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Phenotypic and Phylogenetic Analysis of Lactic Acid Bacteria Isolated from Forage Crops and Grasses in the Tibetan Plateau

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A total of 140 lactic acid bacteria (LAB) strains were isolated from corn, alfalfa, clover, sainfoin, and Indian goosegrass in the Tibetan Plateau. According to phenotypic and chemotaxonomic characteristics, 16S rDNA sequence, and *rec*A gene PCR amplification, these LAB isolates were identified as belonging to five genera and nine species. Corn contained more LAB species than other forage crops. *Leuconostoc pseudomesenteroides*, *Lactococcus lactis* subsp. *lactis*, *Lactobacillus brevis*, and *Weissella paramesenteroides* were dominant members of the LAB population on alfalfa, clover, sainfoin, and Indian goosegrass, respectively. The comprehensive 16S rDNA and *rec*A-based approach effectively described the LAB community structure of the relatively abundant LAB species distributed on different forage crops. This is the first report describing the diversity and natural populations of LAB associated with Tibetan forage crops, and most isolates grow well at or below 10°C. The results will be valuable for the future design of appropriate inoculants for silage fermentation in this very cold area.

Keywords: forage, epiphytic microflora, lactic acid bacteria, 16S rDNA, *rec*A gene

Introduction

Silage making has become a major method of forage conservation throughout the world. Of the many factors that could influence this process, the number and type of microorganisms that dominate the fermentation process often dictate the final quality of the silage (Lin *et al.*, 1992; Cai, 1999). Usually, the epiphytic lactic acid bacteria (LAB) present on forage crops and grasses convert water-soluble carbohydrates (WSC) into lactic acid during the ensiling process, thereby reducing the final pH and enhancing the nutritional value of the silage (McDonald *et al.*, 1991).

The Tibetan Plateau is an area in northwestern China and adjoining countries with an average altitude of over 4,000 m (Duan *et al.*, 2008). Specific geographic and climatic conditions have led to the development of unique species on the Tibetan Plateau. In this distinct ecological region, high biodiversity impacts the formation of the specific microflora found in fermented milk, vegetables, forage crops, and grasses.

Corn (*Zea mays* L.), alfalfa (*Medicago sativa* L.), clover (*Trifolium scabrum* L.), sainfoin (*Onobrychis viciifolia* L.), and Indian goosegrass (*Eleusine indica* L.) are major forage crops and grasses that are widely used to make silage for ruminant feed in the Tibetan Plateau area. However, limited information is available on the characteristics of natural LAB on forage crops and grasses of this region. This study was conducted to identify and detail the predominant LAB on five forage crops and grasses in the Tibetan Plateau using phenotypic and phylogenetic methods. Isolates were identified biochemically, and selected representative strains were identified at the molecular level using the 16S rDNA sequence and *rec*A gene amplification.

Materials and Methods

Samples and bacterial isolates

Corn at milk stage and alfalfa, clover, sainfoin, and Indian goosegrass at flowering stage were obtained from dairy farms in the Tibetan Plateau, northwestern China. Areas of collection and strains used in this study are shown in Table 1.

Samples (10 g) were chopped into 1-cm lengths and shaken well for 60 sec with 90 ml sterile distilled water, then serially diluted from 10^{-1} to 10^{-5} with sterile water. The number of LAB was measured in plates on lactobacilli MRS agar (Difco Laboratories, USA) incubated at 30°C for 48 h in an anaerobic box (TE-HER Hard Anaerobox, ANX-1; Hirosawa Ltd., Japan). About 15–20 strains on MRS agar were selected randomly from each sample; a total of 215 isolates were collected, of which 140 isolates were determined to be LAB by Gram-staining, catalase tests, and lactic acid production. Their physiological properties were then determined using the methods of Kozaki *et al.* (1992). Coliform bacteria were counted on blue light broth agar (Nissui Ltd., Japan) incubated at 30°C for 48 h. Molds and yeasts were counted on potato dextrose agar (Nissui Ltd.) incubated at 30°C for 24 h, and yeasts were distinguished from molds and other bacteria by colony appearance and the observation of cell morphology. Bacilli and aerobic bacteria were counted on

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Table 1. Sample collected places and representative lactic acid bacteria stains

Samples	Collection place	Representative strains
Corn	Linzhi cattle farm, Linzhi, Tibet	CW 40, CW 41, CW 43, CW 44, CW 45, CW 46
Alfalfa	Qinchuan dairy farm, Lanzhou, Gansu	CW 13, CW 14, CW 15, CW 16
Clover	Linzhi cattle farm, Linzhi, Tibet	CW 53, CW 54, CW 55
Sainfoin	Qinchuan dairy farm, Lanzhou, Gansu	CW 11, CW 12
Goosegrass	Linzhi cattle farm, Linzhi, Tibet	CW 47, CW 48, CW 49, CW 51

nutrient agar (Nissui Ltd.) incubated at 30°C for 24 h under aerobic conditions. Colonies were counted as viable numbers of microorganisms in colony-forming units (CFU) per g of fresh matter (FM). Each LAB colony was isolated and purified twice by streaking on MRS agar plates. Pure cultures were grown on MRS agar at 30°C for 24 h, then resuspended in a solution of nutrient broth (Difco Laboratories) and dimethyl sulfoxide at a ratio of 9:1, and stored as stock cultures in a freezer (Sanyo, Japan) at -80°C for further examination.

Morphological, physiological, and biochemical tests

LAB morphology and Gram-staining response were examined after 24 h of incubation on MRS agar. Catalase activity and gas production from glucose were determined using the methods of Kozaki *et al.* (1992). Growth at different temperatures was observed in MRS broth after incubation at 10°C and 15°C for 14 days and at 20, 25, 30, 35, 40, 45, and 50°C for 7 days. Salt tolerance was determined in MRS broth containing 3.0% and 6.5% NaCl. Growth of LAB at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, and 8.0 was determined in MRS broth after incubation at 30°C for 7 days. Carbohydrate fermentation tests were carried out using analytical profile index [API; 50 carbohydrates (CH)] strips (bioMérieux, Japan) for 49 different compounds and one control according to the manufacturer's instructions; reactions were determined after incubation at 30°C for 48 h.

16S rRNA gene sequencing and *rec*A gene PCR amplification

Cells grown at 30°C for 8 h in MRS broth were used for DNA extraction and purification, as described in Saito and Miura (1963). The 16S rRNA gene sequence coding region was amplified by PCR in a PCR thermal cycler (TaKaRa Shuzo Co. Ltd., Japan). The sequences of the PCR products were determined directly with a sequencing kit (ALFexpress AutoCycle; Pharmacia Biotech, USA) using the prokaryotic 16S ribosomal DNA universal primers 27F (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTT ACGACTT-3′) (Cai *et al.*, 1999a). Sequence similarity searches were performed using the DNA Database of Japan (DDBJ) and the Basic Local Alignment Search Tool (BLAST). The sequence information was then imported into the CLUSTAL X software program (Hitachi Software Engineering Co., Japan) for assembly and alignment. The 16S rDNA sequences of China west (CW) strains were compared with sequences from type LAB strains held in the DDBJ (Figs. 1 and 2). Nucleotide substitution rates (*K*nuc values) were calculated (Kimura and Ohta, 1972) and phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987). *Bacillus subtilis* NCDO 1769^T was used as an outgroup organism. The topologies of trees were evaluated using bootstrap analysis of the sequence data with Molecular Evolutionary Genetics Analysis (MEGA) 4 software (Tamura *et al.*, 2007), based on 1,000 random resamplings (Eitan *et al.*, 2006). The sequences were aligned with published sequences of the type strains from the DDBJ, GenBank, and the European Molecular Biology Laboratory (EMBL).

The strains in groups A and C, and type strains, *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum*, were distinguished through partial amplification product comparison of the *rec*A gene according to Torriani *et al.* (2001).

The nucleotide sequences for 16S rDNA described in this report were deposited with the DDBJ/ GenBank/ EMBL under accession nos. AB602799–AB602817 for the strains CW 11, CW 12, CW 14, CW 49, CW 13, CW 15, CW 16, CW 40, CW 41, CW 43, CW 44, CW 45, CW 46, CW 47, CW 48, CW 51, CW 53, CW 54, and CW 55, respectively.

Results

Counts of microorganisms

The counts of microorganisms are shown in Table 2. Overall, 10^3–10^4 CFU/g FM LAB, 10^4–10^6 CFU/g FM coliform bacteria, 10^4–10^6 CFU/g FM aerobic bacteria, and 10^3–10^5 CFU/g FM yeasts were found in all samples. Bacilli were found at 10^5 CFU/g FM in Indian goosegrass and at 10^4 CFU/g FM in corn, but few were counted on other forage crops and grasses. Molds were present at 10^4 CFU/g FM in alfalfa and at 10^2 CFU/g FM in corn.

Morphological, physiological, and biochemical properties

Cell forms, characteristics, and API 50 CH fermentation patterns of representative strains isolated from forage crops and grasses are shown in Tables 3 and 4. A total of 215 strains were isolated from these forage crops and grasses, of which 140 isolated strains (44 strains from corn, 29 strains from alfalfa, 22 strains from clover, 15 strains from sainfoin, and 30 strains from Indian goosegrass) were considered as LAB, based on the Gram-positive reaction, negative catalase reaction, and production of lactic acid as the main fermentation product. No LAB isolate grew at pH 3.0, but all grew at 15–40°C, in 3.0% NaCl, and at pH 4.0–8.0. According to the morphological, physiological, and biochemical properties, these strains were divided into nine groups (A–I). Strains in all groups produced acid from glucose, fructose, N-acetyl-glucosamine, and maltose, but failed to produce acid from erythritol, D-arabinose, L-xylose, inositol, glycogen, D-lyxose, D-fucose, and L-fucose. Strains in groups A (n=7), B (n=6), D (n=8), F (n=7), and G (n=9) had the same growth temperature, salt tolerance, growth

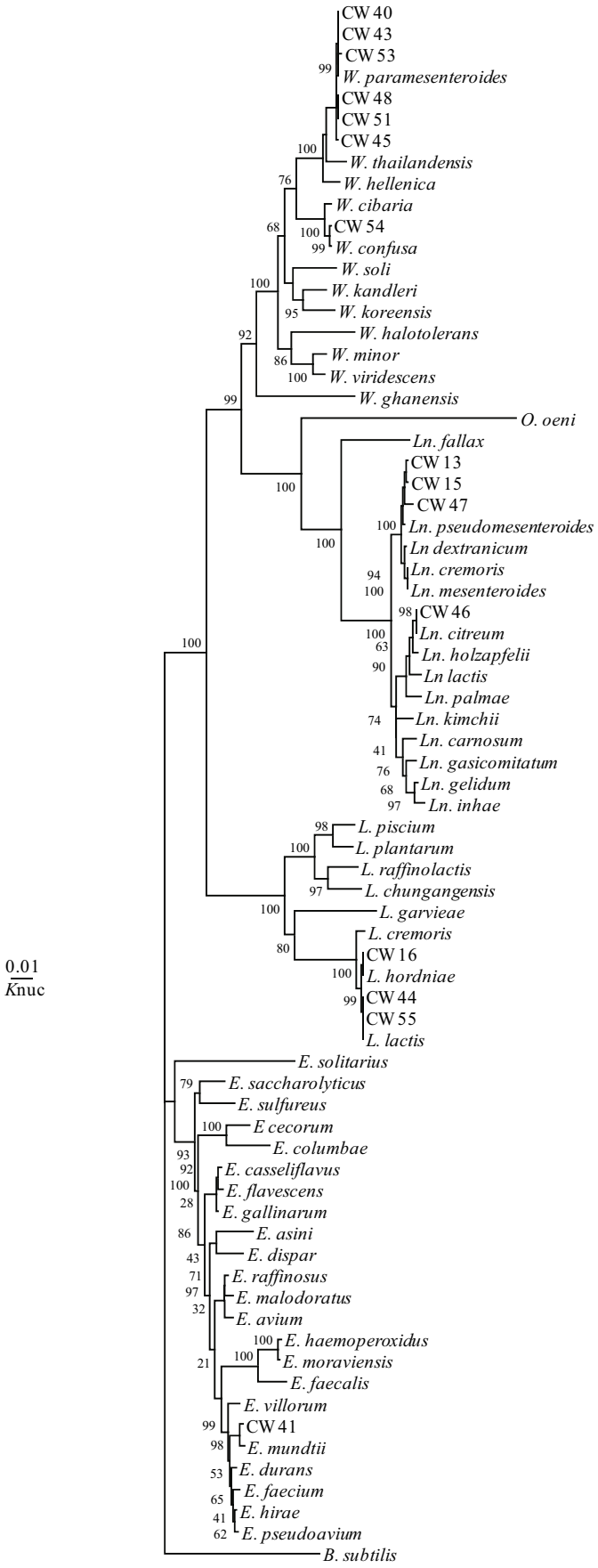

Fig. 1. Phylogenetic tree showing the relative position of *Leuconostoc*, *Weissella*, *Lactococcus* and *Enterococcus* species as inferred by the neighbor-joining method with 16S rRNA gene sequences. *B. subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *Ln.*, *Leuconostocs*; *W.*, *Weissella*, *L.*, *Lactococcus*; *E.*, *Enterococcus*.

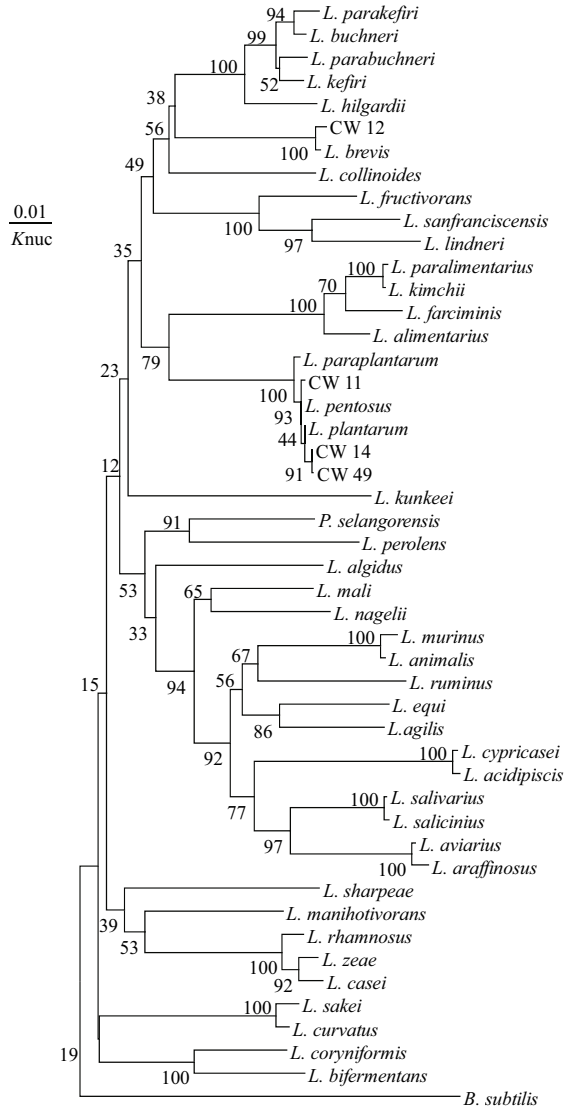

Fig. 2. Phylogenetic tree showing the relative position of *L. plantarum*, *L. brevis*, and *L. paraplantarum* species as inferred by the neighbor-joining method with 16S rRNA gene sequences. Bootdstrap values for a total of 100 replicates are shown at the nodes of the tree. *B. subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *K*nuc, nucleotide substitution rates.

pH, and carbohydrate fermentation patterns. On the other hand, strains in groups C (n=15), E (n=44), H (n=22), and I (n=22) were separated into two, six, three, and three subgroups, respectively, with different properties. Each representative strain from these groups is listed in Table 3 and was used for phylogenetic analysis. Strains in groups A (representative strain: CW 11), B (CW 12), and C (CW 14 and CW 49) were homofermentative rod-shaped bacteria that produced lactic acid as the D (-) isomer and did not produce gas from glucose. Strains in group A grew in 6.5% NaCl, but those in groups B and C did not. Unlike strains in groups A and C, strains in group B did not produce acid from mannose, mannitol, sorbitol, amygdalin, arbutin, esculin, salicin, cellobiose, lactose, saccharose, trehalose, raffinose, or gentiobiose. Groups D (representative strain: CW 41) and I (CW 16, CW 44, and CW 55) were homofermentative cocci that produced lactic acid as L (+) isomers. The other members of groups E (CW 40, CW 43, CW 45, CW

Table 2. Microbiological analysis of forge crop and grasses used in this study

Samples	Counts (CFU g of FM^{-1}) of viable microorganisms					
	Lactica acid bacteria	Bacilli	Coliform bacteria	Aerobic bacteria	Mold	Yeast
Corn	3.5×10^4	1.0×10^4	5.0×10^6	5.1×10^6	5.0×10^2	1.2×10^5
Alfalfa	7.5×10^3	ND	4.5×10^4	1.9×10^6	ND	1.6×10^5
Clover	1.1×10^3	ND	2.4×10^6	3.4×10^6	3.0×10^4	1.1×10^5
Sainfoin	1.0×10^3	ND	3.0×10^6	3.5×10^4	ND	2.5×10^3
Goosegrass	4.1×10^3	1.5×10^5	7.5×10^5	5.0×10^6	ND	5.5×10^4

CFU, colony forming unit; FM, fresh matter. ND, not detected.

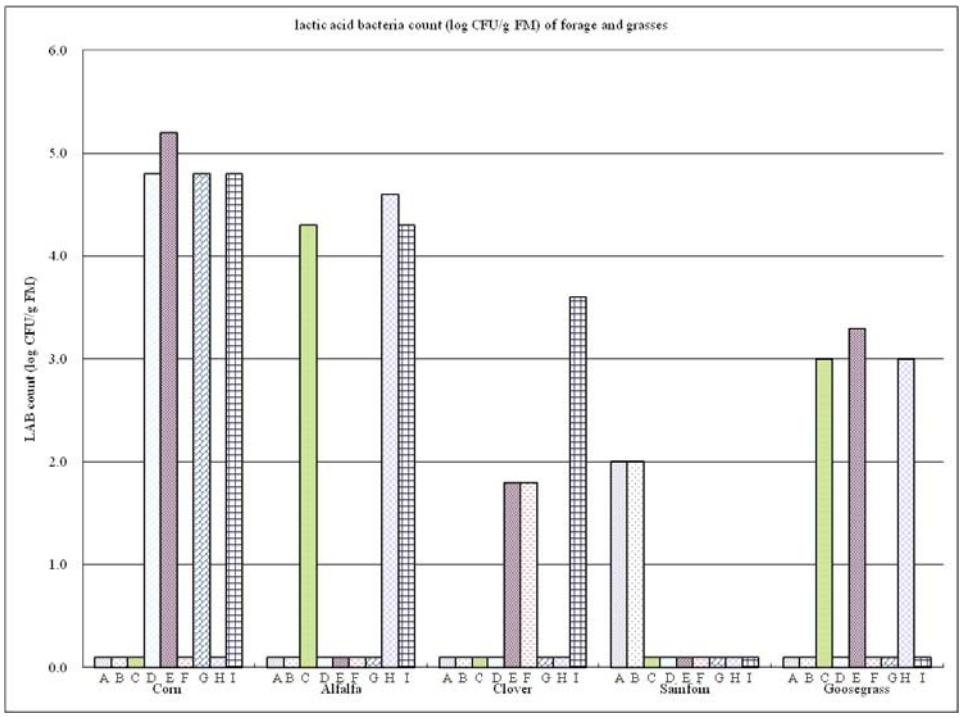

Fig. 3. Distribution of LAB on fresh forage crop and grasses.

A: *Lactobacillus paraplantarum*
B: *Lactobacillus brevis*
C: *Lactobacillus plantarum*
D: *Enterococcus mundtii*
E: *Weissella paramesenteroides*
F: *Weissella confusa*
G: *Leuconostoc citreum*
H: *Leuconostoc pseudomesenteroides*
I: *Lactococcus lactis* subsp. *lactis*

48, CW 51, and CW 53), F (CW 54), G (CW 46), and H (CW 13, CW 15, and CW 47) were heterofermentative cocci that produced lactic acid as D (-) isomers. Unlike strains in group D, those in Group I did not grow at 45°C and did not produce acid from sorbitol or D-tagatose. Most strains in group E did not produce acid from esculin, unlike those in group I, and strains in both groups produced acid from D-xylose, which distinguished them from strains in groups F and G. Unlike strains in groups E, G, and H, group F strains grew at 10°C and produced acid from sorbose, rhamnose, α-methyl-D-mannoside, D-tagatose, D-arabitol, and L-arabitol, but not from L-arabinose or D-turanose. Group G strains did not produce acid from ribose, galactose, mannose, or melibiose, unlike the other heterofermentative cocci groups.

16S rRNA gene sequencing

Phylogenetic trees of representative strains constructed from evolutionary distances using the neighbor-joining method are shown in Figs. 2 and 3. The strains in all groups were placed in a cluster consisting of the genera *Lactobacillus*, *Enterococcus*, *Weissella*, *Leuconostoc*, and *Lactococcus*. Strains in groups A, B, and C were placed in the cluster of genus *Lactobacillus* in the phylogenetic tree (Fig. 2); groups A and C formed a very well-defined cluster with three type strains (*L. plantarum*, *L. pentosus*, and *L. paraplantarum*) and 100% bootstrap values confirmed monophyly. Group B strains were well clustered as *Lactobacillus brevis* with 100% bootstrap support. Strains in group D were placed in the *Enterococcus* cluster, with *E. mundtii* being the most closely related species (99% bootstrap support; Fig. 1). Representative strains of groups E and F were placed in the *Weissella* cluster; both formed a distinct cluster with *W. paramesenteroides* and *W. confusa*, with bootstrap values of 99% and 100%, respectively. Strains in groups G and I were placed in the *Leuconostoc* cluster, and distinctly clustered with *L. citreum* and *L. pseudomesenteroides* with high bootstrap values of 98% and 100%, respectively. The representative strain of

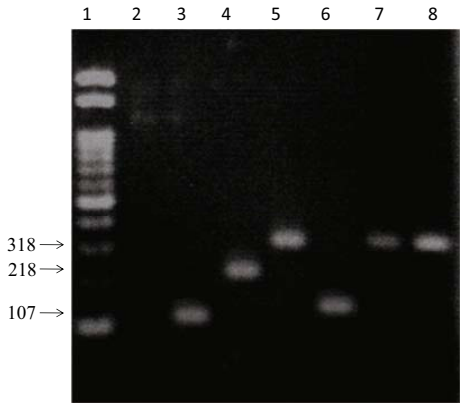

Fig. 4. Amplification products obtained from the *rec*A multiplex assay. Lane 1 contained a 2-kb PLUS DNA ladder (Wako Pure Chemical Industries, Ltd., Japan). Lanes 2, 3, 4 and 5, PCR amplification products from *L. casei* JCM 16167^T (negative control), *L. paraplantarum* JCM 12533^T, *L. pentosus* JCM 1558^T and *L. plantarum* JCM 1149^T, respectively; Lanes 6, 7 and 8, PCR amplification products from CW 11, CW14, and CW 49, respectively.

group I was placed in a cluster of *Lactococcus* and was ascribed to the subspecies *lactis* on the phylogenetic tree, with a 99% bootstrap value supporting its monophyly.

Amplification products obtained from the *rec*A gene multiplex assay

Amplification products obtained from the *rec*A gene are shown in Fig. 4. Group C strains and type strain *Lactobacillus paraplantarum* JCM 12533^T produced 107 bp *rec*A gene amplification products, and representative strains in group A and type strain *Lactobacillus plantarum* JCM 1149^T produced 318 bp products, whereas the negative control *Lactobacillus casei* produced no amplicon. Thus, strains in group A were clearly identified as *L. paraplantarum* and those in group C as *L. plantarum*.

Discussion

The preservation of forage crops and grasses as silage depends on the production of sufficient acid to inhibit the activity of undesirable microorganisms, such as clostridia and molds, under anaerobic conditions. LAB are responsible for the fermentation of silage in the process of forage conversion, in which epiphytic LAB lower the pH of forage crops and grasses to about pH 4.0 (Stiles, 1996); in contrast, unwanted bacteria, such as clostridia, increase the pH through production of acetic acid and *n*-butyric acid, thereby decreasing the nutritional value (McDonald *et al.*, 1991). Cai *et al.* (1998, 1999a, 1999b) found that the predominant LAB were lactic acid-producing cocci and that few were lactobacilli, which play an important role in promoting longer lactic-acid fermentation during silage production. Epiphytic lactobacilli counts in silage crops are usually low and variable, but must reach a level of at least 10^5 CFU/g FM to ensure successful silage storage. Some LAB ($<10^4$ CFU/g FM) were present in the forage crops and grasses examined (Table 2), but further studies and ensiling experiments are required to isolate and identify LAB from laboratory and farm silage in Tibet. This research would provide insight into whether the Tibetan highlands are unique with regard to the species present.

The regional characteristics, low temperatures, and geography of the Tibetan Plateau may allow almost all strains to grow well at 10°C, apart from strains CW 54 and CW 55. The isolates obtained belonged to five different LAB genera: *Lactobacillus*, *Enterococcus*, *Weissella*, *Leuconostoc*, and *Lactococcus*. Lactobacilli were common on sainfoin, alfalfa, and Indian goosegrass; enterococci were found only on corn; *Weissella* spp. appeared on Indian goosegrass, clover, and corn; and *Leuconostoc* spp. and lactococci were mainly on alfalfa, Indian goosegrass, and corn. This study showed that corn contained the most LAB genera and species. In addition, different species of *Lactobacillus*, *Enterococcus*, *Weissella*, *Leuconostoc*, and *Lactococcus* were distributed on different forage crops and grasses. This report is the first to describe most of these LAB species on forage crops and grasses in the Tibetan Plateau area.

Based on phylogenetic analysis, strains in groups B, D, E, F, G, and H were unambiguously identified as *Lactobacillus brevis*, *Enterococcus mundtii*, *Weissella paramesenteroides*, *W. confusa*, *Leuconostoc citreum*, and *Leuconostoc pseudomesenteroides*, respectively (Figs. 2 and 3).

Group A (representative strain: CW 11) and group C (CW 14 and 49) strains were placed on the phylogenetic tree together with *L. pentosus*, *L. plantarum*, and *L. paraplantarum* in a 100% bootstrap cluster (Fig. 2). Strain CW 11 had 16S rRNA sequence similarities of 99.8% and 99.7% to type strains *L. plantarum* and *L. pentosus*, respectively; strain CW 14 showed 99.5% and 99.4% similarity, and CW 49 had 99.8% and 99.7% similarity to these two type strains, respectively. According to Hammes and Vogel (1995), Curk *et al.* (1996), Ennahar *et al.* (2003), and Pang *et al.* (2011), members of the *L. plantarum* group, including *L. pentosus*, *L. plantarum*, and *L. paraplantarum*, have very similar 16S rRNA gene sequences that differ only by 2 bp. We further defined their carbohydrate fermentation patterns, but found ambiguity between our strains and these three type strains that prevented identification to the species level based on the 16S rRNA gene sequence and API 50 CH analysis. Therefore, other phylogenetic analytical methods were required to accurately distinguish these strains (Eisen, 1995). PCR amplification analysis of partial *rec*A gene products permitted a clear distinction among these three type strains and representative strains in groups A and C (Fig. 4). Group A had the same *rec*A gene amplification product (107 bp) as *L. paraplantarum*, and group C had the same product (318 bp) as *L. plantarum*; therefore, strains in groups A and C were identified as *L. paraplantarum* and *L. plantarum*, respectively. This is the first report of *L. paraplantarum* on forage crops and grasses.

The representative strains of group I were clearly identified as a *Lactococcus lactis* cluster containing three subspecies: *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *hordniae* (Fig. 3). However, 16S rDNA sequence analysis could not distinguish strains at the subspecies level or efficiently classify interspecies relationships (Stackebrandt and Goebel, 1994). In this study, all of these strains had carbohydrate fermentation patterns nearly identical to that of the type strain *L. lactis* subsp. *lactis* 5805^T, excepting L-arabinose, but their ribose, mannitol, amygdaline, and D-turanose fermentation patterns differed from those of the other two subspecies (Table 4); therefore, group I strains could be identified as *L. lactis* subsp. *lactis*.

The different species and characteristics of epiphytic LAB can change and influence fermentation losses and silage quality (Lin *et al.*, 1992), and the populations of epiphytic LAB are not always sufficiently large or of suitable composition to promote efficient fermentation under farm conditions (Fenlon *et al.*, 1995). Thus, future studies should seek to obtain good-quality grass silage through the development of additives that stimulate and direct the fermentation process.

Based on biochemical and phylogenetic analyses, the LAB species classified in this study are common inhabitants of several forage crops and silages. Homofermentative species accounted for 42% of the total microflora, and nine LAB species were identified: *L. paraplantarum* (5.3%), *L. brevis* (5.2%), *L. plantarum* (10.5%), *Enterococcus mundtii* (5.5%), *W. paramesenteroides* (31.5%), *W. confusa* (5.4%), *L. citreum*

Table 3. **Characteristics of lactic acid bacteria strains isolated from forge grasses and crop used in this study**[a]

Characteristics	Group A	Group B	Group C		Group D	Group E						Group F	Group G	Group H			Group I		
	CW 11	CW 12	CW 14	CW 49	CW 41	CW 40	CW 43	CW 45	CW 48	CW 51	CW 53	CW 54	CW 46	CW 13	CW 15	CW 47	CW 16	CW 44	CW 55
Shape	rod	rod	rod	rod	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from glucose	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Optical form of lactate	DL	DL	DL	DL	L(-)	D(-)	D(-)	D(-)	D(-)	D(-)	D(-)	D(-)	D(-)	D(-)	D(-)	D(-)	L(-)	L(-)	L(-)
Fermentation type	Homo	Homo	Homo	Homo	Homo	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Homo	Homo	Homo
Growth at temperature (°C):																			
10	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45	+	-	+	+	+	+	+	+	+	w	w	+	+	-	+	-	-	w	-
Growth in NaCl:																			
3.0%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH:																			
3.0	-	-	-	-	w	-	-	-	-	w	-	w	-	w	w	-	-	w	-
3.5	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w
4.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+

[a] All strains were Gram-positive, catalase negative. +, positive; -, negative; w, weakly positive; Homo, homofermentative; Hetero, heterofermentative.

Table 4. **API 50 CH fermentation patters of lactic acid bacteria strains isolated from forge crop and grasses used in this study**[a]

Characteristics	Group A	Group B	Group C		Group D	Group E						Group F	Group G	Group H			Group I			JCM	JCM	JCM	JCM	JCM	JCM
	CW 11	CW12	CW 14	CW 49	CW 41	CW 40	CW 43	CW 45	CW 48	CW 51	CW 53	CW 54	CW 46	CW 13	CW 15	CW 47	CW 16	CW 44	CW 55	5805^T	16167^T	1180^T	1558^T	1149^T	12533^T
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	w
L-Arabinose	-	w	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	-	+	+	+
Ribose	+	+	+	+	+	+	w	w	w	w	+	+	-	+	+	+	+	+	+	+	-	-	+	+	w
D-Xylose	-	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	-	+	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Methyl-Xyloside	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	-	w	w	+	+	+	+	+	+	-	+	+	+
Mannose	+	-	+	+	+	+	+	+	+	+	+	+	-	w	+	+	+	+	+	+	+	+	+	+	+
Sorbose	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	w	-	w	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	w	+	w
Dulcitol	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	-	+	-	+	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	+	+
Sorbitol	+	-	+	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+
α-Methyl-D Mannoside	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	+
α-Methyl-D-Glucoside	-	-	-	-	-	+	+	+	+	+	+	w	w	+	+	+	-	-	w	-	-	-	-	+	+
Amygdalin	+	-	+	-	+	-	-	-	-	-	+	+	w	-	-	w	+	+	+	+	-	-	+	+	+
Arbutin	+	-	+	-	+	-	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	+	+	+	+
Esculin	+	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Salicin	+	-	+	-	+	-	-	-	-	-	+	+	+	-	-	w	+	+	+	+	-	+	+	+	+
Cellobiose	+	-	+	-	+	w	-	-	-	-	+	+	w	-	-	+	+	+	+	+	-	+	+	+	+
Lactose	+	-	+	+	+	+	-	-	w	-	+	w	-	+	+	w	+	+	+	+	+	-	+	+	+
Melibiose	+	w	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	-	+	+	+
Saccharose	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Trehalose	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+
Raffinose	+	-	+	+	+	+	-	w	+	+	+	-	-	+	+	+	-	-	+	-	-	-	-	+	+
Starch	-	-	-	-	-	-	-	-	-	-	w	-	-	w	w	-	w	w	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	+	-	+	-	+	-	-	-	w	w	w	+	w	-	-	w	+	+	w	w	-	-	w	+	+
D-Turanose	-	-	-	-	-	+	w	+	+	+	+	-	+	+	+	+	-	-	+	w	-	-	-	+	+
D-Tagatose	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	w	-	w	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	w	w	+	w	-	w	+	w	w	w	w	+	w	-	-	w	w	w	w	w	w	w	w	+	+
2-Ceto-Gluconate	-	-	-	-	-	-	-	w	w	-	w	-	w	w	w	-	-	-	-	-	-	-	-	-	-
5-Ceto-Gluconate	-	w	-	-	-	-	-	-	w	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	w

+, 90% or more of the strains positive; -, 90% or more of the strains negative; w, weakly positive; Homo, homofermentative; Hetero, heterofermentative

JCM, Japan collection of microorganisms; ^{T} = Type strains; JCM 5805^T, *Lactococcus lactis* subsp. *lactis* JCM 16167^T, *Lactococcus lactis* subsp. *cremoris* JCM 1180^T, *Lactococcus lactis* subsp. *hordniae* JCM 1558^T, *Lactobacillus pentosus* JCM 1149^T, *Lactobacillus plantarum* JCM 12533^T, *Lactobacillus paraplantarum*.

[a] All strains produced acid from glucose, fructose, *N*-acetyl-glucosamine and maltose, but failed to produce acid from erythritol, D-arabinose, L-xylose, inositol, glycogen, D-lyxose, D-fucose, and L-fucose.

(5.1%), *L. pseudomesenteroides* (15.8%), and *L. lactis* subsp. *lactis* (15.7%). These results are in agreement with those of previous studies (Lin *et al*., 1992; Cai *et al*., 1998, 1999a, 1999b; Pang *et al*., 2011), which have reported similar LAB species composition in areas of China, Japan, and the USA. The dominant species were the heterofermentative *Weissella* spp., which may influence silage quality; the relationship between LAB and silage fermentation quality is an interesting research topic now being studied. As shown in Fig. 3, corn contained the most species (*Weissella*, *Leuconostoc*, *Lactococcus*, and *Enterococcus* spp.), but few epiphytic LAB were detected on the other forage crops and grasses examined. Further investigation of the relationship between LAB species and silage fermentation quality is vital.

The special conditions on the Tibetan Plateau have led to unique microorganism communities. In comparison with other regions, forage crops and grasses that survive in this area are adapted to an environment with thin air, low air pressure, and cold weather. Most strains identified in this study can tolerate relatively low temperatures (10°C), and could be used to enhance silage fermentation in other very cold areas and in winter. Trials are underway in our laboratory to screen and apply these isolates to the production of good-quality silage under low temperature conditions.

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