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Multilocus sequence typing of a dairy-associated *Leuconostoc mesenteroides* population reveals clonal structure with intragenic homologous recombination

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ABSTRACT

Leuconostoc mesenteroides strains play an important role in food fermentation. In this study, 136 strains from different dairy products in China and Mongolia were examined by multilocus sequence typing of 9 housekeeping genes. In total, 82 polymorphic sites were detected among the 9 loci. The number of polymorphic nucleotide sites varied between 4 (*dnaA*) and 18 (*uvrC*), whereas the nucleotide diversity per site among the 9 genes varied from 0.00379 in *dnaA* to 0.01195 in *uvrC*, suggesting a relatively low level of sequence diversity. For the recombination measurement, incongruence in the trees based on a single gene and concatenated sequences of all sequencing types were observed, indicative of extensive intragenic homologous recombination. The overall relatedness built by minimum spanning trees showed no clear relationship between the clonal complexes and either isolation source or sampling location of the strains. Our study presents, for the first time, the population structure of *Leuc. mesenteroides* strains of dairy origin.

Key words: *Leuconostoc mesenteroides*, multilocus sequence typing, homologous recombination, clonal structure

INTRODUCTION

Leuconostoc mesenteroides are gram-positive, nonmotile, non-spore-forming facultative anaerobes, which require complex stimulation factors during growth. Their presence is detected in various food-related ecological niches, including beverages, meat products, milk products, and some plant materials (Hemme and Foucaud-Scheunemann, 2004). The first description of *Leuconostoc mesenteroides* was by Van Tieghem in 1878 and it was later proposed as the type strain (Garvie, 1979). In 1983, *Leuconostoc dextranicum* and *Leuconostoc cremoris* were reclassified as subspecies of *Leuc. mesenteroides* because of the common properties they shared with *Leuc. mesenteroides* (Garvie, 1983). These 3 organisms were reported to have similar lactate dehydrogenases and glucose-6-phosphate dehydrogenases (Garvie, 1983). When DNA–DNA hybridization was performed, the 3 strains showed a high degree of relatedness: *Leuc. mesenteroides* had 110 ± 9.3% and 66 ± 10.3% homology with *Leuc. dextranicum* and *Leuc. cremoris*, respectively (Garvie, 1976). In a more recent study, a novel subspecies, *Leuc. mesenteroides* ssp. *suionicum*, was proposed based on the data obtained using a polyphasic approach (Gu et al., 2012).

Taxonomic studies of *Leuc. mesenteroides* initially focused mostly on physiological tests. Unfortunately, concerns have been raised about the phenotypic markers used during the process regarding their instability and irreproducibility, especially when applied to discriminate closely related species (Thunell, 1995). In fact, Barrangou et al. (2002) pointed out that the historical failure to differentiate *Leuconostoc fallax* from *Leuc. mesenteroides* was due to a lack of proper molecular identification methods. To address these serious concerns, molecular methods such as random amplified polymorphic DNA analysis, DNA fingerprinting and restriction of ribosomal DNA were developed and implemented (Villani et al., 1997; Pérez et al., 2002), which proved effective for discriminating *Leuc. mesenteroides* at the species level. However, continuous improvement and standardization of the methods for taxonomic study is still necessary because comparison of results between different laboratories remains challenging (Maiden, 2006).

Multilocus sequence typing (**MLST**), a new generic typing method, has been principally used in studies of *Lactobacillus* within the community of lactic acid bacteria (**LAB**; Tanigawa and Watanabe, 2011; Buhnik-Rosenblau et al., 2012; Chaillou et al., 2013). Herein, we used MLST to probe the evolution and population

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structure of 136 *Leuc. mesenteroides* isolates from different dairy products in China and Mongolia. To our knowledge, this is the first MLST scheme for *Leuc. mesenteroides*. We believe that the development and application of MLST for this species will promote further use of the strains in food fermentation.

MATERIALS AND METHODS

Bacterial Isolates and DNA Extraction

One hundred thirty-six *Leuc. mesenteroides* strains were obtained from the Collection Centre of Lactic Acid Bacteria at Inner Mongolia Agriculture University of China (Hohhot). These strains were isolated from dairy products (whey, yogurt, yak milk, and *qula*, a traditional cheese) in 3 provinces of China and 5 provinces of Mongolia (Supplemental Table S1; http://dx.doi.org/10.3168/jds.2014-9227). The strains were grown in 5.0 mL of M17 broth (CM0817B, Oxoid, Wesel, Germany) at 30°C for 18 to 22 h. Total genomic DNA was extracted from cultures as described by Yu et al. (2012). For further use, purified DNA was diluted to a final concentration of 100 ng/μL.

Selection of MLST Loci

With reference to the genome sequence of *Leuc. mesenteroides* ATCC8293 (GenBank accession number: CP000414.1), 9 housekeeping genes (*dnaA*, *groEL*, *murC*, *murE*, *pepN*, *pheS*, *pyrG*, *rpoB*, and *uvrC*) were selected for MLST analysis. These genes are evenly separated across the entire genome, conserved, and well characterized (Cai et al., 2007; Bilhère et al., 2009). The primers for these 9 genes were designed using the Primer Premier 5.0 program (Premier Biosoft International, Palo Alto, CA) and are listed in Table 1.

PCR Amplification and DNA Sequencing

The PCR amplifications of MLST loci were carried out in an automatic thermal cycler (PTC-200, MJ Research, Waltham, MA). The thermal cycling conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, annealing temperature of each locus for 1 min, and 72°C for 2 min; and a final elongation step of 72°C for 8 min. The PCR mixture (50 μL) for each target contained 150 ng of genomic DNA, 10 m*M* of each deoxynucleotide triphosphates (dNTP), 10 pmol of each primer, and 2.5 U of Taq polymerase in 1× PCR buffer (with Mg^{2+}). The PCR products were first analyzed by electrophoresis in a 1.2% agarose gel and were then sequenced by Shanghai Majorbio Bio-Pharm Technology Corporation (Shanghai, China). The same primers

Table 1. Genes and primers used for multilocus sequence typing

Gene	Length (bp)	Gene function	PCR primer	Sequence (5′–3′)	Temperature, (°C)
dnaA	621	Chromosomal replication initiator protein *dnaA*	*dnaA*_primerF	CGAAGCAAAGCCTACCACT	52
			*dnaA*_primerR	AAGCCATCAGATTCAGACAA	
pyrG	676	CTP synthase	*pyrG*_primerF	ATTACGCCAAATGAAGTCG	50
			*pyrG*_primerR	CAAGCAAATGCCTAAGAAA	
rpoB	597	RNA polymerase β subunit	*rpoB*_primerF	ATATTGAAGCCGACGAGGTT	52
			*rpoB*_primerR	TTCCCTTGTTTCCATGACG	
groEL	451	Chaperonin GroEL	*groEL*_primerF	GTGCTGTCGCATCTCGTGT	55
			*groEL*_primerR	GCGGCAATCATGTCTACCC	
pheS	545	Phenylalanyl-tRNA synthetase subunit α	*pheS* _primerF	ATCAACAGTTATTGGCAGAA	50
			pheS _primerR	TACCTCCACAGATGGCTCA	
murC	524	UDP-*N*-acetyl muramate-L-alanine ligase	*murC*_primerF	GTAACGGACCACACAAGGTAG	50
			*murC*_primerR	GCATTAGTCGGAATTTCTC	
pepN	580	Lysyl-aminopeptidase, aminopeptidase N	*pepN*_primerF	ACGATGAAATGGTGGGATG	53
			*pepN*_primerR	TCACTACACGCTTACTGGAT	
uvrC	615	Excinuclease ABC subunit C	*uvrC*_primerF	GTAACGGACCACAAGGTAG	50
			*uvrC*_primerR	GCATTAGTCGGAATTTCTC	
murE	619	UDP-*N*-acetylmuramoylalanyl-D-glutamate-L-lysine ligase	*murE*_primerF	ACGATGAAATGGTGGGATG	51
			*murE*_primerR	TCACTACACGCTTACTGGAT	