtained at different culture temperatures (4°C and 25°C) (Fig. 1A). Eighty-three and 747 strains of bacteria were isolated from these media at 4°C and 25°C, respectively. The results showed that X1, R, F1, M1, M5, M8, and GS culture media had a better effect on isolating bacteria at 25°C; however,
X1, R, and M5 culture media had a better effect on isolating bacteria at 4°C, none of the bacteria was isolated from the F2, M7, HV, and GS media at 4°C.

**Phylogenetic analysis of cultivable strains by the 16S rRNA gene sequence.** According to the morphological characteristics of bacteria, 330 strains were screened for the 16S rRNA gene sequence analysis using the universal primers 27F/1492R, and 98.65% of the 16S rRNA gene sequences were used as the species boundary of prokaryotes. After combining more than 98.65% of the 16S rRNA gene sequences with the same species, the sequences of 77 species were obtained, which belonged to 42 genera and four phyla (Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes), as shown in Table II. Phylogenetic tree based on the 16S rRNA gene sequences of representative bacteria strains were shown (Fig. 2).

There were 53 species and 25 genera in Actinobacteria, accounting for 68.82% of the species' total number. The predominant genus was *Streptomyces* (22.08%, 17 species), followed by *Micromonospora* (5.19%, four species), *Microbacterium* (5.19%, four species), and *Kribbella* (3.90%, three species). Some rare Actinobacteria were also isolated, for example, *Leifsonia, Longispora, Nocardioides, Nocardia, Nocardiooides, Terrabacter, Umezawaea*, and *Kribbella*. There were 12 species and ten genera in Proteobacteria, accounting for 15.59% of the total number of species, but no dominant genus was found in Proteobacteria. There were 11 species and six genera in Firmicutes, accounting for 14.29% of the total number of species; the predominant genus was *Hymenobacter* (22.08%, three species).

### Table I

**Isolation media.**

| Media | Composition |
|-------|-------------|
| X1    | peptone 2.0 g, yeast extract 0.5 g, FePO₄·4H₂O 0.1 g, MgSO₄·7H₂O 0.5 g, CaCO₃ 0.2 g, NaCl 0.5 g, agar 18.0 g, ddwater 1,000 ml, pH 7.0 |
| R     | peptone 10.0 g, yeast extract 5.0 g, maltose extract 5.0 g, casein amino acid 5.0 g, beef extract 2.0 g, glycerol 2.0 g, Tween-80 50.0 mg, MgSO₄·7H₂O 1.0 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.6 |
| L1    | NaCl 100.0 g, K₂HPO₄ 5.0 g, MgSO₄·7H₂O 7.5 g, hydrolyzed casein 1.0 g, yeast extract 5.0 g, Na₂C₃H₂O₇·2H₂O 3.0 g, FeSO₄·7H₂O 0.1 g, MnCl₂·4H₂O 0.1 g, ZnSO₄·7H₂O 0.1 g, agar 18.0 g, ddwater 1,000 ml, pH 7.0–8.0 |
| ISP2  | NaCl 100.0 g, dextrose 4.0 g, yeast extract 4.0 g, maltose extract 10.0 g, MgSO₄·7H₂O 0.5 g, CaCO₃ 2.0 g, FeSO₄ 10 mg, agar 18.0 g, ddwater 1,000 ml, pH 7.0–8.0 |
| GW1   | NaCl 100.0 g, casein 0.3 g, mannanitol 1.0 g, NaHCO₃ 2.0 g, CaCO₃ 0.2 g, (NH₄)₂SO₄ 2.0 g, KNO₃ 2.0 g, K₂HPO₄ 1.0 g, MgSO₄·7H₂O 2.0 g, FeSO₄ 10.0 mg, Trace-salt 10.0 mg/l, Agar 18.0 g, ddwater 1,000 ml, pH natural |
| DSM372| NaCl 100.0 g, hydrolyzed casein 5.0 g, yeast extract 5.0 g, Na₂C₃H₂O₇·2H₂O 3.0 g, Na₂CO₃·10H₂O 8.0 g, NaC₃H₇NO₃ 1.0 g, KCl 2.0 g, MgSO₄·7H₂O 2.0 g, agar 18.0 g, ddwater 1,000 ml, pH natural |
| F1    | glycerol 5.0 g, alanine 3.0 g, arginine 1.0 g, (NH₄)₂SO₄ 2.64 g, KH₂PO₄ 2.38 g, K₂HPO₄ 5.65 g, MgSO₄·7H₂O 1.0 g, CuSO₄·5H₂O 0.0064 g, FeSO₄·7H₂O 0.0011 g, MnCl₂·4H₂O 0.0079 g, ZnSO₄·7H₂O 0.0015 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
| F2    | MgSO₄·7H₂O 0.5 g, CaCO₃ 0.2 g, FeSO₄ 10.0 mg, NaCl 0.5 g, MnCl₂·4H₂O 1.4 g, Na₂MoO₄·2H₂O 0.39 g, Co(NO₃)₂·6H₂O 0.025 g, ZnSO₄·7H₂O 0.222 g, NaHCO₃ 2.0 g, Na₂HPO₄·2H₂O 0.05 g, agar 18.0 g, ddwater 1,000 ml, pH natural (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
| M1    | soluble starch 10.0 g, casein 0.3 g, KNO₃ 2.0 g, K₂HPO₄ 2.0 g, MgSO₄·7H₂O 0.05 g, FeSO₄·7H₂O 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
| M5    | yeast extract 4.0 g, soluble starch 15.0 g, K₂HPO₄ 1.0g, FeSO₄·7H₂O 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.6 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
| M6    | raffinose 10.0 g, L-histidine 1.0 g, MgSO₄·7H₂O 0.5 g, FeSO₄·7H₂O 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
| M7    | L-aspartic acid 0.1 g, peptone 2.0 g, sodium propionate 4.0 g, FeSO₄·7H₂O 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
| M8    | glycerine 6.0 ml, arginine 1.0 g, MgSO₄·7H₂O 0.5 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
| HV    | humic acid 1.0g, Na₂HPO₄·5H₂O 0.2 g, KCl 1.7 g, MgSO₄·7H₂O 0.5 g, FeSO₄·7H₂O 0.01 g, CaCO₃ 0.02 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
| GS    | soluble starch 20.0 g, NaCl 0.5 g, KNO₃ 1.0 g, K₂HPO₄·3H₂O 0.5 g, MgSO₄·7H₂O 0.5 g, FeSO₄·7H₂O 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.4–7.6 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin) |
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A

B

C

25°C and 4°C
Culturable bacteria diversity in highland cultivation soil

by R (14.55%, 48 strains), M8 (13.64%, 45 strains), X1 (12.73%, 42 strains), M5 (12.73%, 42 strains), GS (10.00%, 33 strains), F1 (4.55%, 15 strains), L1 (1.52%, five strains), F2 (1.52%, five strains), HV (1.52%, five strains), IsPT2 (1.21%, four strains), M6 (1.21%, four strains), M7 (1.21%, four strains), GW1 (1.21%, four strains), and DSM372 (0.61%, two strains). The number of bacteria isolated from M1, M8, and GS was larger, while the main genus was only Streptomyces. The R, X1, and M5 yielded higher genera diversity (21 genera, 20 genera, and 17 genera, respectively). Meanwhile, media R, X1, and M5 were more useful than other media for isolation of rare genera of bacteria, such as Nocardioiides, Leifsonia, Terrabacte, Umezawaea, Vari­vorax, Neorhizobium, and Pararhizobium (Fig. 1B).

Here, we presumed that single-nutrition was the main reason, especially when non-monosaccharide was used as the carbon source (Zhang et al. 2010b; Kurm et al. 2019). This study demonstrated that it is necessary to use various isolation media types to increase the number and diversity of bacteria from highland barley cultivation soil samples.

Diversity of culturable strains at different temperature. There were 62 species and 33 genera bacteria isolated at 25°C, accounting for 80.52% of the species' total number. The predominant genus was Streptomyces (22.08%, 17 species), followed by Bacillus (6.49%, five species), Micromonospora (5.19%, four species), Kribbella (3.90%, three species), and Paenarthrobacter (3.90%, three species). There were 23 species and 18 genera bacteria isolated at 4°C, accounting for 29.87% of the total species, but no dominant genus was found. Meanwhile, only eight species and six genera of bacteria could be isolated at 25°C and 4°C (Fig. 1C). Most common bacteria could be isolated at 25°C, but some rare bacteria could be isolated at 4°C without

Fig. 1. The number and diversity of bacteria.
A) The numbers of bacteria isolated from different media at 4° and 25°. B) Diversity of bacteria isolated from different culture media. C) Diversity of bacteria isolated from different temperature. D) The numbers of dominant species isolated from 4° and 25°.

| Genera distributed in each of the four phyla. |
|-----------------------------------------------|
| **Actinobacteria** | **Proteobacteria** | **Firmicutes** | **Bacteroidetes** |
| Actinoplanes | Micrococcus | Kaistia | Bacillus | Hymenobacter |
| Aeromicrobium | Micromonospora | Luteimonas | Exiguobacterium |
| Agromyces | Nocardia | Neorhizobium | Macroccos |
| Arthrobacter | Nocardioiides | Pararhizobium | Paenibacillus |
| Dietzia | Paenarthrobacter | Phyllobacterium | Peribacillus |
| Glycomyces | Promicromonospora | Pseudomonas | Staphylococcus |
| Gordonia | Pseudarthrobacter | Pseudoxanthomonas |
| Kocuria | Rhodococcus | Skermanella |
| Kribbella | Streptomyces | Sphingopyxis |
| Kyococcus | Terrabacter | Variorax |
| Leifsonia | Umezawaea |
| Longispora | Yinghuangia |
| Microbacterium |
the inhibitory effect of dominant species, promoting the diversity of bacteria (Margesin 2012; Collins and Margesin 2019). The numbers of dominant species mainly isolated at 4°C were *Arthrobacter humicola* (7.25%, 24 strains), while the numbers of dominant species mainly isolated at 25°C were *Streptomyces flavovirens* (8.19%, 27 strains), *Streptomyces xanthophaeus* (7.58%, 25 strains), *Streptomyces canus* (6.36%, 21 strains), and *Bacillus sianensis* (4.24%, 14 strains) (Fig. 1D). The species of culturable bacteria and the

| Strain number | Name of strain having the highest 16S rRNA gene similarity | The highest similarity (%) | Strain number | Name of strain having the highest 16S rRNA gene similarity | The highest similarity (%) |
|---------------|----------------------------------------------------------|-----------------------------|---------------|----------------------------------------------------------|-----------------------------|
| T74*          | Actinoplanes digitatis IFO 12512                          | 98.82                       | T608          | Paenarthrobacter aurescens NRBC 12136                     | 99.07                       |
| T203          | Aeromicrobium ginsengioli Gsoil 098                       | 99.82                       | T236          | Paenarthrobacter nitroguajalicus G2-1                   | 100                         |
| T96*          | Agromyces binzhuoensis OAC1353                           | 98.62                       | T808*         | Pararhizobium herbae CCBAU 83011                        | 98.79                       |
| T229*         | Agromyces humatus CD5                                    | 98.74                       | T209          | Peribacillus simplex NRBC 15720                        | 100                         |
| T805          | Arthrobacter crystallopoides DSM 20117                   | 99.85                       | T811          | Phyllobacterium ifrigiensen STM 370                     | 100                         |
| T763          | Arthrobacter humicola KV-651                             | 100                         | T274*         | Phyllobacterium zundukense Tri-48                       | 98.57                       |
| T65           | Bacillus sianensis KCTC 13613                            | 100                         | T63           | Promicromonospora alba 1C-HV12                         | 100                         |
| T94           | Bacillus cereus ATCC 15479                               | 100                         | T193*         | Pseudarthrobacter sicciolerans 4J27                    | 99.34                       |
| T228*         | Bacillus drentensis LMG 21831                            | 99.34                       | T755          | Pseudomonas latysulfativorans AP3_22                    | 99.73                       |
| T95           | Bacillus pumilus ATCC 7061                               | 100                         | T776          | Pseudomonas lini CFBP 5737                             | 100                         |
| T115          | Bacillus selenat arsenatis SF-1                           | 99.6                        | T174*         | Pseudoxanthomonas sacheonensis BD-c54                   | 99.34                       |
| T822          | Dietzia kunjamenensis subsp DSM 44907                    | 99.86                       | T127*         | Rhodococcus jostii DSM 44719                           | 99.32                       |
| T230          | Exiguobacterium mexicanum 8NT                            | 100                         | T788          | Rhodococcus qingshengi JCM 15477                       | 100                         |
| T183*         | Glycomyces algeriensis NRRL B-16327                      | 98.9                        | T185*         | Skermanella aerolata 5416T-32                          | 98.86                       |
| T64           | Gordonia otiitidis NRBC 100426                           | 100                         | T93           | Sphingopyxis fribergensis Kp.5.2                       | 99.87                       |
| T830*         | Hymenobacter humi DG31A                                  | 98.60                       | T45           | Staphylococcus capratue ATCC 35538                      | 100                         |
| T769*         | Kaistia defluvii B6-12                                   | 99.72                       | T61           | Staphylococcus cohini subsp ATCC 49330                 | 100                         |
| T144          | Kocuria seda ATCC FCS-11                                 | 99.43                       | T666          | Streptomyces albigerinolus NRRL B-1305                  | 100                         |
| T145          | Kribbella albertainiae BC640                             | 100                         | T313          | Streptomyces atrolovicans NRRL ISP-5137                | 100                         |
| T214*         | Kribbella catacombae DSM 19601                           | 99.6                        | T234          | Streptomyces botropensis ATCC 25435                    | 99.87                       |
| T422          | Kribbella kawaraoi Q411                                  | 99.87                       | T130          | Streptomyces caniferus NRBC 15389                      | 99.87                       |
| T823          | Kytopsectus schroeteri DSM 13884                         | 99.73                       | T235          | Streptomyces canus DSM 40017                           | 99.73                       |
| T781          | Leifsonia flavis YSP-B2174                               | 99.73                       | T690          | Streptomyces dissocia A217                             | 99.47                       |
| T416          | Longispora urticae NEAU-PCY-3                            | 99.88                       | T532          | Streptomyces flavovirens NRBC 3716                     | 99.85                       |
| T181*         | Luteimonas composti CC-YY255                             | 98.9                        | T674*         | Streptomyces humidus NRBC 12877                        | 98.8                        |
| T156          | Macrooccus canis KM 45013                                | 99.86                       | T296          | Streptomyces hydrogenans NRBC 13475                    | 99.46                       |
| T489          | Microbacterium maritypicum DSM 12512                     | 99.55                       | T219          | Streptomyces hypholiticus HSM10                        | 99.46                       |
| T773          | Microbacterium natoriense TNJIL43-2                      | 99.87                       | T426          | Streptomyces kusarianovi DSM 13192                     | 99.6                        |
| T804          | Microbacterium phyllophaeae DSM 13468                    | 99.73                       | T569          | Streptomyces lunalectis MM109                          | 99.2                        |
| T133          | Microbacterium thalassium IFO 16060                      | 98.93                       | T348          | Streptomyces niveus NRRL 2466                          | 99.46                       |
| T226          | Micrococcus luteus NCTC 2665                             | 99.63                       | T84           | Streptomyces phaseolitrophicus MRIL 41896             | 99.6                        |
| T47           | Micromonospora cremaea DSM 45599                         | 99.87                       | T581          | Streptomyces turgidiscabies ATCC 700248                | 100                         |
| T206          | Micromonospora lutefulsa GUI2                             | 99.87                       | T110*         | Streptomyces xanthochromogenes NRRL B-5410             | 98.97                       |
| T197*         | Micromonospora palomenae NEAU-CX1                       | 98.74                       | T100          | Streptomyces xanthophaeus NRRL B-5414                  | 99.71                       |
| T92           | Micromonospora saelcenes Lupa 09                        | 100                         | T111*         | Terrabacter ginsengioli Gsoil 653                     | 99.19                       |
| T786*         | Neorhizobium vignae CCBAU 05176                          | 98.70                       | T160          | Umezawaa tangerina NRRL B-24465                       | 99.18                       |
| T62           | Nocardia salmonicida subsp R89                           | 99.47                       | T812          | Varioroxor boronicumulans BAM-48                       | 99.47                       |
| T105*         | Nocardioidea caeni MN8                                   | 98.01                       | T134*         | Yinghuangia seraminata YIM 45720                      | 98.73                       |
| T218          | Paenibacillus odorifer DSM 15391                          | 99.63                       |               |                                                          |                             |

* – shown that the full length 16S rRNA gene of this bacterium was sequenced
Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of soil isolates and related species.
numbers of dominant species were significantly different at 4°C and 25°C in this study.

Potential new species information. Among the 77 species, four bacterial strains exhibited low 16S rRNA gene sequence similarities (< 98.65 %) with validly described species based on the results of the BLAST search in EzBiocloud (Table IV), which indicated that these isolates could represent novel taxa. *Neorhizobium* gen. nov. was a new genus of rhizobia established by Mousavi et al. (2014); so far, only five species had been published. The T786 strain had 98.70%, 98.47%, 98.24%, 98.16%, 97.79%, and 96.55% sequence similarity with *Neorhizobium vignae* CCBAU 05176T (GU128881), *Neorhizobium alkalisoli* CCBAU01393T (EU074168), *Neorhizobium tumejilense* T17_20T (PVBG01000052), *Neorhizobium huautlense* S02T (AF025852), *Neorhizobium galegae* ATCC43677T(D11343), and *Neorhizobium lilium* 24NRT (MK386721), respectively (Fig. 3). Further data analysis suggested that the dDDH and ANI values between strain T786 and *N. vignae* CCBAU 05176T, *N. alkalisoli* CCBAU 01393T, *N. tumejilense* T17_20T, *N. huautlense* S02T, and *N. galegae* ATCC 43677T were 20.20–20.50% and 76.64–80.01%, respectively, which were lower than the threshold values of 70% and 95–96% for species discrimination (unpublished). *Pararhizobium* gen. nov. was a new genus of rhizobia also established by Mousavi et al. (2015); so far, only seven species had been published. The T808 strain had high similarity with *Pararhizobium herbae* CCBAU83011T (GU565534) (98.79%), *Pararhizobium polonicum* F5.1T (GLV01000030) (98.65%), and *Pararhizobium giardini* H152T (ARBG01000149) (98.50%). The 16S rRNA sequence of strain T808 had about 40 more bases in the V1-V2 region than the seven validly published species of *Pararhizobium*, while the NCBI database showed that T808 had 98.54–99.15% similarity with Uncultured bacterium clone barrow_FF_26 (JX668750.1), *Rhizobium* sp.Ia8 (KF444807), and Rhizobiaceae bacterium strain FW305-C-27(MN067584), all of which were uncultured bacteria without lacking the 40 bases in V1-V2 region (Fig. 4). Based on the above analysis, T808 might be a potentially new species of *Pararhizobium*. *Neorhizobium* and *Pararhizobium* were important non-symbiotic species of rhizobia with poor nodulation or nitrogen fixation genes, which have the important microbial niche value (Shen et al. 2018; Soenens et al. 2019).

Three potential new species were isolated from media M5, and one species was isolated from media M8, R, and F1, respectively. Half of six potential new species were cultured at 4°C, while others were cultured at 25°C. The culture medium and temperature have a significant influence on the separation of new species. All six potential new species will be further identified with a polyphasic approach (including chemotaxonomic properties, DNA-DNA hybridization analysis) to determine their taxonomic positions.

Discussion

Together with the incubation of the highland barley cultivation soil sample using fifteen kinds of media at 25°C and 4°C, a total of 830 individual strains were purified. The 16S rRNA gene sequence analysis results are consistent with a previous report, in which Actinobacteria, Proteobacteria, Firmicutes were found to be dominant phyla in the arctic-alpine area, especially in the Qinghai-Tibet plateau (Jiang et al. 2006; Kumar et al. 2016a; Tang et al. 2016). The predominant genus was *Streptomyces*, followed by *Bacillus*, *Micromonospora*, and *Microbacterium*. The most diverse isolates belonged to high the G+C Gram-positive group; in particular, the *Streptomyces* genus is a dominant genus in the high G+C Gram-positive group. The bacteria in arctic-alpine areas are mainly the spore producing, stress-resistant, and thick cell walls microorganisms (Zhang et al. 2010b; Rao et al. 2016).

The Actinobacteria are widely dispersed throughout the highland barley cultivation soil, while few studies are on it. The bacteria in highland barley cultivation soil in Lhasa analyzed by high-throughput sequencing technology showed that the main actinomycetes were *Gaiella*, *Arthrobacter*, and *Nocardioides* (Liu et al.
Fig. 3. Phylogenetic tree based on the 16S rRNA gene sequences of new candidates and related species.
Culturable bacteria diversity in highland cultivation soil

2020), which was quite different from our study using the culturable technique. In other previous reports, the main genus in the highland barley cultivation soil was *Streptomyces*, *Arthrobacter*, and *Nocardioides*. Most Actinomycetes had a wide spectrum of inhibitory activity against pathogenic bacteria, highly IAA production, and phosphate solubilization, which were in similarity with our study (Qi et al. 2017; Yin et al. 2017; Gao et al. 2019). As the most well-known genus in Actinobacteria, *Streptomyces* contains 960 species (http://www.bacterio.net/streptomyces) and 4227 genome assemblies available (https://www.ncbi.nlm.nih.gov/genome/streptomyces) at the time of writing. Members of the genus *Streptomyces* are well known as the primary sources of antibiotics with diverse biological activities and chemical structures (Jones and Elliot 2017; Li et al. 2018). In this study, 17 species of *Streptomyces* were found in the highland barley cultivation soil, the larger numbers of dominant species of *Streptomyces* were *Streptomyces flavovirens*, *Streptomyces xanthophaeus* and *Streptomyces canus*, which were mainly isolated at 25°C. The Qamdo region's temperature is between 20°C and 28°C from June to September, which is also a critical growth period for highland barley. We believe that these *Streptomyces* that can produce many biological activities have an essential role in the growth of highland barley in this period. The other dominant isolates in highland barley cultivation soil were *Arthrobacter humicola* and *Bacillus saniensis*, which are important plant growth-promoting rhizobacteria (PGPR) (Bai et al. 2015).

Meanwhile, *Arthrobacter humicola* was mainly isolated at 4°C, producing cold lipase and biopolymeric flocculant (Agunbiade et al. 2017). The low-temperature adaptation and ecological function of *A. humicola* in highland barley cultivation soil need to be studied in-depth. Some rare Actinobacteria were also isolated from the soil sample, for example, *Leifsonia*, *Longispora*, *Nocardia*, *Nocardioides*, *Terrabacter*, *Umezawae*, and *Kribbella*. Rare Actinobacteria are also important sources in discovering novel antibiotics and have been seldom studied (Cai et al. 2018; Bundale et al. 2019).

In summary, this study has demonstrated a rich diversity of bacteria (especially Actinobacteria) and some undiscovered bacteria species in the highland barley cultivation soil of Qinghai-Tibet plateau it suggests that these strains might represent a valuable source of new taxa for further microbial development and utilization. Additionally, this study indicates that cultivating Actinobacteria in highland barley cultivation soil of Qinghai-Tibet plateau could be interesting for further study.

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| T808 | Uncultured bacterium clone barrow FF26 | Rhizobiaceae bacterium strain FW305-C-27 | Rhizobium sp. IA86 | Pseudomonas sp. IFAM 1004 | Consensus |
|------|----------------------------------------|------------------------------------------|-------------------|----------------------------|-----------|
| A     | G                      | G                      | C                 | G                        | A**C**    |
| T     | C                      | A                      | G                 | A                        | T**G**    |
| C     | A                      | G                      | C                 | C                        | A**C**    |
| T     | G                      | C                      | C                 | A                        | G**TA**   |

Fig. 4. The Clustal X analysis of strain T808.
Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Agunbiade MO, Van Heerden E, Pohl CH, Ashafa AT. Flocculating performance of a biocldulant produced by Arthrobacter lumicola in sewage waste treatment. BMC Biotechnol. 2017 Dec;17(1):51. https://doi.org/10.1186/s12898-017-0375-0

Bai Y, Müller DB, Srinivas G, Garrido-Otero R, Potthoff E, Rott M, Dombrowski N, Münch PC, Spaepen S, Remus-Emsermann M, et al. Functional overlap of the Arabidopsis leaf and root microbiota. Nature. 2015 Dec;528(7582):364–369. https://doi.org/10.1038/nature16192

Bailly A, Weiskopf I. The modulating effect of bacterial volatiles on plant growth: current knowledge and future challenges. Plant Signal Behav. 2012 Jan;7(1):79–85. https://doi.org/10.4161/psb.7.1.18418

Bodelier PLE. Toward understanding, managing, and protecting microbial ecosystems. Front Microbiol. 2011;2:80. https://doi.org/10.3389/fmicb.2011.00080

Bundale S, Singh J, Begde D, Nashikkar N, Upadhyay A. Rare actinobacteria: a potential source of bioactive polyketides and peptides. World J Microbiol Biotechnol. 2019 Jun;35(6):92.

Cai Y, Tao WZ, Ma YJ, Cheng J, Zhang MY, Zhang YX. Leifsonia flava sp. nov., a novel actinobacterium isolated from the rhizosphere of Aquilegia viridiflora. J Microbiol. 2018 Aug;56(8):549–555. https://doi.org/10.1007/s12275-018-8061-x

Collins T, Margesin R. Psychrophilic lifestyles: mechanisms of adaptation and biotechnological tools. Appl Microbiol Biotechnol. 2019 Apr;103(7):2857–2871. https://doi.org/10.1007/s00253-019-09659-5

Delgado-Baquerizo M, Oliverio AM, Brewer TE, Benavent-González A, Eldridge DJ, Bardgett RD, Maestre FT, Singh BK, Fierer N. A global atlas of the dominant bacteria found in soil. Microbiome. 2017 Dec;5:73. https://doi.org/10.1186/s40168-017-0289-5

Deng N, Zheng B, Li T, Liu RH. Two novel alkalophiles, Bacillus alkalisoli sp. nov., and Bacillus solitudinis sp. nov., isolated from saline-alkali soil. Extremophiles. 2017 Jul;21(4):1175. https://doi.org/10.1007/s10227-017-01127-2

Distantly sampled microorganisms in alpine soils. In: Florschütz J, Blackall L., editor. Plants in Alpine regions. Vienna (Austria): Springer; 2012. p. 187–198.

Furlong MA, Singleton DR, Coleman DC, Whitman WB. Psychrophilic lifestyles: mechanisms of the genetic diversity and promoting functions of the culturable actinobacteria and their potential for plant growth promotion. Environ Microbiol. 2004 Dec;6(12):1244–1251. https://doi.org/10.1111/j.1462-2920.2004.00658.x

Gao X, Gu YF, Nyima T, Liu GY, Liu T, Liu Y, Pubu G. Isolation and characterization of soybean-associating bacteria and their potential for plant growth promotion. Environ Microbiol. 2014 Feb;16(4):346–351. https://doi.org/10.1007/s00299-015-2702-3

González A, Eldridge DJ, Bardgett RD, Maestre FT, Singh BK, Collins T, Margesin R. Psychrophilic lifestyles: mechanisms of adaptation and biotechnological tools. Appl Microbiol Biotechnol. 2019 Apr;103(7):2857–2871. https://doi.org/10.1007/s00253-019-09659-5

He Q, Wang X, He L, Yang L, Wang S, Bi Y. Alternative respiration pathway is involved in the response of highland barley to salt stress. Plant Cell Rep. 2019 Mar;38(3):295–309. https://doi.org/10.1007/s00299-018-2366-6

Jiang H, Dong H, Zhang G, Yu B, Chapman LR, Fields MW. Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China. Appl Environ Microbiol. 2006 Jun;72(6):3832–3845. https://doi.org/10.1128/AEM.02869-05

Jones SE, Elliot MA. Streptomyces exploration: competition, volatility, and antibiotic production. Trends Microbiol. 2017 Jul;25(7):522–531. https://doi.org/10.1016/j.tim.2017.02.001

Kim M, Oh HS, Park SC, Chun J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol Microbiol. 2014 Feb;64(1 Pt 2):346–351. https://doi.org/10.1099/ijs.0.05977-0

Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ Microbiol. 2004 Dec;6(12):1244–1251. https://doi.org/10.1111/j.1462-2920.2004.00658.x

Kumar M, Männistö MK, van Elsas JD, Nissinen RM. Plants impact structure and function of bacterial communities in Arctic soils. Plant Soil. 2016a Feb;391(1–2):319–332. https://doi.org/10.1007/s11104-015-2702-3

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016b Jul;33(7):1870–1874. https://doi.org/10.1093/molbev/msw054

Kurma V, van der Putten WH, Hol WHG. Cultivation–sustenance of rare soil bacteria is not influenced by incubation time and growth medium. PLoS One. 2019 Jan 10;14(1):e0210073. https://doi.org/10.1371/journal.pone.0210073

Li F, Liu S, Lu Q, Zheng H, Osterman IA, Lukyanov DA, Sergiev PV, Donselova OA, Liu Y, Ye J, et al. Studies on antibacterial activity and diversity of cultivable actinobacteria isolated from manure soil in Futian and Maoweihai of China. Evid Based Complement Alternat Med. 2019 Jun 09;2019:1–11. https://doi.org/10.1155/2019/347686

Li L, Wei K, Zheng G, Liu X, Chen S, Jiang W, Lu Y. CRISPR-Cpf1-assisted multiplex genome editing and transcriptional repression in Streptomyces. Appl Environ Microbiol. 2018 Jul 06;84(18):e00827-18

Liu GH, Narsing Rao MP, Dong ZY, Chen JP, Chen Z, Liu B, Li WJ. Two novel alkaliphiles, Bacillus alkalisoli sp. nov., and Bacillus solitudinis sp. nov., isolated from saline-alkali soil. Extremophiles. 2019 Nov;23(6):759–764. https://doi.org/10.1007/s00792-019-01127-2

Liu QH, Pan H, Da WZM, Tian Y, Liu HH, Wang C, Lu XY, Bai JP. Microbial commu...
Qi SS, Zhou LH, Hu JP, Liu M, Zhao H, Xiong Y. [Isolation, identification and diversity of soil bacteria in multiple regions from Tibetan Plateau] (in Chinese). Xi Nan Nong Ye Xue Bao. 2017;30(7):1629–1635.

Rao S, Chan OW, Lacap-Bugler DC, Pointing SB. Radiation-tolerant bacteria isolated from high altitude soil in Tibet. Indian J Microbiol. 2016 Dec;56(4):508–512. https://doi.org/10.1007/s12088-016-0604-6

Řeháková K, Chroňáková A, Kristůfek V, Kuchtová B, Čapková K, Scharfen J, Čapek P, Doležal J. Bacterial community of cushion plant Thylacospermum caespitosum on elevational gradient in the Himalayan cold desert. Front Microbiol. 2015 Apr 16;6:304. https://doi.org/10.3389/fmicb.2015.00304

Shen X, Li Y, Zhao Z, Han YE, Zhang WW, Yu XY, Zhang CY, Sun C, Wu M. Polyphasic taxonomic characterisation of a novel strain as Pararhizobium haloflavum sp. nov., isolated from soil samples near a sewage treatment tank. Antonie van Leeuwenhoek. 2018 Apr;111(4):485–491. https://doi.org/10.1007/s10482-017-0969-5

Shen Y, Zhang H, Cheng L, Wang L, Qian H, Qi X. In vitro and in vivo antioxidant activity of polyphenols extracted from black highland barley. Food Chem. 2016 Mar;194:1003–1012. https://doi.org/10.1016/j.foodchem.2015.08.083

Soenens A, Gomila M, Imperial J. Neorhizobium tomejilense sp. nov., first non-symbiotic Neorhizobium species isolated from a dryland agricultural soil in southern Spain. Syst Appl Microbiol. 2019 Mar;42(2):128–134. https://doi.org/10.1016/j.syapm.2018.09.001

Tang JY, Ma J, Li XD, Li YH. Illumina sequencing-based community analysis of bacteria associated with different bryophytes collected from Tibet, China. BMC Microbiol. 2016 Dec;16(1):276. https://doi.org/10.1186/s12866-016-0892-3

Yin MY, He IQ, Zhang GJ. [Biological activity and diversity of psychrophilic Actinomycetes in Tibet] (in Chinese). J Northwest Agric Forest Univer (Nat Sci Ed). 2017;45(6):221–234.

Yu Y, Guo Z, Wu H, Kahmann JA, Oldfield F. Spatial changes in soil organic carbon density and storage of cultivated soils in China from 1980 to 2000. Global Biogeochem Cy. 2009;23:GB2021. https://doi.org/10.1029/2008GB003428

Yuan Y, Si G, Wang J, Luo T, Zhang G. Bacterial community in alpine grasslands along an altitudinal gradient on the Tibetan Plateau. FEMS Microbiol Ecol. 2014 Jan;87(1):121–132. https://doi.org/10.1111/1574-6941.12197

Zhang G, Niu F, Ma X, Liu W, Dong M, Feng H, An L, Cheng G. Phylogenetic diversity of bacteria isolates from the Qinghai-Tibet Plateau permafrost region. Can J Microbiol. 2007 Aug;53(8):1000–1010. https://doi.org/10.1139/W07-031

Zhang K, Yang J, Qiao Z, Cao X, Luo Q, Zhao J, Wang F, Zhang W. Assessment of β-glucans, phenols, flavor and volatile profiles of hulless barley wine originating from highland areas of China. Food Chem. 2019 Sep;293:32–40. https://doi.org/10.1016/j.foodchem.2019.04.053

Zhang S, Yang G, Wang Y, Hou S. Abundance and community of snow bacteria from three glaciers in the Tibetan Plateau. J Environ Sci (China). 2010a Sep;22(9):1418–1424. https://doi.org/10.1016/S1001-0742(09)60269-2

Zhang Y, Dong S, Gao Q, Liu S, Zhou H, Ganjurjav H, Wang X. Climate change and human activities altered the diversity and composition of soil microbial community in alpine grasslands of the Qinghai-Tibetan Plateau. Sci Total Environ. 2016 Aug;562:353–363. https://doi.org/10.1016/j.scitotenv.2016.03.221

Zhang YQ, Liu HY, Chen J, Yuan LJ, Sun W, Zhang LX, Zhang YQ, Yu LY, Li WJ. Diversity of culturable actinobacteria from Qinghai-Tibet plateau, China. Antonie van Leeuwenhoek. 2010b Aug;98(2):213–223. https://doi.org/10.1007/s10482-010-9434-4

Zhao NN, Guggenberger G, Shibistova O, Thao DT, Shi WJ, Li XG. Aspect-vegetation complex effects on biochemical characteristics and decomposability of soil organic carbon on the eastern Qinghai-Tibetan Plateau. Plant Soil. 2014 Nov;384(1–2):289–301. https://doi.org/10.1007/s11104-014-2210-x