Relationships between functional genes in *Lactobacillus delbrueckii* ssp. *bulgaricus* isolates and phenotypic characteristics associated with fermentation time and flavor production in yogurt elucidated using multilocus sequence typing

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ABSTRACT

Lactobacillus delbrueckii ssp. *bulgaricus* (*L. bulgaricus*) is well known for its worldwide application in yogurt production. Flavor production and acid producing are considered as the most important characteristics for starter culture screening. To our knowledge this is the first study applying functional gene sequence multilocus sequence typing technology to predict the fermentation and flavor-producing characteristics of yogurt-producing bacteria. In the present study, phenotypic characteristics of 35 *L. bulgaricus* strains were quantified during the fermentation of milk to yogurt and during its subsequent storage; these included fermentation time, acidification rate, pH, titratable acidity, and flavor characteristics (acetaldehyde concentration). Furthermore, multilocus sequence typing analysis of 7 functional genes associated with fermentation time, acid production, and flavor formation was done to elucidate the phylogeny and genetic evolution of the same *L. bulgaricus* isolates. The results showed that strains significantly differed in fermentation time, acidification rate, and acetaldehyde production. Combining functional gene sequence analysis with phenotypic characteristics demonstrated that groups of strains established using genotype data were consistent with groups identified based on their phenotypic traits. This study has established an efficient and rapid molecular genotyping method to identify strains with good fermentation traits; this has the potential to replace time-consuming conventional methods based on direct measurement of phenotypic traits.

Key words: *Lactobacillus delbrueckii* ssp. *bulgaricus*, dairy starter, acidification, multilocus sequence typing, functional gene

INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* (*L. bulgaricus*) is widely used as a starter culture to convert milk into yogurt, often in combination with *Streptococcus thermophilus* (van de Guchte et al., 2006). In homemade yogurts, *L. bulgaricus* and *S. thermophilus* nominally exhibited antimicrobial activity against different bad bacteria (Akpınar and Yerlikaya, 2011). In industrial application, *L. bulgaricus* strain 2038 is a well-known starter isolate in Meiji Milk Products Co., Tokyo, Japan (Terahara et al., 2001; Sasaki et al., 2014). Furthermore, a wild-type starter *L. bulgaricus* ND02 was isolated from naturally fermented yak milk in Qinghai, China (Sun et al., 2010) and screened as a yogurt starter culture for milk fermentation (Gao et al., 2013). This strain has been used in the industrial production of dairy starter cultures by Inner Mongolia Yili Industrial Group Company Limited, the largest dairy corporation in China (Sun et al., 2011). However, fast acidification during fermentation is regarded as a useful property of *L. bulgaricus* in industrial applications (Ongol et al., 2007), and persistent acid production during storage is a major cause of postacidification (Shah, 2000). In the dairy industry acidification and flavor production are the most important phenotypic production characteristics of a starter culture. Speed of fermentation is the most important factor determining production costs and the product development cycle in modern yogurt production. However, not only should starter cultures acidify the milk rapidly, but the time to achieve the required pH should not vary substantially from batch to batch (Tamime, 2006). Acidification rate, acid concentration, and fermentation time are the key

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indicators used for selection of starter cultures with the best fermentation characteristics (Cachon et al., 2002; Kristo et al., 2003; Oliveira et al., 2009). Fermentation time is the time it takes from inoculation of lactic acid bacteria (**LAB**) into milk (initiation of fermentation) until the pH has decreased to 4.5 (end of fermentation). Acidification rate can be calculated by pH decrease or titratable acidity (in °T) increase per unit time (t) [dpH/dt (pH unit/h) or dT/dt (°T unit/h)] according to Cachon et al. (2002). Maximum acidification is determined by the difference in pH or titratable acidity of yogurt from the start to the end of fermentation.

Sensory characteristics play an important role in product acceptance by consumers. Key sensory characteristics of fermented dairy products are flavor, mouth feel, and texture, among which flavor is regarded as the most crucial for fermented milk, or yogurt (Kalviainen et al., 2003). The distinct flavor of yogurt is due to lactic acid and a complex mixture of aroma compounds, the latter including volatiles already present in the milk and specific compounds produced from the components of milk during fermentation (Tamime and Deeth, 1980; Imhof and Bosset, 1994; Ott et al., 1997). To date more than 90 flavor compounds have been identified from milk products (Lubbers et al., 2004; Routray and Mishra, 2011). The most important aromatic components are acetaldehydes, acetone, acetoin, diacetyl, and lactic acid (Imhof and Bosset, 1994; Routray and Mishra, 2011). The typical aroma of yogurt is characterized chiefly by acetaldehydes, making them the most important flavor compound. Flavor compounds produced by LAB are derived through various metabolic pathways responsible for degrading milk fats, milk proteins (caseins), and milk sugars (lactose) into alcohols, aldehydes, and carboxylic acids during fermentation (Cheng, 2010; Routray and Mishra, 2011).

It is possible to select functional LAB using molecular technology and, when their genome sequences are available, to select particular characteristics of LAB at the gene level that increase their industrial application traits. Forty complete and 142 incomplete genomes for *Lactobacillus* species have been reported (Holzapfel and Wood, 2014) among which 5 complete and 7 draft genome sequences of *L. bulgaricus* are available online (<ftp://ftp.ncbi.nlm.nih.gov/genbank/genomes/>). Complete genome sequencing and comparative genomics analysis of LAB can reveal the genetic basis of phenotypic characteristics essential for industrial yogurt production. However, significant problems can be encountered while reconstructing flavor-forming routes based on the genomic information stored in the public databases because the functional annotation for many of the relevant genes is inconsistent (Liu et al.,

2008). It is possible to identify the correlations between genotypes (i.e., presence or absence of genes based on comparative genomic hybridization) and phenotypes (i.e., growth on carbohydrates, NO_2 production, and stress tolerance) using a web tool—PhenoLink (Siezen, and van Hylckama Vlieg, 2011; Bayjanov et al., 2012).

In the present study, we compared multilocus sequence typing (**MLST**) analysis of 7 functional genes with phenotypic characteristics (acidification rate, fermentation time, and acetaldehyde content) of 35 strains of *L. bulgaricus* to reveal the relationship between phenotypic variability and the evolution of functional genes.

MATERIALS AND METHODS

Strains and Culture Conditions

Thirty-five strains of *L. bulgaricus* were characterized in this study (Table 1). These strains were isolated from naturally fermented dairy products (e.g., fermented cow milk, goat milk, and yak milk) by using de Man, Rogosa, and Sharpe (**MRS**) medium (Difco Lactobacilli MRS Agar, Difco/Becton Dickinson, Franklin Lakes, NJ) in China or Mongolia including one reference strain ND02, for which the genome sequence is complete. Stock cultures were stored at -80°C in 20% (vol/vol) glycerol. Working cultures were prepared from frozen stock by 2 transfers in MRS Broth (CM0359B, Oxoid, Basingstoke, UK), each for 16 to 18 h at 37°C and without agitation.

Substrates and Chemical Reagents

Acetaldehyde, diacetyl, and organic acids (formic acid, acetic acid, propionic acid, lactic acid) were purchased as standards from Sigma Chemical Co. (St. Louis, MO). Skim milk powder was purchased from NZMP Ltd. (Wellington, New Zealand). The medium for culturing *L. bulgaricus* was MRS Broth (CM0359B, Oxoid) purchased from Thermo Fisher Biochemicals (Beijing) Ltd. (Beijing, China).

Quantification of Flavor and Acidification Traits During Fermentation and Storage

The fermentation characteristics of isolates of *L. bulgaricus* were studied in reconstituted skim milk. This milk was prepared by adding 1.5% glucose (wt/wt) into 10% skim milk (wt/wt). The milk was sterilized at 95°C for 10 min, cooled to the incubation temperature (37°C), and inoculated with 1×10^7 cfu/mL each test

or reference strain individually as starter cultures to initiate fermentation. Fermentation was carried out at 37°C until the samples achieved a pH of 4.5. Samples were taken every 2 h following introduction of the starter cultures, and the pH, titratable acidity, and total viable counts of bacteria were determined for each isolate as described by Liu et al. (2013). Fermentation was considered to have finished when the pH value had fallen below 4.5 or the titratable acidity had reached 80°T; this time was recorded as the fermentation time for that isolate. Acidification rate was calculated as titratable acid/fermentation time.

Once fermentation was considered to have finished, the resulting samples of yogurt for each isolate were stored at 4°C for 48 h. During this storage period further samples were taken at 0, 12, 24, 36, and 48 h, and the pH; titratable acidity; acetaldehyde, diacetyl, and organic acid concentrations; and the total viable counts of bacteria were determined for each strain as described above. All the tested experiments were performed in triplicate.

Determining pH, Titratable Acidity, and Concentration of Flavor-Producing Compounds

Acetaldehyde concentration was determined spectrophotometrically using an acetaldehyde determination kit K-ACHYD (Megazyme, Wicklow, Ireland) based on the enzymatic (acetaldehyde dehydrogenase) reduction of NAD to NADH and recording light absorbance at 340 nm (Chaves et al., 2002). Titratable acidity was determined by titration with NaOH using phenolphthalein as the indicator (National Standards of the People's Republic of China, 1996). The viable counts of each strain were enumerated according to the description of Liu et al. (2013).

Statistical Analyses

The fermentation time and acetaldehyde concentration were expressed as mean values $\pm$ standard error. Mann-Whitney test with Bonferroni correction for multiple testing was used to evaluate the sample difference in a pairwise manner. Two sample groups with

Table 1. Background information on experimental strains

Numbers	16S rRNA GeneBank	Habitat	Geographical origin of isolates
IMAU20136	HM057874	Fermented cow milk	Sükhbaatar province, Mongolia
IMAU20216	HM057951	Fermented cow milk	Khentii province, Mongolia
IMAU20220	HM057954	Fermented cow milk	Dronod province, Mongolia
IMAU20222	HM057956	Fermented goat milk	Dronod province, Mongolia
IMAU20227	HM057961	Fermented cow milk	Dronod province, Mongolia
IMAU20234	HM057968	Fermented cow milk	Dronod province, Mongolia
IMAU20240	HM057974	Fermented cow milk	Khentii province, Mongolia
IMAU20277	HM058487	Fermented cow milk	Ulaanbaatar, Mongolia
IMAU20295	HM058026	Fermented cow milk	Selenge province, Mongolia
IMAU20342	HM058071	Fermented cow milk	Dzahan province, Mongolia
IMAU20360	HM058088	Fermented cow milk	Dzahan province, Mongolia
IMAU20396	HM058120	Fermented cow milk	Bulgan province, Mongolia
IMAU20401	HM058125	Fermented cow milk	Hovsgol province, Mongolia
IMAU20403	HM058127	Fermented cow milk	Hovsgol province, Mongolia
IMAU20404	HM058128	Fermented cow milk	Hovsgol province, Mongolia
IMAU20410	HM058134	Fermented cow milk	Hovsgol province, Mongolia
IMAU20421	HM058145	Fermented cow milk	Hovsgol province, Mongolia
IMAU20423	HM058147	Fermented cow milk	Hovsgol province, Mongolia
IMAU205631	HM058345	Fermented cow milk	Ovorhangay province, Mongolia
IMAU20595	HM058312	Fermented cow milk	Hovsgol province, Mongolia
IMAU20632	HM058346	Fermented cow milk	Arhangay, Mongolia
IMAU20758	HM058468	Fermented cow milk	Ulaanbaatar, Mongolia
IMAU20760	HM058470	Fermented cow milk	Ulaanbaatar, Mongolia
IMAU20762	HM058472	Fermented cow milk	Ulaanbaatar, Mongolia
IMAU20763	HM058473	Fermented cow milk	Ulaanbaatar, Mongolia
IMAU20769	HM058479	Fermented cow milk	Ulaanbaatar, Mongolia
IMAU20775	HM058485	Fermented cow milk	Ulaanbaatar, Mongolia
IMAU20776	HM058486	Fermented cow milk	Ulaanbaatar province, Mongolia
IMAU40157	FJ915695	Fermented goat milk	Haixi county, Qinhai province, China
IMAU40160	FJ915697	Fermented goat milk	Haixi county, Qinhai province, China
IMAU40166	FJ915703	Fermented goat milk	Haixi county, Qinhai province, China
IMAU40167	FJ915704	Fermented goat milk	Haixi county, Qinhai province, China
IMAU80319	HM058599	Qula	Aba county, Sichuan province, China
ND02	FJ749353	Fermented yak milk	Hainan county, Qinghai province, China

a corrected P -value of less than 0.05 were considered significantly different from each other. Differences in fermentation time were considered significant at the 5% level (i.e., $P \leq 0.05$) and highly significant at the 1% level (i.e., $P \leq 0.01$) and allowed the strains to be grouped into those with similar (not significantly different) and contrasting (significantly different) fermentation times.

MLST Analysis of Functional Genes: DNA Extraction

The DNA was extracted from each strain according to the protocol of Liu et al. (2012). In brief, all strains were cultured in MRS medium, and DNA was extracted using the CTAB (cetyltrimethyl ammonium bromide) method. The quality of the extracted DNA was assessed by 0.8% agarose gel electrophoresis, and the optical density 260/280 was measured by spectrophotometry. All of the DNA samples were stored at -20°C before further processing.

MLST Analysis

Functional Gene Selection and Primer Design.

Intragenic regions of 7 functional genes involved in acetaldehyde synthesis were selected for MLST analysis. These genes are found in all strains as a single copy, and the functions of the encoded proteins are conserved and well characterized (van de Guchte et al., 2006; Hao et al., 2011; Sun et al., 2011). The PCR primers of 7 functional genes were designed using Premier 6.0 software (Premier Biosoft International, Palo Alto, CA) on the basis of the genome sequence of *Lactobacillus delbrueckii* ssp. *bulgaricus* ND02 (Sun et al., 2011) and were listed in Table 2.

PCR Amplification and MLST Sequencing.

Based on the concentration of genomic DNA, the DNA stock suspension was diluted to 100 ng/ μL in Tris-EDTA buffer and used as the template for PCR amplification. The reaction volume for PCR amplification was 50 μL and contained 150 ng of genomic DNA, 10 mM of each deoxynucleotide triphosphate, 10 pmol of each primer, and 2.5 U of Taq polymerase in 1 $\times$ PCR buffer (with Mg^{2+}). The cycle parameters were 94 $^{\circ}\text{C}$ predegeneration for 5 min, degeneration at 94 $^{\circ}\text{C}$ for 1 min, annealing for 1 min (annealing temperature varied depending on what was appropriate for the particular gene), extension at 72 $^{\circ}\text{C}$ for 2 min, and 30 cycles with a final extension of 10 min. Sequencing of the PCR products was done by the Shanghai Majorbio Bio-pharm Technology Corporation. The same primers were used for PCR and sequencing of both DNA strands.

Data Analysis of MLST. For each MLST locus, an allele number was designated to each distinct combi-

nation of alleles of the 7 genes using BioNumerics v. 6.0 (Applied-Maths, Sint-Martens-Latem, Belgium). Each allele number was assigned a sequence type (ST). The same ST was used for several strains if they shared the same allelic profiles. After calibration and alignment, allelotype affirmation of functional gene sequences was carried out using BioNumerics 6.6 software. Different allelic sequences (with at least one nucleotide difference) were assigned arbitrary numbers. For each strain, the combination of 7 alleles defined its allelic profile, and a unique allelic profile was designated a ST. A minimum spanning tree was built based on the sequences of functional genes and phenotypic data including acetaldehyde concentration and fermentation time using the same software to analyze the evolution of genetically different strains and their relationships to each other.

The phylogenetic tree was constructed based on the joint-plant-bacteria gene, 7 genes connected sequentially (*ald-bgl-gpd-htp-ldh-pdc-pdh*), and Applications START 2.0 software (K. Jolley; <http://www.mlst.net/links/software.asp>), using the neighbor-joining evolution method of cluster analysis. The combination of the 7 allele numbers defined the allelic profile of each isolate, and each different allelic profile was assigned as a ST.

Nucleotide Sequence Accession Numbers. The sequences of all alleles were deposited in the EMBL/GenBank databases, and the accession numbers are listed as follows: KR258857 to KR258891 for *ald* genes; KR258892 to KR258926 for *bgl* genes; KR258927 to KR258961 for *gpd* genes; KR258962 to KR258996 for *htp* genes; KR258997 to KR259031 for *ldh* genes; KR259032 to KR259066 for *pdc* genes; and KR259067 to KR259101 for *pdh* genes.

RESULTS

Variability in Fermentation Times Among Strains of *L. bulgaricus*

The fermentation time of the 35 strains of *L. bulgaricus* evaluated ranged from 8.5 to 24.5 h and allowed the strains to be separated into 6 distinct groups (Figure 1) with significantly different fermentation times ($P \leq 0.05$, shown in Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2015-10209>).

Variability in Flavor and Acidification Traits of Strains of *L. bulgaricus* During Fermentation and Storage

The average acidification rate of 35 strains of *L. bulgaricus* during the milk fermentation period ranged

Table 2. Function and primers of functional genes used in multilocus sequence typing analysis

Gene	Function of enzymes coded by these genes	Primer ¹	Sequences (5'-3')	T _m ² (°C)	Length (bp)	Size of amplicon (bp)
<i>ald</i> (alcohol-acetaldehyde dehydrogenase)	This gene encodes an enzyme alcohol dehydrogenase that oxidizes ethanol into acetaldehyde.	<i>ald</i> F <i>ald</i> R	GACAGCGGACATTTGAACGA GCGAAGGCAAACTTACGG	54	19 18	519
<i>ldh</i> (lactate-dehydrogenase)	This gene encodes an enzyme that catalyzes the conversion of lactate to pyruvate.	<i>ldh</i> F <i>ldh</i> R	CGGCATCTTCGTCGTTTC GTGGCTGATCCCTTGTC	54	18 18	483
<i>pdh</i> (pyruvate dehydrogenase)	This gene encodes a complex of 3 enzymes that convert pyruvate into acetyl-CoA by a process called pyruvate decarboxylation.	<i>pdh</i> F <i>pdh</i> R	CAAGGCTGACCTGGAAAGA GTCACGCAGGGCTGTAA	54	19 17	553
<i>bgl</i> (β-galactosidase)	This enzyme is a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides.	<i>bgl</i> F <i>bgl</i> R	GTATTCCTCCGCCCTCCTG TCCTCTTCGGTGATAGCC	54	18 18	510
<i>gpd</i> (glycerol-3-phosphate-dehydrogenase)	This gene encodes an enzyme that catalyzes the reversible redox conversion of dihydroxyacetone phosphate.	<i>gpd</i> F <i>gpd</i> R	ATCAACCCCGCTGAACAAT AATGAAGGCACCAGACAGA	54	18 19	465
<i>htp</i> (ATPases, H ⁺ -transporting)	This gene encodes a protein that is associated with ATPases. Proton-translocating ATPases have fundamental roles in energy conservation.	<i>htp</i> F <i>htp</i> R	GGTGGTAAGGTCGGTCTG GTCCAGGTAGGCGAAATG	54	18 18	528
<i>pdc</i> (pyruvate decarboxylase)	This gene encodes a homotetrameric enzyme (EC 4.1.1.1) that catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide.	<i>pdc</i> F <i>pdc</i> R	CAAGTGGTGCCCTTGTATTG CGTAGCCTGGAAGAAGTATC	54	19 20	579

¹F = forward; R = reverse.²Melting temperature.

Figure 1. Boxplot depicting variability in fermentation times of 35 strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

from 1.75 to 5.15°T/h (Table 3). The strain with the fastest acidification rate was IMAU20396 (acidification rate was 5.15°T/h), and the strain with the slowest acidification rate was IMAU20227 (acidification rate was 1.75°T/h). The maximum acid production of each strain ranged from 24.08 to 69.14°T, and the different strains differed significantly ($P \leq 0.01$).

The acetaldehyde content of yogurt produced by 35 strains of *L. bulgaricus* during the storage period ranged from 0.28 to 56.81 mg/L, and the strains differed significantly ($P \leq 0.01$). These strains could be divided into 3 groups according to variation of acetaldehyde concentration during the 48-h storage period (Figure 2). A high-yielding group existed (greater than 20 mg/L), which included most strains in group A (Figure 2A). The acetaldehyde concentrations of strains in group A were significantly higher ($P \leq 0.01$) than those in group B (shown in Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2015-10209>), which was a medium-yielding group (10–20 mg/L; Figure 2B). Acetaldehyde significantly differed between strains from group A, group B, and group C at all storage times: 0 h ($P = 0.0029, 0.0008$), 12 h ($P = 0.0002, 0.0001$), 24 h ($P = 0.0003, 0.0001$), 36 h ($P = 0.0001, 0.0001$), and 48 h ($P = 0.0001$). A low-yielding group (less than 10 mg/L) included most strains in group C (Figure 2C). Acetaldehyde concentration did not significantly

differ between strains in group B and group C during storage except after 24 h ($P = 0.0843$ for 0 h, 0.1052 for 12 h; 0.0197 for 24 h; 0.1890 for 36 h; 0.1679 for 48 h; Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2015-10209>).

The acetaldehyde concentration of yogurt produced by strains in group A reached a peak at 0 or 8 h of storage and thereafter decreased; acetaldehyde concentration in yogurt produced by most strains in this group was greater than 15.83 mg/L. The highest yielding strain of all those evaluated was IMAU20769; acetaldehyde content of yogurt produced by this strain reached 56.81 mg/L after 8 h of storage. The acetaldehyde content of yogurt produced by strains in group B ranged from 2.36 to 20.28 mg/L, reaching a peak after 24 or 36 h storage and thereafter decreased. The acetaldehyde content of yogurt produced by most strains in this group ranged between 5.56 and 15.70 mg/L, and thus this group could be considered as an intermediate group. The acetaldehyde concentration of yogurt produced by strains in group C ranged from 0.28 to 11.39 mg/L, gradually reaching a peak at 36 or 48 h of storage. This group was regarded as a low acetaldehyde-producing group.

Grouping of *L. bulgaricus* Strains by Functional Gene Sequences and Phenotypic Characteristics: Gene Diversity and Evolution of *L. bulgaricus*

Thirty-five *L. bulgaricus* strains were analyzed for MLST typing based on 7 key functional genes associated with acid production and flavor-compound formation in fermented milk. The 35 strains could be divided into 20 ST (Table 4). The ST15 contained the largest number of strains (5), followed by ST2, which included 4 strains; ST1, ST6, and ST11, each of which contained 3 strains; and ST7, ST9, and ST13, each of which contained 2 strains. All the remaining ST were represented by a single strain. The number of alleles for each functional gene varied from 1 (at least one allele in 7 genes among different strains) to 7 (*ald*). The numbers of alleles that changed demonstrated genetic change in strains under long-term genetic evolution through environmental adaptability and selection pressure.

A neighbor-joining phylogenetic tree was constructed to identify the phylogenetic relationships among the 35 *L. bulgaricus* strains (Figure 3). The 35 strains were divided into 4 major clusters (cluster 1, cluster 2, cluster 3, cluster 4), and 6 subclusters (from c1 to c6) based on key functional gene sequence typing and the acetaldehyde content of the yogurt produced by each strain after different storage times. More than 90% of strains belonged to cluster 1 and were high acetaldehyde-

yielding isolates (>10 mg/L). The 4 strains in cluster 2 were generally low yielding (<5 mg/L). Acetaldehyde concentration was less than 5 mg/L for more than 90% of cluster 3 strains; these strains belong to the low-yield acetaldehyde group. Acetaldehyde content of cluster 4 strains was in the range 5 to 10 mg/L, making them intermediate.

Cluster Formation Based on Fermentation Time

The 35 *L. bulgaricus* strains formed 20 ST based on fermentation time and functional genes sequences, each containing between 1 and 6 strains. A minimum spanning tree was constructed according to fermentation time and functional genes (Figure 4). The ST could also be associated into 4 groups.

Cluster Formation Based on Functional Genes and Acetaldehyde Content

Phylogenetic evolution analysis was done on all 35 strains based on sequence typing combined with acetaldehyde content of yogurt produced by each strain, and a minimum spanning tree was constructed (Figure 5). Each colored circle denotes an ST, and the color of the circle denotes whether the ST contains strains that are high, medium, or low acetaldehyde yielding. The size of the colored circles is relative to the number of strains present in the ST, and the dashed lines contain ST that fall into the same groups. The 35 *L. bulgaricus* strains formed 20 ST, each containing between 1 and 6 strains. The ST could also be associated into 4 groups. Group 1 contained 2 low acetaldehyde-yielding strains. Group 2 contained low and intermediate acetaldehyde-

Table 3. Mean fermentation time and acid-producing characteristics of *Lactobacillus delbrueckii* ssp. *bulgaricus* isolates $\pm$ SE of means

Isolate reference number	Fermentation time (h)	pH at the end of fermentation	Titrateable acidity ($^{\circ}$ T) at the end of fermentation	Maximum amount of acid produced ($^{\circ}$ T)	Acidification rate (Δ° T/h)
IMAU20136	24.5 $\pm$ 0.42	4.73 $\pm$ 0.45	64.84 $\pm$ 0.64	51.23	2.09
IMAU20216	18.7 $\pm$ 0.08	4.15 $\pm$ 0.14	83.28 $\pm$ 0.42	69.14	3.70
IMAU20220	11.6 $\pm$ 0.04	4.47 $\pm$ 0.04	57.15 $\pm$ 1.32	45.16	3.89
IMAU20222	16.0 $\pm$ 0.11	4.51 $\pm$ 0.01	51.06 $\pm$ 3.31	31.67	1.98
IMAU20227	24.2 $\pm$ 1.24	4.67 $\pm$ 0.02	56.88 $\pm$ 0.68	42.43	1.75
IMAU20234	15.2 $\pm$ 0.12	4.56 $\pm$ 0.02	47.5 $\pm$ 12.05	33.73	2.22
IMAU20240	16.3 $\pm$ 0.72	4.50 $\pm$ 0.05	53.59 $\pm$ 0.26	40.10	2.46
IMAU20277	13.0 $\pm$ 0.46	4.52 $\pm$ 0.01	ND ¹	24.08	1.85
IMAU20295	12.0 $\pm$ 0.62	4.26 $\pm$ 0.14	54.34 $\pm$ 5.29	41.23	3.44
IMAU20342	8.5 $\pm$ 1.32	4.48 $\pm$ 0.01	49.19 $\pm$ 0.66	33.73	3.97
IMAU20360	12.0 $\pm$ 0.01	4.50 $\pm$ 0.04	43.10 $\pm$ 14.57	27.64	2.30
IMAU20396	11.6 $\pm$ 1.20	4.47 $\pm$ 0.04	ND	59.78	5.15
IMAU20401	9.8 $\pm$ 0.82	4.4 $\pm$ 0.03	65.12 $\pm$ 13.11	48.26	4.92
IMAU20403	13.8 $\pm$ 0.64	4.56 $\pm$ 0.01	45.16 $\pm$ 3.44	33.17	2.40
IMAU20404	23.3 $\pm$ 0.42	4.70 $\pm$ 0.42	66.62736	52.48	2.25
IMAU20410	11.5 $\pm$ 0.02	4.50 $\pm$ 0.02	51.16 $\pm$ 1.85	36.17	3.15
IMAU20421	18.9 $\pm$ 0.02	4.55 $\pm$ 0.04	60.23 $\pm$ 0.17	42.22	2.23
IMAU20423	14.2 $\pm$ 0.41	4.46 $\pm$ 0.00	50.41 $\pm$ 1.85	37.11	2.61
IMAU205631	15.1 $\pm$ 0.41	4.49 $\pm$ 0.01	47.6 $\pm$ 12.19	34.29	2.27
IMAU20595	15.2 $\pm$ 0.32	4.56 $\pm$ 0.04	60.91 $\pm$ 0.02	31.11	2.05
IMAU20632	18.8 $\pm$ 0.42	4.67 $\pm$ 0.02	62.64 $\pm$ 0.64	48.40	2.57
IMAU20755	18.5 $\pm$ 0.22	4.50 $\pm$ 0.06	63.58 $\pm$ 0.93	48.19	2.60
IMAU20758	17.0 $\pm$ 0.49	4.53 $\pm$ 0.04	50.6 $\pm$ 0	37.01	2.18
IMAU20760	14.1 $\pm$ 0.82	4.55 $\pm$ 0.02	56.69 $\pm$ 1.18	46.38	3.29
IMAU20762	13.6 $\pm$ 0.05	4.52 $\pm$ 0.02	55.28 $\pm$ 0.53	42.91	3.16
IMAU20763	19.5 $\pm$ 0.01	4.44 $\pm$ 0.01	62.33 $\pm$ 0.22	46.62	2.39
IMAU20769	13.6 $\pm$ 2.62	4.47 $\pm$ 0.01	58.56 $\pm$ 0.66	44.69	3.29
IMAU20775	12.4 $\pm$ 0.08	4.45 $\pm$ 0.01	47.97 $\pm$ 1.59	32.98	2.66
IMAU20776	14.0 $\pm$ 0.22	4.49 $\pm$ 0.00	58.19 $\pm$ 2.51	43.95	3.14
IMAU40157	17.0 $\pm$ 2.50	4.46 $\pm$ 0.03	54.35 $\pm$ 0	40.76	2.40
IMAU40160	17.8 $\pm$ 0.62	4.75 $\pm$ 0.10	50.88 $\pm$ 3.57	39.17	2.20
IMAU40166	14.8 $\pm$ 2.10	4.33 $\pm$ 0.01	63.25 $\pm$ 4.63	50.88	3.44
IMAU40167	16.3 $\pm$ 1.82	4.63 $\pm$ 0.02	51.16 $\pm$ 1.85	40.01	2.45
IMAU80319	12.8 $\pm$ 0.02	4.54 $\pm$ 0.04	59.96 $\pm$ 2.65	45.91	3.59
ND02	15.6 $\pm$ 1.02	4.51 $\pm$ 0.02	43.29 $\pm$ 1.32	29.61	1.90

¹Not detected.

Figure 2. Variability in acetaldehyde content of yogurt produced by 35 *Lactobacillus delbrueckii* ssp. *bulgaricus* strains during storage. Error bars represent standard errors, based on triplicate experiments.

producing strains, and group 3 contained all the high acetaldehyde-yielding strains and several low- and intermediate-yielding strains. Group 4 contained only one low-yielding strain. These results are consistent with the results of the analysis using neighbor-joining techniques.

DISCUSSION

The ability of a starter culture to initiate rapid acidification of raw milk is essential to improve the flavor, texture, and safety of fermented milk and other products (Daly et al., 1998; Grattepanche et al., 2008). Process parameters such as pH, titratable acidity, fermentation time, and acidification ability are regarded as key factors that strongly influence the quality and reliability of the products (Rault et al., 2009). Acidification rate, pH, titratable acidity, fermentation time, and acetaldehyde content of fermented milk produced by 35 strains of *L. bulgaricus* were determined in the present study.

The fermentation time of most strains in the present study ranged from 10 to 15 h, and the strains differed significantly ($P \leq 0.01$) in their fermentation times. Short fermentation times were correlated with rapid acidification rates and good acid-producing properties. Of course, the fermentation time of starter cultures can also be influenced by the amount of inoculum (Sahlin, 1999). The fermentation times of *L. bulgaricus* in this study were longer than the commercial starter culture, in which fermentation times are generally less than 8 h. In general, the starter culture contains *S. thermophilus* and *L. bulgaricus*. It is said that a symbiotic relationship exists between *S. thermophilus* and *L. bulgaricus*, and this decreases fermentation time (Hayek and Ibrahim, 2013). Thus, the fermentation time of the strains studied here could be even shorten if they were combined with *S. thermophilus* when used as a starter culture for dairy products.

Fast acidification, high acid-producing ability, and short fermentation times are regarded as the primary

Table 4. Number of alleles of each functional gene, sequence type (ST) number, and cluster number of each of 35 isolates of *Lactobacillus delbrueckii* ssp. *bulgaricus*

Isolate reference number	Functional gene							ST	Cluster
	<i>ald</i>	<i>bgl</i>	<i>gpd</i>	<i>htp</i>	<i>ldh</i>	<i>pdh</i>	<i>pdh</i>		
IMAU20136	7	1	1	1	1	3	1	19	C1
IMAU20216	1	1	1	1	1	1	2	2	C2
IMAU20220	1	1	1	1	1	1	1	1	C2
IMAU20222	1	1	1	1	1	1	2	2	C2
IMAU20227	1	1	1	1	1	1	2	2	C2
IMAU20234	1	1	1	1	1	1	2	2	C2
IMAU20240	6	5	4	1	2	5	2	16	C4
IMAU20277	1	1	1	1	1	1	1	1	C3
IMAU20295	2	2	1	1	2	1	1	3	C3
IMAU20342	3	1	1	1	1	2	1	4	C3
IMAU20360	1	3	1	1	1	3	1	5	C1
IMAU20396	1	2	1	1	1	1	1	6	C1
IMAU20401	1	2	1	1	1	1	1	6	C3
IMAU20403	1	2	1	1	1	1	3	7	C3
IMAU20404	1	2	1	1	1	1	3	7	C3
IMAU20410	1	2	1	1	1	1	1	6	C3
IMAU20421	1	1	1	1	1	1	1	1	C3
IMAU20423	3	2	1	1	1	3	1	8	C4
IMAU205631	3	3	2	1	1	1	1	9	C1
IMAU20595	4	2	3	2	3	3	4	10	C4
IMAU20632	3	3	2	1	1	1	1	9	C1
IMAU20755	1	1	2	1	2	1	1	11	C1
IMAU20758	1	1	2	1	2	1	1	11	C1
IMAU20760	1	3	1	1	1	1	1	12	C1
IMAU20762	1	3	1	1	2	1	1	13	C1
IMAU20763	3	1	1	1	1	1	1	20	C1
IMAU20769	1	1	2	1	2	1	1	11	C1
IMAU20775	1	3	1	1	2	1	1	13	C1
IMAU20776	2	1	2	1	2	1	1	14	C1
IMAU40157	5	4	4	3	4	4	5	15	C5
IMAU40160	5	4	4	3	4	4	5	15	C5
IMAU40166	5	4	4	3	4	4	5	15	C5
IMAU40167	5	4	4	3	4	4	5	15	C5
IMAU80319	6	6	4	1	5	5	6	17	C4
ND02	5	4	4	3	4	4	5	15	C5

Figure 3. Phylogenetic tree dendrogram of *Lactobacillus delbrueckii* ssp. *bulgaricus* strains based on acetaldehyde content from 0 to 48 h and functional gene sequences. C = cluster; c = subcluster. Data are means of triplicate determinations ± standard deviation.

criterion for selection of a good starter culture (Liu and Wang, 2010). Fermentation time, the maximum quantity of acid produced, and the acidification rate can be used as important indices for measuring fermentation and acid-producing characteristics of a starter culture during the yogurt fermentation process (Oliveira et al., 2001). Rapid acidification rates, short fermentation times, and production of high concentrations of acid by LAB could be used as key indicators to study the characteristics of acid-production dynamics (Pette and Lolkema, 1950; Oliveira et al., 2001). In our study, the fermentation time, maximum concentration of acid pro-

duction, and the average acidification rate were used as indicators of fermentation and acid-producing characteristics of *L. bulgaricus*. Previous studies suggested that the peak acidification rate was the best indicator to measure the characteristics and kinetics of acid production in starter cultures (Cachon et al., 2002). We argue that the lactic acid fermentation process is a dynamic process and that the peak acidification rate only reflects the acid-production properties of an isolate over a transient or short period of time and does not accurately reflect the acid-production characteristics of isolates during the entire fermentation process. This is

Figure 4. Minimum spanning tree analysis of 35 *Lactobacillus delbrueckii* ssp. *bulgaricus* strains based on allelic profiles at 7 functional genes combined with fermentation time. Each circle corresponds to a sequence type, and the circle is related to the number of strains found with that profile. To illustrate the concordance of multilocus sequence typing data with phenotypic data, circles were colored based on grouping of fermentation time. Each distant group corresponds to a distinct color. The strength of the link (bold, plain, or discontinuous) is related to the genetic similarity (number of common alleles) between profiles. ST = sequence type.

why we chose the average acidification rate as a better indicator to measure fermentation and acid-production characteristics in *L. bulgaricus* strains.

Since Pette and Lolkema (1950) first suggested that acetaldehyde was the most important constituent of yogurt aroma, many researchers have quantified acet-

aldehyde production by bacterial isolates during fermentation and storage. Gorner et al. (1968) found that ripening of yogurt was characterized by the appearance

of acetaldehyde during the first 2 h of storage, rising to between 23 and 55 mg/L in ripe yogurt. In the present study, the concentration of acetaldehyde produced by

Figure 5. Minimum spanning tree analysis of 35 *Lactobacillus delbrueckii* ssp. *bulgaricus* strains based on allelic profiles at 7 functional genes combined with acetaldehyde content. Each circle corresponds to a sequence type, and the circle is related to the number of strains found with that profile. To illustrate the concordance of multilocus sequence typing data with phenotypic data, circles were colored based on grouping of acetaldehyde content. Each distant groups corresponds to a distinct color. The strength of the link (bold, plain, or discontinuous) is related to the genetic similarity (number of common alleles) between profiles. ST = sequence type.

L. bulgaricus strains ranged from 0.28 to 56.81 mg/L, varying significantly between different strains; this level of variability was much greater than had been observed in previous studies on other LAB (Sieuwerds, 2009; Routray and Mishra, 2011). As for some strains, the acetaldehyde concentration decreased as storage time was extended. It is well known that acetaldehyde is volatile and decreases in concentration with extended storage time, which is usual and has been observed in other studies (Liu, 2010).

In LAB the degradation of carbohydrate, caseins, fats, and proteins is undoubtedly the most important pathway for flavor formation in yogurt. Among these metabolic substrates, carbohydrates metabolism are regarded as the most important metabolic processes for milk fermentation and flavor formation (Routray and Mishra, 2011); therefore, in this study we select 7 functional genes in the carbohydrate metabolism pathway as the study target. Different enzymes produced by LAB are responsible for this degradation and each contributes to the production of specific flavors depending on whether alcohols, aldehydes, or esters are the breakdown product (Smit et al., 2005). The metabolic processes of LAB can vary in response to genetic and environmental change. For example, an altered pyruvate metabolism results in different volatile flavor compounds being produced as end products.

The genomic and molecular mechanisms of flavor formation by LAB are well understood for many species now that complete genome sequences have been published and methods for comparative genomic analysis have been developed (Liu, 2010; Holzapfel and Wood, 2014). However, because of the high cost and time-consuming nature of whole genomic sequence analysis, analysis of key functional genes scattered within the genome is regarded as a better alternative opportunity to elucidate industrial and fermentation characteristics. The classification method based on the web tool PhenoLink allowed identification of correlations between genotypes (i.e., presence or absence of genes based on comparative genomic hybridization) and phenotypes (i.e., growth on carbohydrates, NO₂ production, and stress tolerance) of *L. plantarum* strains (Siezen and van Hylckama Vlieg, 2011). In the present study, we combined MLST analysis of 7 functional genes with data on fermentation and storage characteristics such as acid production, fermentation time, and acetaldehyde concentration of *L. bulgaricus* isolates to elucidate the relationships between functional genes and phenotypic characteristics. In addition to describing the phylogenetic relationships among 35 *L. bulgaricus* isolates using neighbor-joining methods, minimum spanning trees were constructed to reveal phylogenetic relation-

ships between groups of isolates divided by functional genes and by phenotypic characteristics; the minimum spanning tree method provided better resolution than neighbor-joining methods for phylogenetic grouping (Chaves et al., 2002).

The groups that were separated based on phenotypic characteristics were clustered in corresponding groups that were separated based on functional gene MLST analysis. Because the groups divided by functional genes were consistent with phenotypic characteristics such as fermentation time, acidification rate, and acetaldehyde concentration, it is likely that genetic differentiation of functional genes in *L. bulgaricus* had occurred under the genetic and environmental selection pressures imposed during domestic yogurt production. Based on phylogenetic grouping and minimum spanning tree analysis, these 2 kinds of analysis on the acetaldehyde production, and functional genes evolution (Figure 4) in 35 strains of *L. bulgaricus*, we can infer the following: the original strains IMAU80319 and IMAU20340 belong to group 1 with a mixture of acetaldehyde-producing characteristics (including low-yielding and normal-yielding acetaldehyde strains), which produced group 2, including strains with low and middle acetaldehyde-producing characteristics when they evolved in the long evolutionary process under natural-selection pressure and acid environmental stress conditions of yogurt. This evolution maybe formed by mutations or genetic recombination of key metabolic genes concerning milk fermentation and flavor formation. Others progeny strains of group 1 emerged genetic differentiation resulting in the formation of group 3, which includes mixed acetaldehyde-concentration phenotype strains including low or high acetaldehyde-producing characteristics. After long-term natural evolution, the strains of group 3 formed evolutionary descendant group 4; the strains in such group are purely low-yielding acetaldehyde strains.

From the results of this study we believe that functional gene sequence typing and genotype data analysis accurately reflect phenotypic characteristics and could be used as a rapid screen to select isolates most likely to produce yogurt with a good flavor.

CONCLUSIONS

The result of the present study showed that *L. bulgaricus* with different acetaldehyde-producing characteristics evolved from ancestral strains with intermediate acetaldehyde-producing capabilities under genetic evolution and natural selection pressures imposed during yogurt production. These functional genes played vital roles (positive or negative) in acetaldehyde production.

Typing of functional gene sequences reflects an ongoing genetic evolutionary process, and the level of acetaldehyde production of previously unstudied isolates could be predicted from where their functional genes clustered during phylogenetic analysis. From the results of this study, we expect that genetic screening based on MLST could be used as a promising approach for identifying *L. bulgaricus* with excellent flavor-producing properties.

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