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Multilocus sequence typing of *Lactococcus lactis* from naturally fermented milk foods in ethnic minority areas of China

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ABSTRACT

To determine the genetic diversity and phylogenetic relationships among *Lactococcus lactis* isolates, 197 strains isolated from naturally homemade yogurt in 9 ethnic minority areas of 6 provinces of China were subjected to multilocus sequence typing (MLST). The MLST analysis was performed using internal fragment sequences of 12 housekeeping genes (*car*B, *clp*X, *dna*A, groEL, *mur*C, *mur*E, *pep*N, *pep*X, *pyr*G, *rec*A, *rpo*B, and *phe*S). Six (*dna*A) to 8 (*mur*C) different alleles were detected for these genes, which ranged from 33.62 (*clp*X) to 41.95% (*rec*A) GC (guanine-cytosine) content. The nucleotide diversity (π) ranged from 0.00362 (*mur*E) to 0.08439 (*car*B). Despite this limited allelic diversity, the allele combinations of each strain revealed 72 different sequence types, which denoted significant genotypic diversity. The d_N/d_S ratios (where d_S is the number of synonymous substitutions per synonymous site, and d_N is the number of nonsynonymous substitutions per nonsynonymous site) were lower than 1, suggesting potential negative selection for these genes. The standardized index of association of the alleles (I_A^S = 0.3038) supported the clonality of *Lc. lactis*, but the presence of network structure revealed by the split decomposition analysis of the concatenated sequence was strong evidence for intraspecies recombination. Therefore, this suggests that recombination contributed to the evolution of *Lc. lactis*. A minimum spanning tree analysis of the 197 isolates identified 14 clonal complexes and 23 singletons. Phylogenetic trees were constructed based on the sequence types, using the minimum evolution algorithm, and on the concatenated sequence (6,192 bp), using the unweighted pair-group method with arithmetic mean, and these trees indicated that the evolution of our *Lc. lactis* population was correlated with geographic origin. Taken together, our results demonstrated that MLST could provide a better understanding of *Lc. lactis* genome evolution, as well as useful information for future studies on global *Lc. lactis* structure and genetic evolution, which will lay the foundation for screening *Lc. lactis* as starter cultures in fermented dairy products.

Key words: traditional fermented milk food, *Lactococcus lactis*, multilocus sequence typing, housekeeping gene

INTRODUTION

Lactococcus lactis is a species of lactic acid bacteria (**LAB**) used in dairy starter cultures, especially hard and semihard cheeses. This species is composed of 4 known subspecies: *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *hordniae*, and *Lc. lactis* ssp. *tructae* (Schleifer, 1987; Pérez et al., 2011). *Lactococcus lactis* ssp. *hordniae* isolated from the leafhopper *Hordnia circellata* has not been detected in dairy products (Schleifer et al., 1985), and *Lc. lactis* ssp. *tructae* was isolated from the intestinal mucus of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) species (Pérez et al., 2011). *Lactococcus lactis* ssp. *lactis* is found in a variety of environments, including animal sources, dairy products, and plant surfaces (Klijn et al., 1995; Nomura et al., 2006), whereas *Lc. lactis* ssp. *cremoris* is isolated primarily from raw milk and other dairy products (Urbach et al., 1997; Nomura et al., 2006). *Lactococcus lactis* ssp. *lactis* and ssp. *cremoris* are widely used for industry and research and play a key role in the determination of shelf life, preservation, and organoleptic quality, thereby influencing the quality and safety of these fermented products (Smit et al., 2005). In addition, many strains of *Lc. lactis* ssp. *lactis* and ssp. *cremoris* carry plasmids encoding important traits, such as lactose catabolism, citrate utilization, proteinase production, bacteriocin production and immunity, bacteriophage resistance, exopolysaccharide production, as well as heavy metal resistance (McKay, 1983; Davidson et al., 1996). These broad applications

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might be attributed to their remarkable ecological adaptability to diverse habitats.

Lactococcus lactis ssp. *lactis* and ssp. *cremoris* differ by no more than 0.7% in their 16S rDNA sequences (Salama et al.,1991) but display an average of only 85% DNA identity at the genome level (Wegmann et al., 2007). Some strains of *Lc. lactis* showing an *Lc. lactis* ssp. *lactis* phenotype according to classical distinction criteria, show an *Lc. lactis* ssp. *cremoris* genotype (*cremoris* genotype; Jarvis and Jarvis, 1981), meanwhile, phenotypic *Lc. lactis* ssp. *cremoris* showing an *Lc. lactis* ssp. *lactis* genotype (*lactis* genotype) have also been reported (Kelly et al., 2010; Tanigawa et al., 2010). Therefore, *Lc. lactis* has an unusual structure with 2 phenotypically distinct groups, *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*, which may differ in genotype. Consequently, it has been difficult to accurately distinguish the 2 subspecies. Numerous methods, including DNA-DNA hybridization, small subunit rRNA gene sequencing, and PCR fingerprint analyses (Salama et al., 1991; Erlandson and Batt, 1997; Mangin et al., 1999; Pu et al., 2002), have been used for identification of *Lc. lactis* subspecies. However, their resolutions were low and the resulting data proved difficult for public database curation.

Multilocus sequence typing (**MLST**) is considered the gold standard for characterization of bacterial isolates and is based on the partial nucleotide sequences of multiple housekeeping genes (Maiden, et al., 1998; Urwin and Maiden, 2003; Aanensen and Spratt, 2005). It was recently shown to be a powerful technique for bacterial typing (Enright and Spratt, 1999), providing critical information for evolutionary history, population structure, and long-term epidemiology of bacterial species (Maiden, 2006; Turner and Feil, 2007). Although MLST has been used principally to study major bacterial pathogens, several recent MLST schemes were developed for LAB species, including *Oenococcus oeni* (de las Rivas et al., 2004), *Lactobacillus plantarum* (de las Rivas et al., 2006), *Lactobacillus casei* (Cai et al., 2007), *Streptococcus thermophilus* (Delorme et al., 2010), and *Lactobacillus sanfranciscensis* (Picozzi et al., 2010).

We examined the diversity and relationships of 197 *Lc. lactis* strains isolated from traditional fermented dairy products in different regions of China by characterizing population structure using an MLST scheme. The main aims of the present study were to apply MLST to assess phylogenetic relationship and evolutionary characteristics of these isolates and further reexamine the subspecies composition of *Lc. lactis*, which will contribute to screen starter cultures in the future.

MATERIALS AND METHODS

Bacterial Strains and Genomic DNA Extraction

A total of 197 *Lc. lactis* strains were selected from Collection Centre of Lactic Acid Bacteria of Inner Mongolia Agriculture University in China. Those included strains isolated from naturally fermented yogurt made by the Mongolian peoples in Chifeng, Xilin Gol, Hulunbeir, Bayan Nur of Inner Mongolia, by the Bai peoples in Dali of YunNan, and by the Tibetan peoples in Qinghai, Szechwan, Gansu, and Tibet of China from 2005 to 2009. Other than that, all strains had been achieved by 16S rDNA sequences. Details information of strains are listed in Table 1.

Lactococcus lactis strains were maintained in M17-broth (Oxoid, CM0817B, Wesel, Germany) supplemented with 5.0 g/L (wt/vol) of lactose at 30°C for 18 to 24 h, and then strains were harvested by centrifugation and cell pellets were used for DNA extraction. Total genomic DNA was extracted from cultures by using a previously reported method (Yu et al., 2012). Purified DNA was diluted to a final concentration of 100 ng/μL for application.

DNA Amplification and Sequencing for MLST

The MLST analyses traditionally focus on allelic diversity of housekeeping genes, which match admitted criteria, including their presence as a single copy in all strains, their conserved sequence, their wide distribution across the chromosome, and their mutually unlinked location. The final selection included 12 housekeeping genes: *dna*A, *pyr*G, *rpo*B, *gro*EL, *rec*A, *clp*X, *car*B, *mur*C, *pep*N, *pep*X, *mur*E, and *phe*S. In addition, *pyr*G, *gro*EL, and *rec*A were selected based on the results of a previous study on *Lactobacillus delbrueckii* (Kana and Watanabe, 2011), and *pep*N, *pep*X, and *phe*S were selected based on a study with *Lc. lactis* (Rademaker et al., 2007). Selection of the remaining loci (*dna*A, *rpo*B, *clp*X, *car*B, *mur*C, and *mur*E) was based on the presence of SNP between *Lc. lactis* ssp. *lactis* IL-1403 and *Lc. lactis* ssp. *cremoris* SK11. These SNP were identified using comparative genome microarrays. These primers of 12 housekeeping genes were designed by Premier 5.0 (Premier Biosoft International, Palo Alto, CA) based on the known genome of *Lc. lactis* ssp. *lactis* IL1403, and the information of primers is listed in Table 2.

For each strain, the genomic DNA was used as a template for PCR amplification of MLST loci on the automatic thermal cycler (PTC-200, MJ Research,