A survey of the relationship between functional genes and acetaldehyde production characteristics in *Streptococcus thermophilus* by multilocus sequence typing

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ABSTRACT

Streptococcus thermophilus is an important bacterium used in the production of fermented dairy products. Yogurt with good flavor is preferred by consumers; thus, variation in flavor-formation characteristics among isolates is attracting attention. Here, acetaldehyde production characteristics of 30 isolates were evaluated in parallel with genotyping and multilocus sequence typing of key functional genes involved in acetaldehyde production. The results showed that isolates could be divided into 3 phenotypically distinct groups: high-acetaldehyde-yielding isolates (>10 mg/L), medium-acetaldehyde-yielding isolates (5–10 mg/L) and low-acetaldehyde-yielding (<5 mg/L) based on evaluation of acetaldehyde production during yogurt storage. These groups, distinguishable by phenotypic characteristics, were clustered in corresponding groups based on functional gene multilocus sequence typing analysis. Combining functional gene sequence analysis of 30 *Strep. thermophilus* isolates with phenotypic evaluation of their flavor-related characteristics (specifically acetaldehyde production) demonstrated that groups of isolates established using genotype data analysis corresponded with groups identified based on their phenotypic traits. Interestingly, the 30 isolates of *Strep. thermophilus* showed significant phylogenetic clustering in acetaldehyde content by functional gene and acetaldehyde content analysis. A corresponding relationship exists between functional gene phylogenetic clustering and acetaldehyde content variation.

Key words: *Streptococcus thermophilus*, multilocus sequence typing, functional genes, flavor production

INTRODUCTION

Consumption of yogurt, an ancient and nutritious food, is increasing as a result of its beneficial effects on human health (Astrup, 2014; El-Abbadi et al., 2014; Rizzoli, 2014). Biochemically, yogurt is a complex gel comprising proteins, polysaccharides, and lipids. It is a mixture of milk (whole, low fat, or nonfat) and sometimes cream, that has been fermented by the lactic acid bacteria (**LAB**) *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Mater et al., 2005). Flavor (taste and odor) is a characteristic property of food that influences consumer acceptance and is also associated with feelings of wellbeing (Routray and Mishra, 2011).

Several flavor compounds have been isolated from natural yogurts. The predominant ones are lactic acid, which imparts an acidic, refreshing taste, and a mixture of various carbonyl compounds such as acetaldehyde, ethanol, acetone, diacetyl, and 2-butanone. Among these, acetaldehyde is considered to be the major flavor compound contributing to the typical yogurt aroma reported by several researchers (Ott et al., 1997; Chaves et al., 2002).

Although each of these carbonyl compounds constitutes a recognizable aroma in their own right, yogurt flavor is determined by a balanced mixture of the most important volatile compounds. Both the ratio of different flavor compounds and their overall quantity affect yogurt flavor. For example, a ratio of 2:8 for acetaldehyde and acetone is considered optimum (Gardini et al., 1999; Chaves et al., 2002). The effect of a particular compound on the character of the aroma is determined by its concentration in the end product and its threshold value in any given matrix (Imhof et al., 1995).

Lactobacillus delbrueckii ssp. *bulgaricus* and *Strep. thermophilus* are known to be the most important species producing volatile organic aroma compounds in yogurt (Smit et al., 2005). These 2 microorganisms

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act together to produce the volatile molecules associated with flavor development (Courtin and Rul, 2004). Despite this, a great number of volatile organic compounds identified in yogurt originate from the milk and are not produced by the starter culture (Beshkova et al., 1998). Production of acetaldehyde by both bacterial species is isolate dependent; some authors report that *Strep. thermophilus* produces more acetaldehyde than *L. delbrueckii* ssp. *bulgaricus* (Ott et al., 1997), and other authors find the opposite (Pette and Lolkema, 1950; Schirch et al., 1985; Gezgin et al., 2015).

Flavor compounds are produced by carbohydrate fermentation in LAB and some potential pathways for acetaldehyde synthesis have been proposed (Cheng, 2010; Steele et al., 2013; Chen et al., 2017). Some flavor compounds are also produced by AA conversion in LAB via 2 steps: proteolysis and AA degradation. The third production pathway for flavor compounds in LAB is from lipids. It is possible that several metabolic pathways could lead to the formation of flavor compounds: (1) directly via pyruvate decarboxylase or pyruvate oxidase during fermentation or (2) indirectly by the formation of the intermediate acetyl coenzyme A by pyruvate dehydrogenase or pyruvate formate lyase or (3) via another pathway.

Previous studies have shown that acetaldehyde formation in *Strep. thermophilus* is catalyzed by threonine activity enzyme activity (interconversion of threonine into acetaldehyde and glycine) of serine hydroxyl methyl transferase (EC 2.1.2.1), encoded by the *glyA* gene (Chaves et al., 2002). Many LAB NADH oxidases, encoded by the *nod* gene, can switch metabolic route to produce different end compounds (Hugenholtz and Kleerebezem, 1999). Glucose was shown to be the main precursor of acetaldehyde, accounting for >90% of labeled acetaldehyde in *L. delbrueckii* ssp. *bulgaricus* and close to 100% in *Strep. thermophilus* (Ott et al., 2000).

To increase consumer acceptance and the competitiveness of production, scientific research to optimize selection of starter cultures is required. For fermented milk products it is particularly important to select LAB starter cultures with excellent flavor characteristics. However, phenotypic analysis of fermentation characteristics and other functional traits is time consuming, especially when a large number of bacteria are to be tested at the same time and when several different production characteristics (e.g., fermentation time, viable counts, volatile components, sensory characteristics of yogurt, synthesis of compounds of interest) are to be determined.

With an increasing number of sequenced LAB genomes available, the functional properties of LAB isolates can be studied more easily at the molecular

level. To the best of our knowledge 22 complete genome sequences of *Strep. thermophilus* have been published to date (<http://www.ncbi.nlm.nih.gov/genome/420>). These provide an opportunity to comprehensively understand the relationship between functional genes and phenotypic characteristics.

In the present study, a total of 30 isolates of *Strep. thermophilus* with different acetaldehyde production characteristics were investigated; specifically, the phenotypic characteristics of each isolate were quantified and related to phylogenetic groupings of key functional genes thought to be involved in flavor formation. Among the many functional genes associated with yogurt fermentation and acid production described in the literature, we selected 7 genes for which key function analysis had already been performed on the metabolic pathways associated with flavor formation; these genes are involved in acidification rate, flavor formation, and survival of *Strep. thermophilus* during yogurt storage (Chaves et al., 2002; Smit et al., 2005; Sander, 2009; Cheng, 2010; Sieuwerts et al., 2010). Using multilocus sequence typing (MLST) analysis we aimed to relate the phylogeny of these genes to the observed phenotypes of the isolates. The results of this study provide an indication of whether genetic screening based on MLST could be used as a rapid screening process to identify isolates of *Strep. thermophilus* with optimal acetaldehyde production properties. We also aimed to elucidate the relationship between acetaldehyde production and functional genes in phylogenetic clusters.

MATERIALS AND METHODS

Substrates and Chemical Reagents

Acetaldehyde was purchased from Sigma Chemical Co. (St. Louis, MO). Skim milk powder was purchased from NZMP Ltd. (Wellington, New Zealand). Media for culturing *Strep. thermophilus* were M17 Agar (CM0785B, Oxoid, UK) and M17 Broth (CM0817B, Oxoid) purchased from Thermo Fisher Biochemicals (Beijing) Ltd. (Beijing, China).

Isolates and Culture Conditions

A total of 30 wild *Strep. thermophilus* isolates were selected and characterized in this study (Table 1). They originated from 18 geographical locations in China and Mongolia, and one was a reference isolate (L822) used as a commercial starter culture in China (unknown origin). Stock cultures were stored at -80°C in 20% (vol/vol) glycerol. Working cultures were prepared from frozen stock in M17 Broth (Oxoid); each isolate was

Table 1. Information on sequence type (ST) and alleles of functional genes and clustering groups of 30 *Streptococcus thermophilus* isolates

Strain reference	Isolation source	Isolation site	No. of alleles by functional gene							ST	
			ak	glyA	lld	nod	pdh	pfl	ST (no.)	Cluster type	Group
IMAU20713	Spontaneous yogurt	Tov province, Mongolia	1	1	1	2	1	2	7	I a	6
IMAU20313	Spontaneous yogurt	Dzahan province, Mongolia	1	1	1	2	2	2	5	I a	6
IMAU20418	Spontaneous yogurt	Hovsgol province, Mongolia	1	1	1	2	2	2	7	I a	6
IMAU20141	Spontaneous yogurt	Sukhbaatar Province, Mongolia	1	1	1	4	1	1	1	I b	4
IMAU20588	Spontaneous yogurt	Ovorhangay, Mongolia	1	1	1	4	1	1	1	I b	4
IMAU80545	Kurut	Xiahe county, Sichuan Province, China	1	1	1	4	1	1	1	I b	4
IMAU20438	Spontaneous yogurt	Hovsgol province, Mongolia	1	1	1	4	5	1	1	I b	4
L822	—	Unknown	2	1	1	1	4	1	4	II b	3
IMAU20246	Spontaneous yogurt	Khentii province, Mongolia	2	1	4	5	1	1	1	III a	1
IMAU80715	Sour whey	Luqu, Gansu province, China	2	1	4	5	7	8	3	III a	1
IMAU10632	Koumiss	Hulunbeier, Inner Mongolia	3	1	1	1	4	1	1	II a	3
IMAU10636	Spontaneous yogurt	Hulunbeier, Inner Mongolia	3	1	1	1	4	1	1	II a	3
IMAU20765	Spontaneous yogurt	Ulaanbaatar, Mongolia	3	1	1	1	4	6	6	II a	3
IMAU20764	Spontaneous yogurt	Ulaanbaatar, Mongolia	3	2	1	1	4	1	1	II a	3
IMAU80285	Qula	Aba of Hongyuan County, Sichuan Province, China	3	2	2	1	1	1	1	II a	2
IMAU80287	Qula	Aba of Hongyuan County, Sichuan Province, China	3	2	2	1	1	1	1	II a	2
IMAU80809	Qula	Luqu, Gansu province, China	3	2	2	1	1	1	1	II a	2
IMAU80840	Kurut	Maqu, Gansu province, China	3	2	2	1	1	1	1	II a	2
IMAU80842	Kurut	Maqu, Gansu province, China	3	2	2	1	1	1	1	II a	2
IMAU80844	Qula	Maqu, Gansu province, China	3	2	2	1	1	1	1	II a	2
IMAU80846	Qula	Maqu, Gansu province, China	3	2	2	1	1	1	1	II a	2
IMAU80843	Kurut	Maqu, Gansu province, China	3	2	2	1	6	1	1	II a	2
IMAU20796	Spontaneous yogurt	Ulaanbaatar, Mongolia	4	1	1	2	2	2	5	I a	6
IMAU20728	Spontaneous yogurt	Tov province, Mongolia	4	1	3	3	3	3	1	I c	5
IMAU20729	Spontaneous yogurt	Tov province, Mongolia	4	1	3	3	3	3	1	I c	5
IMAU20738	Spontaneous yogurt	Tov province, Mongolia	4	1	3	3	3	3	1	I c	5
IMAU20448	Spontaneous yogurt	Hovsgol province, Mongolia	4	3	1	3	2	5	8	I a	6
IMAU20594	Spontaneous yogurt	Ovorhangay, Mongolia	4	3	2	2	2	4	1	I a	6
IMAU40133	Kurut	Haibei, Qinghai, China	5	1	1	6	3	3	2	I a	6
IMAU20350	Spontaneous yogurt	Bulgan province, Mongolia	6	1	1	7	1	7	1	I c	4

incubated at 37°C for 16 to 18 h, without shaking, and subcultured twice at 16- to 18-h intervals before use.

Milk Fermentation

Fermentation characteristics of all isolates were individually evaluated in milk prepared by adding 1.0% glucose to 10% reconstituted skim milk. The milk was sterilized at 95°C for 10 min and cooled to 42°C. For each isolate evaluated 5 mL of a 1×10^7 cfu/mL suspension was inoculated, as a starter culture, into each of 5 flasks containing 250 mL of the prepared milk. Fermentation was allowed to proceed at 42°C until either the pH reached 4.6 to 4.7 or the titratable acidity reached 80°T; the elapsed time was recorded (Wang et al., 2009). Experiments were repeated 3 times. The resulting yogurts were then stored at 4°C for 48 h. The pH and titratable acidity were measured during fermentation and storage time. In addition, the acetaldehyde concentration was measured during storage, as described below.

Analysis of pH and Titratable Acidity. Samples (25 mL) were taken every 2 h during fermentation and the pH and titratable acidity determined in triplicate to determine how fermentation was progressing. During the storage time, the pH and titratable acidity was also detected every 12 h. The pH was measured using a pH meter (Mettler Toledo, Zurich, Switzerland) and titratable acidity (°T) was determined after mixing the sample (9.0 g) with 10 mL of distilled water and titrating it with 0.1 N NaOH using a 0.5% phenolphthalein indicator to an end point (faint pink color: National Standards of the People's Republic of China, 1996).

Analysis of Acetaldehyde Content. Samples of fermented milk (5 g) were taken at the end of fermentation (0 h) and after 12, 24, 36, and 48 h of storage. Citric acid (20%, wt/vol) was added (0.5 mL) to each sample and the suspension diluted with sterile distilled water to a final volume of 15 mL. After mixing, the suspension was centrifuged at 4°C at $4,200 \times g$ for 10 min. The acetaldehyde concentration in the supernatant was determined spectrophotometrically using an acetaldehyde determination kit K-ACHYD (Megazyme, Bray, Ireland) following the manufacturer's instructions. This method is based on the enzymatic (acetaldehyde dehydrogenase) reduction of NAD to NADH and recorded as the absorbance at 340 nm (Chaves et al., 2002). Based on acetaldehyde levels, we allocated isolates to high-, medium-, and low-producing groups as described in previous studies (Sandine et al., 1972; Saint-Eve et al., 2006; Routray and Mishra, 2011). The isolates in the high groups produced more than 10 mg/L of acetaldehyde after 24 or 36 h of storage; medium groups

produced 5 to 10 mg/L; and low groups produced less than 5 mg/L.

Statistical Analyses. Statistical analysis of data was done using SAS 16.0 (SAS Institute Inc., Cary, NC). Acetaldehyde concentration was evaluated in triplicate and results expressed as means $\pm$ standard deviation. Statistical analysis was performed by a *t*-test. For each isolate grouping, 3 pairwise Mann-Whitney comparisons were done at each different storage time.

Comparing Genetic Variability in 7 Functional Genes in Different Isolates of *Strep. thermophilus*

DNA Extraction. The DNA was extracted from each isolate according to the protocol of Liu et al. (2012). The quality of the extracted DNA was assessed by 0.8% agarose gel electrophoresis, and the optical density at 260/280 nm was measured by spectrophotometry. All DNA samples were stored at -20°C before processing.

Functional Gene Selection and Primer Design. Primers for MLST analysis were based on the intragenic regions of 7 genes selected for their functional involvement in acetaldehyde synthesis and pyruvate metabolism (Table 2). Seven selected functional genes were evenly distributed on the chromosome of *Strep. thermophilus*. The amplicon by these primers were used for MLST analysis of *Strep. thermophilus* strains.

PCR Amplification and MLST Sequencing. The reaction volume for PCR amplification was 50 μ L and contained 150 ng of genomic DNA, 10 mM of each dNTP, 10 pmol of each primer, and 2.5 U of Taq polymerase in $1 \times$ PCR buffer (with Mg^{2+}). Parameters for thermal cycling were 94°C for 5 min; 30 cycles of 94°C for 1 min; 55 to 60°C for 0.5 min (optimal annealing temperature varied depending on what was appropriate for the particular gene); extension at 72°C for 1 min; and a final extension of 72°C for 10 min. Sequencing of the PCR products was done on the Genome Sequencer FLX454 Titanium System (454 Life Sciences) by the Shanghai Majorbio Bio-Pharm Technology Corporation (Shanghai, China). The same primers were used for PCR and sequencing of both DNA strands.

MLST Data Analysis. Both forward and reverse sequences from each isolate were trimmed, aligned, and analyzed. For each MLST locus, a number was allocated to each distinct combination of alleles for each of the 7 genes using BioNumerics v. 6.0 (Applied Maths, Sint-Martens-Latem, Belgium). Different allelic sequences (with at least one nucleotide difference) were assigned arbitrary numbers. For each isolate, the combination of 7 alleles defined its allelic profile, and a unique allelic profile was designated as a sequence type

Table 2. Functional gene primers for multilocus sequence typing analysis

Gene	Primer ¹	Sequence of primer	Size of amplicon (bp)
<i>ak</i> (acetate kinase)	<i>ak</i> F	CAAAGTATTGAGCGATGT	431
	<i>ak</i> R	CAAGGAGCTTCTACCAGA	
<i>lld</i> (L-lactate dehydrogenase)	<i>lld</i> F	TAGCCACGCACTTCCTTT	470
	<i>lld</i> R	CGTAGTATGTAGCACCTTTT	
<i>pdh</i> (pyruvate decarboxylase)	<i>pdh</i> F	ATTTGCGTCGTTATGTAG	461
	<i>pdh</i> R	CTTGAAGACTTTGGCTGA	
<i>glyA</i> (serine hydroxy methyl transferase)	<i>glyA</i> F	TCACTCAGGTAGCCAAGC	542
	<i>glyA</i> R	TTCTTTAAGAGCGACAGC	
<i>nod</i> (NADH oxidase)	<i>nod</i> F	TCATTCTTGGGTTGTGGT	519
	<i>nod</i> R	CTGTTACGATGCGTTCTA	
<i>pfl</i> (pyruvate formate-lyase)	<i>pfl</i> F	GGTATCTTCCGTGCTTAT	323
	<i>pfl</i> R	TTGTGATGAATGTTGGGT	
<i>pdh</i> (pyruvate dehydrogenase)	<i>pdh</i> F	ACAGGATTTGGTGGTAAG	474
	<i>pdh</i> R	GTAACGGTGTTCCTCTTT	

¹F = forward; R = reverse.

(ST). The mean G+C content of the DNA, the number of polymorphic sites, and the nucleotide diversity were calculated using Dna SP v. 5.1 (Librado and Rozas, 2009). This software was also used to calculate the ratio of the number of synonymous changes per synonymous site (d_S) to the number of nonsynonymous changes per nonsynonymous site (d_N ; d_S/d_N ratio) and to perform Tajima's D test.

Linkage equilibrium analysis of the 7 alleles of I_A value and SI_A was performed using the LIAN-linkage analysis tool on the MLST website (<https://pubmlst.org/databases.shtml>; Haubold and Hudson, 2000). To probe evolution and phylogenetic relationships among isolates based on the functional genes for differential acetaldehyde production, a minimum spanning tree was constructed with BioNumerics v 6.0 (Applied Maths). The recombinant analysis of 7 functional genes was done using SplitsTree v 4.10 software (Huson, 1998) on the MLST website (<http://eburst.mlst.net/>).

A phylogenetic tree was constructed based on sequences of 7 functional genes connected sequentially (*ak-lld-pdc-glyA-nod-pfl-pdh*) using the neighbor-joining evolution method (Sneath and Sokal, 1973) in the MEGA 4 program (Tamura et al., 2007). The sequences

of all alleles were deposited in the EMBL/GenBank databases, and the accession numbers are listed in Table 3. All unique sequences in the 7 genes were assigned an allele number. Clonal complexes (CC) corresponded to groups of ST that differed by only 1 (the single-locus variants) or 2 (the double-locus variants) alleles. In our description we call these CC "groups" because we assigned them by combining genotypic and phenotypic characteristics.

RESULTS

Change of pH and Titratable Acidity During Yogurt Storage

The pH of yogurt produced by the different isolates declined slightly (from 4.5 to 4.2) during storage (Figure 1). The titratable acidity increased to a small extent (Figure 1).

Acetaldehyde Production by Different *Strep. thermophilus* Isolates in Yogurt During Storage

The acetaldehyde concentration in yogurt produced by different isolates of *Strep. thermophilus* varied greatly,

Table 3. Allelic variation in 7 functional genes

Locus	Fragment size (bp)	G+C content (mol %)	No. of polymorphic sites	π /site ¹	d_N/d_S ²	Tajima's D value	No. of alleles
<i>ak</i>	431	43.09	6	0.00634	0.0000	-1.29615	6
<i>glyA</i>	470	44.41	2	—	—	—	3
<i>lld</i>	461	43.33	4	0.00470	0.3636	-0.06501	4
<i>nod</i>	542	39.78	7	0.00439	0.3762	-0.86284	7
<i>pdh</i>	519	38.61	13	0.00881	0.3359	-0.75827	7
<i>pfl</i>	323	43.31	9	0.00614	0.5057	-0.70456	8
<i>pdh</i>	474	42.69	22	0.01281	0.1196	-1.65431	8

¹Mean pairwise nucleotide differences per site.

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

Figure 1. Changes in pH and titratable acidity of yogurt produced by *Streptococcus thermophilus* during storage for 48 h. Box = 25th and 75th percentiles; bars = minimum and maximum value; solid line = mean; dotted line = median.

ranging from 1.67 to 16.67 mg/L during storage. Based on acetaldehyde production, isolates were allocated to a high-yielding group (greater than 10 mg/L for most of the storage time; Figure 2A); medium-yielding group (5–10 mg/L for most of the storage time; Figure 2B); and low-yielding group (less than 5 mg/L for most of the storage time; Figure 2C). The acetaldehyde concentration of yogurt produced by group A was greater than 10 mg/L throughout storage with the exception of only one isolate (L822), which had a value less than 10 mg/L at some storage time points. In group B (Figure 2B), which contained 10 isolates, the acetaldehyde content of the yogurt produced ranged from 5 to 10 mg/L throughout storage, with the exception of only 2 isolates, IMAU80844 and IMAU20713, which produced more than 10 mg/L at some storage time point. In group C (Figure 2C), which contained 10 isolates, the

acetaldehyde concentration varied little during storage, although for isolates IMAU80715 and IMAU20728, values did exceed 5 mg/L at some storage time points. Three pairwise Mann-Whitney comparison corrected *P*-values are shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-16203>). The acetaldehyde concentration of isolates in group A was higher than that of group B and group C at 0 h of storage ($P = 0.0001$, 0.0003, 0.0016), at 12 h of storage ($P = 0.0006$, 0.0001, 0.0009), at 24 h of storage ($P = 0.0002$, 0.0001, 0.0003), at 36 h of storage ($P = 0.0004$, 0.0001, 0.0211), and at 48 h of storage ($P = 0.0018$, 0.0001, 0.0681).

Cluster Analysis of *Strep. thermophilus* Based on Functional Genes and Acetaldehyde Content

The number of alleles per locus ranged from 3 (*glyA*) to 8 (*pdh*, *pfl*; Table 1). The 30 isolates were assigned to 19 ST. The phylogenetic tree constructed showed that the 30 isolates were separated into 3 clusters and 6 subclusters (Figure 3A). Cluster I had 3 subclusters (subcluster Ia, Ib, and Ic) and contained 15 isolates; cluster II had 2 subclusters (subcluster IIa and IIb) and contained 13 isolates; and cluster III had a single subcluster (subcluster IIIa) and contained 2 isolates.

These clusters were distinguishable from each other by combining genetic analysis (functional gene sequences) and the phenotypic characteristic of acetaldehyde production during storage. Cluster I included low-yielding isolates (Figure 3B); they produced less than 10 mg/L throughout storage with the exception of IMAU20594, IMAU20713, and IMAU20588, where maximum values exceeded 10 mg/L at some storage time points (mean $\pm$ SD): 11.67 ± 0.32 (8 h), 10.70 ± 0.16 (24 h), 11.14 ± 0.04 (36 h), 11.70 ± 0.04 (8 h), 13.17 ± 0.16 (24 h), and 14.34 ± 0.16 (36 h). Isolates in cluster II were high-yielding isolates (more than 10 mg/L throughout storage) with the exception of IMAU80809, which produced maximum values of only 8.47 ± 0.20 (8 h) and 8.92 ± 0.02 (36 h) at some storage time points. Cluster III comprised only 2 isolates: one was a medium-yielding isolate, with maximum values of 7.78 (8 h) and 9.48 ± 0.43 (24 h), and the other was a low-yielding isolate, with a maximum value of 4.67 ± 0.40 (24 h).

MLST Loci and Allelic Diversity

Nonsynonymous substitutions per nonsynonymous site were relatively rare compared with synonymous changes per synonymous site (Table 3); the d_N/d_S ranged from 0.000 (for *ak* gene) to 0.5057 (for *pdh* gene) in this study. All of these genes showed negative Tajima's *D* values (Tajima, 1989), and no significant differences among them existed, which indicated selec-

Figure 2. Variability in acetaldehyde production by *Streptococcus thermophilus* isolates in yogurt during storage for 48 h. Panel A represents the high-acetaldehyde-content group, panel B represents the medium-acetaldehyde-content group, and panel C represents the low-acetaldehyde-content group. Isolates were classified as high-producing (>10 mg/L, $n = 10$), medium-producing (5–10 mg/L, $n = 10$), and low-producing strains (<5 mg/L, $n = 10$). Box = 25th and 75th percentiles; bars = minimum and maximum value; solid line = mean; dotted line = median.

tion was not based on observed allelic diversity, as is typically observed for functional genes.

Phylogenetic Relationships Among *Strep. thermophilus* Isolates in Relation to Acetaldehyde Content

The 30 *Strep. thermophilus* isolates were characterized based on genotype (functional gene sequences) and phenotype (acetaldehyde content) after different storage times. Although acetaldehyde production by *Strep. thermophilus* varied with storage time, the clusters formed based on gene sequence followed the same trend at each time point. Because the trends of phylogenetic development were similar, and acetaldehyde production was generally greatest after 24 h of storage, here we only describe the genetic evolutionary trends for *Strep. thermophilus* as a minimum-spanning tree constructed from functional genes and acetaldehyde production after 24 h (Figure 4). The 30 isolates were divided into 19 ST; every circle represents an ST (Figure 4). Of these, 15 ST were each represented by a single isolate; 2 ST (ST18, ST4) were represented by 3 isolates; ST8 was represented by 5 isolates; and ST5 was represented by 2 isolates. These ST could be placed into 4 different CC: CC1, CC2, CC3, and CC4. Isolates belonging to the same CC were more closely related to each other genetically. Based on a combination of functional gene sequences and the acetaldehyde production analysis, these CC were grouped into different acetaldehyde-producing isolates. Thus, the 30 isolates could be further separated into 6 groups. Group 1 included ST1 and ST2, which were predicted to be ancestral genotypes as they lay at the root of the minimum-spanning tree. Group 4 included ST3, ST4, and ST11, with isolates that had a variety of traits; some were high-yielding isolates and some were low-yielding isolates. In terms of phylogenetic tree development, group 4 belonged to an intermediate node. Groups 3 and 6 included 4 and 6 ST, respectively, and, similar to group 4, both of these groups had a mixture of high- and low-yielding isolates. In contrast, group 5, which included ST17 and ST18, contained mainly low-yielding isolates, and group 2, which included ST8 and ST9, contained mainly high-yielding isolates. From these results we were able to determine the genetic evolution of functional genes in *Strep. thermophilus*; the ancestral isolates (positioned at the intermediate nodes of the minimum split tree) with medium acetaldehyde production attributes led to the formation of isolates with a range of phenotypic characteristics through genetic differentiation of functional genes under environmental stress acclimation or genetic selection pressure during natural yogurt production.

Figure 3. Relationships between phylogeny and phenotypic characteristics of *Streptococcus thermophilus* isolates using the neighbor-joining method. (A) The phylogenetic tree was constructed based on the functional gene sequences; IMAU is the strain number. C = cluster. (B) The acetaldehyde concentration (mg/L; mean ± SD) produced by each isolate. The concentrations were more than 10 mg/L (red) or less than 5 mg/L (blue), or ranged from 5 to 10 mg/L (green).

Nucleotide Variation in Alleles and Changes of AA Sequence of the Protein

A total of 63 polymorphic sites existed in 7 genes; among these, there were 30 missense mutations in 5 genes. These mutations occurred in only a few isolates (1, 2, or 3 isolates) and were not formed as a specific rule. Interestingly, 3 nonsynonymous mutation sites existed in 3 genes (G to T in *glyA*; G to A in *lld*; and G to T in *nod*) from all the high-yielding isolates, except IMAU80809. These changes in nucleotide sequence lead

to the different AA sequence variation (namely A to S; E to K; and G to T; Table 4).

DISCUSSION

Flavor is one of the most important properties of any food product and an important factor determining acceptability and preference. The production of acetaldehyde by *Strep. thermophilus* is a very important characteristic in dairy product production. The acetaldehyde content of yogurt during storage varied

significantly among the isolates we studied. This means that changes in acetaldehyde content are isolate specific. Most studies in the literature report levels of acetaldehyde in yogurt that range between 10 and 15 $\mu\text{g/g}$ (equivalent to mg/L ; Beshkova et al., 1998; Routray and Mishra, 2011), although Tamime and Deeth (1980) reported levels between 2.5 and 37.5 ppm (equivalent to mg/L). Many researchers believe that the acetaldehyde content of yogurt could range from 8 to 50 mg/L (Ršić and Kurmann, 1978; Imhof and Bosset, 1994; Chen et al., 2017). Highest concentrations of acetaldehyde (in the range of 5 to 21 mg/L) are due to its low utilization rate in LAB, which lack the alcohol dehydrogenase enzyme responsible for conversion of acetaldehyde to ethanol (Chaves et al., 2002). Yogurt with poor flavor

has less than 4.0 mg/L of acetaldehyde (which is considered to be a suboptimal amount), whereas yogurt with good flavor has greater than 8.0 mg/L of acetaldehyde (Sandine et al., 1972). Beshkova et al. (1998) measured the production of flavor compounds when mixed starter cultures were used for lactic acid fermentation and found that the maximum concentration of flavor compounds was achieved within 22 to 31 h of the cooling stage. Thus, we analyzed the relationship between acetaldehyde content and functional genes of *Strep. thermophilus* at 36 and 48 h. In the present study the 30 isolates could be divided into 3 groups based on the acetaldehyde content of the yogurt during storage. This methodology for grouping may better reflect their contribution to the flavor of yogurt.

Figure 4. Minimum split tree for *Streptococcus thermophilus* based on functional gene sequences and acetaldehyde concentration in yogurt during storage. IMAU is the strain number. Green circles correspond to medium-acetaldehyde-production isolates (approximately 10 mg/L), red circles to high-acetaldehyde-production isolates (approximately 15 mg/L), and blue circles to low-acetaldehyde-production isolates (approximately 5 mg/L). The size of the circles is proportional to the number of isolates belonging to each sequence type (ST). Clonal complexes (CC) are indicated in a different color. Combining CC and acetaldehyde production allowed further separation into different groups. Group 1 is predicted to be the ancestral genotype of the major CC.

Differences in the flavor-active compounds in yogurt compared with milk are most likely to be due to the metabolism of the LAB. These culture-generated aroma compounds result from microbial, enzymatic, or chemical transformations of lactose, lipids, citric acid, and proteins or AA present in milk (Tamime and Robinson, 1999; McGorin, 2001; Boelrijk et al., 2003). During storage, the volatile constituents of yogurt can change depending on the starter culture, formulation, and storage conditions (Tamime and Deeth, 1980; Kneifel et al., 1992). However, with respect to acetaldehyde content, the most important determining factors are LAB isolate characteristics, particularly their genetic basis. In general, there are 3 major pathways for the production of flavor constituents in yogurt: (1) degradation pathways of milk fat; (2) lactate metabolic pathways; and (3) citrate metabolic pathways (McSweeney and Sousa, 2000; Cheng, 2010). During fermentation, bacteria perform 3 major biochemical conversions of milk components: first, conversion of carbohydrate into lactic acid or other metabolites (glycolysis); second, hydroly-

sis of caseins into peptides and free AA (proteolysis); and third, breakdown of milk fat into free fatty acids (lipolysis; Steele et al., 2013; Smid and Kleerebezem, 2014; Chen et al., 2017).

In recent years, it has become possible to screen microorganisms based on genotype using microbial genome sequencing. In general, the complete genome of the microorganism is sequenced and then functional genes and gene clusters located through comparative genomics sequence analysis tools, to elucidate the relationships between genes or gene clusters and phenotypic characteristics. However, to our knowledge no studies until ours relate specific phenotypic traits with multiple genes active in the metabolic pathway of interest. No multiple gene screening methods have been developed, and genetic evolution and phylogenetic clustering analysis based on the characteristics and functional gene sequences has not been described previously.

In the present study, 7 functional genes associated with acetaldehyde production during pyruvate metabolism were selected and evaluated using MLST in 30

Table 4. Nucleotide variation in alleles and change in AA sequences of proteins¹

Item	<i>gly</i>				<i>lld</i>				<i>nod</i>			
	Nucleotide, site			Protein, site	Nucleotide, site			Protein, site	Nucleotide, site			Protein, site
	211	212	213	71	421	422	423	138	43	44	45	15
IMAU10632	G	C	A	A	G	A	A	E	G	T	C	V
IMAU10636	G	C	A	A	G	A	A	E	G	T	C	V
IMAU20141	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20246	G	C	A	A	G	A	A	E	G	T	C	V
IMAU20313	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20350	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20418	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20438	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20448	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20588	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20594	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20713	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20728	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20729	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20765	G	C	A	A	G	A	A	E	G	T	C	V
IMAU20738	G	C	A	A	G	A	A	E	G	G	C	G
IMAU20769	G	C	A	A	G	A	A	E	G	G	C	G
IMAU40133	G	C	A	A	G	A	A	E	G	G	C	G
IMAU80545	G	C	A	A	G	A	A	E	G	T	C	V
IMAU80715	G	C	A	A	G	A	A	E	G	T	C	V
L822	G	C	A	A	G	A	A	E	G	T	C	V
IMAU80287	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V
IMAU20764	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V
IMAU80285	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V
IMAU80809	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V
IMAU80840	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V
IMAU80842	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V
IMAU80843	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V
IMAU80844	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V
IMAU80846	T	C	A	<i>S</i>	A	A	A	<i>K</i>	G	T	C	V

¹Bold text means that the sequence nucleotide has been mutated. Italic text means that the AA has been changed in proteins.

isolates. These selected genes were present as a single copy in all isolates, and the functions of their encoded proteins were preferentially conserved and well characterized (Chaves et al., 2002; Bolotin et al., 2004; Smit et al., 2005; Sander, 2009; Cheng, 2010). From the resulting minimum split-tree, it was possible to differentiate and genetically group isolates with similar phenotypic acetaldehyde production traits and elucidate the functional genetic evolutionary history of the isolates. It is very interesting that the coding sequence of the *gly*, *lld*, and *nod* genes from high-yielding isolates was identical (at base position of 211, 421, and 43), but these bases had been mutated in most of the low- and medium-yielding isolates (Table 4). This base variation leads to changes in AA sequence. Therefore, it is believable that the variation in these functional gene sequences is an important cause of the phenotypic variation, specifically acetaldehyde production by different isolates.

The isolate clusters that could be separated based on sequence differences in functional genes corresponded well with the group separation achieved based on acetaldehyde production. Hence, different isolates with different acetaldehyde production characteristics could be distinguished based on their functional gene sequence analysis. For this reason, the results of this study may provide a methodological foundation for the development of a rapid molecular screening method to select isolates with the best flavor-related phenotypes. Our results suggest that key functional genes related to acetaldehyde synthesis in *Strep. thermophilus* have undergone genetic differentiation leading to gene-driven variation in metabolism, through a process of genetic selection. We believe that the functional gene sequence data analysis of isolates accurately reflects the phenotypic characteristics of those isolates.

The ratio of d_N/d_S was less than one, which indicates that nonsynonymous mutations are harmful and that purifying selection reduces their retention speed. The results were confirmed by evaluation of Tajima's D value. The DNA G+C content observed for all alleles from the 7 genes ranged from 38.6 mol % (*pdc*) to 44.4 mol % (*glyA*), which was a little higher than that of the complete genomes of *Strep. thermophilus* isolate LMG18311^T (39.1 mol %) and *Strep. thermophilus* isolate ND03 (39.0 mol %).

CONCLUSIONS

Multilocus sequence typing analysis was used to elucidate genetic evolution and phylogenetic relationships among 30 isolates of *Strep. thermophilus* based on variability in the sequences of 7 functional genes associated with acetaldehyde production. Groups of isolates separated by MLST were consistent with anal-

ysis of phenotypic characteristics. Specifically, high-, medium-, and low-acetaldehyde-yielding isolates were clustered in corresponding groups based on functional gene MLST analysis. The result of the present study shows that *Strep. thermophilus* with different acetaldehyde production characteristics evolved from ancestral isolates with medium-acetaldehyde-yielding capabilities through genetic evolution and natural selection pressures during yogurt production. Typing of functional gene sequences reflects the ongoing genetic evolutionary process; also, 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 to identifying *Strep. thermophilus* with excellent acetaldehyde production properties. It is interesting that we found the clusters of *Strep. thermophilus* isolates with similar acetaldehyde production characteristics corresponded with the functional gene phylogenetic groups.

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