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, non-motile, 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 $110 \pm 9.3\%$ and $66 \pm 10.3\%$ homology with *Leuc. dextranicum* and *Leuc. cremoris*, respectively (Garvie, 1976). In a more recent study, a novel subspecies, *Leuc. mesenteroides* ssp. *sui-unicum*, 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 *wvrC*) 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 mM of each deoxynucleotide triphosphates (dNTP), 10 pmol of each primer, and 2.5 U of Taq polymerase in 1× PCR buffer (with Mg²⁺). 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')	Annealing temperature (°C)
<i>dnaA</i>	621	Chromosomal replication initiator protein <i>dnaA</i>	<i>dnaA</i> _primerF	CGAAGCAAAGCCTACCACT	52
			<i>dnaA</i> _primerR	AAGCCATCAGATTGAGACAA	
<i>pyrG</i>	676	CTP synthase	<i>pyrG</i> _primerF	ATTACGCCAAATGAAGTCG	50
			<i>pyrG</i> _primerR	CAAGCAAATGCCTAAGAAA	
<i>rpoB</i>	597	RNA polymerase β subunit	<i>rpoB</i> _primerF	ATATTGAAGCCGACGAGGTT	52
			<i>rpoB</i> _primerR	TTCCCTTGTTCCTCAIGACG	
<i>groEL</i>	451	Chaperonin GroEL	<i>groEL</i> _primerF	GTGCTGTGCATCTCGTGT	55
			<i>groEL</i> _primerR	GCGGCAATCATGCTACCC	
<i>pheS</i>	545	Phenylalanyl-tRNA synthetase subunit α	<i>pheS</i> _primerF	ATCAACAGTTATTGGCAGAA	50
			<i>pheS</i> _primerR	TACCTCCACAGATGGCTCA	
<i>murC</i>	524	UDP-N-acetyl muramate-L-alanine ligase	<i>murC</i> _primerF	GTAACGGACCAAGGTAG	50
			<i>murC</i> _primerR	GCATTAGTCGGAATTTCTC	
<i>pepN</i>	580	Lysyl-aminopeptidase, aminopeptidase N	<i>pepN</i> _primerF	ACGATGAATGTTGGGATG	53
			<i>pepN</i> _primerR	TCACTACACGCTTACTGGAT	
<i>wvrC</i>	615	Excinuclease ABC subunit C	<i>wvrC</i> _primerF	GTAACGGACCAAGGTAG	50
			<i>wvrC</i> _primerR	GCATTAGTCGGAATTTCTC	
<i>murE</i>	619	UDP-N-acetylmuramoylalanyl-D-glutamate-L-lysine ligase	<i>murE</i> _primerF	ACGATGAATGTTGGGATG	51
			<i>murE</i> _primerR	TCACTACACGCTTACTGGAT	

from the PCR amplifications were used for sequencing on both DNA strands.

Data Analysis

For MLST analysis, forward and reverse sequences were trimmed, aligned, and analyzed using MEGA 5.0 (<http://www.megasoftware.net/>). Definition of alleles using a nonredundant data set and minimum spanning tree analysis were based on BioNumerics 6.6 (Applied-Maths, Sint Maartens-Latem, Belgium). Allelic sequences having more than one nucleotide difference were assigned different arbitrary numbers. For each of the 9 MLST loci or alleles, a unique nucleotide sequence was assigned. The sequence types (**ST**) were defined by unique allelic profiles (consisting of the allele numbers at each of the 9 MLST loci). The same ST was used for several strains if they have common allelic profiles. Groups of isolates with closely related allelic profiles were regarded as clonal complexes (**CC**). The ST were grouped into CC using the eBURST (Based Upon Related Sequence Types) program (<http://eburst.mlst.net/>; Feil et al., 2004) and were identified in the BioNumerics program. In our study, an ST was defined as a subgroup founder that had > 4 single locus variants (**SLV**).

Descriptive evolutionary analyses such as G+C content (mol %); d_N/d_S ratio, where d_N is the number of nonsynonymous substitutions per nonsynonymous site, and d_S is the number of synonymous substitutions per synonymous site; nucleotide diversity (π) per site; number of polymorphic sites; Tajima's D test, and Fu and Li's D and F tests (the values from all 3 tests that did not significantly deviate from zero indicate potential negative selection in the loci) were calculated using DnaSP 5.0 (Librado and Rozas, 2009). With the program LIAN3.5, linkage equilibrium between alleles at the 9 housekeeping genes was assessed, based on the standardized index of association (I_A^S ; Haubold and Hudson, 2000). To evaluate the degree of tree-like structure for alleles of each locus and all ST, the split decomposition method was performed with SplitsTree 4.0 (Huson and Bryant, 2006). Phylogenetic trees were constructed by the neighbor-joining method using MEGA 5.0 software package (<http://www.megasoftware.net/>), with 1,000 replicates in bootstrap analysis.

Nucleotide Sequence Accession Numbers

Allele sequences of the 9 MLST loci have been deposited in the GenBank under accession numbers KP035238 to KP036325.

RESULTS

PCR Amplification and Sequencing and Definition of ST

Nine genes fulfilling the criteria of MLST analysis were amplified and sequenced for the present study. The length of the genes ranged from 321 to 496 bp. Sixty-eight different ST were identified among 136 *Leuc. mesenteroides* strains (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2014-9227>). Of these, ST-14 was the most represented and was composed of 13 strains, followed by ST-29 (10 strains), ST-11 (9 strains), ST-15 (6 strains), ST-55 (6 strains), ST-9 (4 strains), and other ST with fewer than 4 strains.

Allelic Variation at the MLST Loci

The nucleotide diversity at each locus of the strains is described in Table 2. Among the 9 loci, 82 polymorphic sites were detected in the 136 isolates of *Leuc. mesenteroides*. The number of polymorphic nucleotide sites varied between 4 (*dnaA*) and 18 (*uvrC*). The mean G+C content (mol %) observed for different gene fragments was 40.5%, within a range from 40.48 to 40.67%. The nucleotide diversity per site (π) among the 9 genes varied from 0.00379 in *dnaA* to 0.01195 in *uvrC*. The d_N/d_S ratios of 9 loci ranged from 0.0219 to 2.4379. Except for that of *murC*, most d_N/d_S ratios were <1 (Table 2), suggesting that no strong positive selective pressure was present. Accordingly, the values from Tajima's D test, and Fu and Li's D and F tests did not show a significant deviation from 0 (Table 2).

Detection for Recombination

Evidence for recombination was determined using 2 different approaches. The result based on I_A^S analysis revealed a value of 0.1661 based on the 9 chosen loci. The observed variance was greater than the maximum variance obtained after 1,000 trials ($P = 0.000$); therefore, the population was in complete linkage equilibrium. This finding also shows that all the studied isolates together form an underlying clonal population structure.

For further measurement of intragenic recombination, split-decomposition analysis was performed on each locus separately and on the concatenated sequences of all ST. As shown in the split graphs (Figure 1), 7 loci (*groEL*, *murE*, *pepN*, *pheS*, *pyrG*, *rpoB*, and *uvrC*) showed classical tree-like structures, whereas a possible parallelogram structure was detected for loci *dnaA* and *murC*. In contrast, the concatenated sequence of

Table 2. Descriptive analysis of genetic variability at *Leuconostoc mesenteroides* loci

Gene	No. of alleles	Polymorphic sites (%)	π^1 /site	Tajima's D value	Fu and Li's D* value	Fu and Li's F* value	G+C content (mol %)	d_N/d_S^2
<i>dnaA</i>	6	4	0.00379	0.3554	0.0713	0.1396	38.80	0.0648
<i>groEL</i>	6	5	0.00582	-0.8258	-0.7915	-0.8507	43.54	0.0717
<i>murC</i>	8	7	0.00539	-1.0445	-0.8937	-1.0262	39.57	2.4379
<i>murE</i>	8	11	0.00919	0.135	-0.0474	-0.0054	40.29	0.1033
<i>pepN</i>	9	11	0.00722	-0.8936	-0.8005	-0.9198	43.06	0.6677
<i>pheS</i>	10	10	0.00657	-0.7264	-0.7678	-0.852	39.24	0.1995
<i>pyrG</i>	6	6	0.00493	-0.4961	-0.4164	-0.4635	38.57	0.2049
<i>rpoB</i>	8	10	0.00814	-0.2287	-0.2019	-0.2307	42.29	0.0219
<i>uvrC</i>	11	18	0.01195	-0.1597	-0.2822	-0.2843	39.75	0.1546

¹Mean pairwise nucleotide difference per site.

² d_N/d_S represents the ratio of nonsynonymous to synonymous substitutions.

the 9 genes displayed a typical extensive network-like structure (Figure 2), suggesting that most sections among the 9 genes had pervasive and associative recombination (Phi test, $P = 1.434E-6$). No apparent difference was observed on the overall topology if data from either *dnaA* or *murC* were removed before tree construction (Supplemental Figures S1 and S2; <http://dx.doi.org/10.3168/jds.2014-9227>).

Population Structure of *Leuc. mesenteroides* Strains

A snapshot of the population structure was obtained by eBURST analysis, with a stringent group definition of 7 of 9 shared alleles. As showed in Figure 3, 8 CC (CC1 to CC8) and 16 singletons were observed for the 52 ST. The number of the strains contained in these CC accounted for 70.59% of the strain collection.

Among the 8 CC, CC1 was the largest and comprised 16 linked ST; namely, ST24, ST15, ST55, ST68, ST62, ST54, ST57, ST60, ST65, ST38, ST37, ST3, ST5, ST6, ST49, and ST66. These ST included 11 strains isolated from Inner Mongolia, 8 isolates from Gansu province and 4 isolates from Sichuan province in China. Clonal complex 2, representing 7 ST, consisted of 12 strains, with 5 of them isolated from Inner Mongolia, 4 from Gansu province, and 3 from Sichuan province. Clonal complex 3 contained 17 strains; most were isolated from Sichuan province (10 strains). Clonal complex 4 had 6 strains, 4 from Inner Mongolia, 1 from Sichuan province, and 1 from Mongolia. Clonal complex 5 had 21 stains, most of which were isolated from Inner Mongolia (17 strains), 2 from Sichuan province, and 2 from Gansu province. The rest of the CC were small clusters that had fewer than 5 ST with limited strains isolated from one geographical location. Moreover, CC1, which had 12 SLV, appeared to be more complicated than any other CC. We therefore assigned it as the primary founder of the CC.

In the minimum spanning tree featured with isolates from different regions (Figure 4), we found 4 main branches. Interestingly, strains isolated from Mongolia and Inner Mongolia showed closer relationships than strains originating from other regions. Branch 1 corresponded to CC1, which contained strains isolated from Inner Mongolia, Gansu, and Sichuan. Branch 2 was nearly identical to CC5, having most strains isolated from Inner Mongolia. Branch 3 consisted of the strains from CC2, which were isolated from Gansu, Sichuan, and Inner Mongolia. Branch 4 included CC7, CC3, and CC6, with most strains from Gansu province, Inner Mongolia, and Mongolia. When the minimum spanning tree was constructed based on the dairy sources of the isolates (Supplemental Figure S3; <http://dx.doi.org/10.3168/jds.2014-9227>), no clear relationship was seen between the clusters and the isolation sources, even considering the 2 main sources that included most strains.

A neighbor-joining tree was constructed using the concatenated sequences of the 9 genes from the 136 *Leuc. mesenteroides* strains (Supplemental Figure S4; <http://dx.doi.org/10.3168/jds.2014-9227>). However, no major clusters were identified with acceptable bootstrap values. Most branches of the tree were poorly supported by bootstrap values of <70%, which means that the corresponding clades were not likely to be real (Hillis and Bull, 2004).

DISCUSSION

Multilocus sequence typing is a powerful technique to characterize bacteria at the molecular level. Because it is a sequence-based method, comparisons of the experimental results from different labs become more convenient. Moreover, data can be retrieved through open databases via the Internet. In the present study, MLST was used to explore the population structure

Figure 1. Split-decomposition analysis of alleles for 9 individual multilocus sequence typing (MLST) loci (*dnaA*, *groEL*, *murC*, *murE*, *pepN*, *pheS*, *pyrG*, *rpoB*, and *uvrC*) of 136 *Leuconostoc mesenteroides* strains.

and evolution of 136 *Leuc. mesenteroides* isolates from different ecological and geographical origins, which may provide better information concerning their biological properties.

To initiate analysis, the sequence diversity of the 9 housekeeping genes was calculated. This step was carried out to measure whether these selected loci had sufficient typing discrimination. The number of alleles in these gene loci ranged from 6 to 11. This observation corroborates the previously described genetic heterogeneity of *Leuconostoc lactis* (Dan et al., 2014). Compared with nucleotide sequence diversities

reported in *Lactobacillus*, such as *Lactobacillus sakei* and *Lactobacillus casei* (Cai et al., 2007; Chaillou et al., 2013), *Leuc. mesenteroides* housekeeping genes have similar but lower π values in general, reflecting that these genes are conserved. Consistently, the 9 loci were polymorphic, and 2 were relatively less diverse (polymorphic sites ≤ 5) among the examined isolates.

By analyzing the synonymous and nonsynonymous changes in the allele sequences, a small d_N/d_S ratio of $\ll 1$ was obtained, which implies that the selected genes were subjected to negative selection. This trend of divergences in the selected genes made them suitable

Figure 2. Combined split-decomposition analysis of alleles for the 9 sequence type (ST) loci (*dnaA*, *groEL*, *murC*, *murE*, *pepN*, *pheS*, *pyrG*, *rpoB*, and *wurC*) of the 136 *Leuconostoc mesenteroides* strains.

for analysis by MLST. One exception was found with gene *murC*, which had a d_N/d_S ratio >1 , which is not typically observed for housekeeping genes. Nevertheless, this gene has been used in the molecular typing of *Lactococcus lactis*, which showed abundant polymorphisms (Xu et al., 2014). However, after sequencing the fragment 194 bp distant from the 5' end of *murC*, only 7 polymorphic sites, equivalent to 1.33% of all sites, were observed. The apparent low level of polymorphisms

led us to believe that this gene was not genetically as diverse as those of other LAB species. Similar observations were reported for the polymorphism of *gtfB* gene in *Streptococcus mutans* isolated from 5-yr-old children and their mothers (Toi et al., 2005).

Several studies have questioned the discriminatory power of single or multiple gene-based phylogeny (Simpson et al., 2004). Because of potential horizontal gene transfer and extensive homologous recombination,

Figure 3. Population structure of 136 *Leuconostoc mesenteroides* isolates by eBURST analysis (Based Upon Related Sequence Types; <http://eburst.mlst.net/>). CC = clonal complex.

phylogenetic trees built based on a limited number of genes could result in misleading inferences. For example, homologous recombination can occur even in the highly conserved rRNA sequences, which reduces the reliability of using the rRNA sequences as traditional markers in phylogenetic analysis (Schouls et al., 2003). The house-keeping genes used here for MLST analysis had similar G+C contents compared with the overall genome of the type strain *Leuc. mesenteroides* ATCC8293, suggesting that they originated from the bacterial genome rather than being acquired via horizontal gene transfer events. For the recombination measurement, incongruence in the trees based on single gene and concatenated sequences of all ST was observed, indicating extensive intragenic recombination. Accordingly, the phylogeny established using the neighbor-joining method failed

to show reliable topology with poorly supported bootstrapping values. This fact implied that the neighbor-joining method was unsuitable for analyzing MLST data of the current *Leuc. mesenteroides* strains. In a previous study, Diancourt et al. (2007) pointed out that approaches such as eBURST and minimum spanning that are less sensitive to the effects of recombination might be preferable.

As indicated by the large number of the strains involved in CC, our study presented a clonal structure of *Leuc. mesenteroides* strains isolated from dairy-associated niches. One interesting application of the MLST technique is identification of associations between strains and their origin. For example, Cai et al. (2007) reported the first MLST study of LAB, in which a collection of 40 *Lactobacillus casei* strains were

Figure 4. Minimum-spanning tree analysis of the 136 *Leuconostoc mesenteroides* strains from different regions. Each circle corresponds to a sequence type (ST), and the circle size denotes the number of strains sharing the same ST. Numbers between the circles indicate the number of allelic differences between the profiles. The strength of the link (solid, bold, or dashed) is related to the genetic similarity (number of common alleles) between profiles.

grouped into a cheese cluster, a silage cluster, and a mixed cluster primarily consisting of the human gastrointestinal tract and cheese isolates. Some correlation was observed between the minimum spanning tree and the pulsed-field gel electrophoresis tree, suggesting high specificity of the individual ecological niches. However, in this study, no strong relationship between the CC and the origins of the strains included in them could be identified, based either on the dairy source or on sampling location. Often, only some strains of the same source or location were clustered together, whereas the rest were dispersed across other clusters. One possible explanation for the weak correlation was the open market for international and regional trading of dairy products, which might have increased the occurrence of the genetic exchange between strains. The absence of such a relationship was reported in typing analyses of *Lactobacillus delbrueckii*, *Lactococcus lactis*, and *Lactobacillus sakei* (Passerini et al., 2010; Tanigawa and Watanabe, 2011; Chaillou et al., 2013).

CONCLUSIONS

Leuconostoc mesenteroides is important in food fermentation, and the species can be detected in a variety of fermented food-related ecological niches. We developed an MLST scheme for its typing analysis and investigated the population biology within a set of 136 strains. Our results showed that intragenic homologous recombination plays a key role in *Leuc. mesenteroides* diversity. However, the strains studied here were entirely of dairy origin, which may introduce bias in representing the genetic heterogeneity of this species, because dairy strains may display lower diversity compared with nondairy strains. It would be interesting to analyze the population structure of *Leuc. mesenteroides* from various niches by combined use of MLST and whole-genome resequencing techniques.

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