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Identification of Thermoduric *Leuconostoc mesenteroides* BD5H That Can Grow at Over 42°C

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ABSTRACT

Lactic acid bacteria was isolated from Chonggak and Baechu kimchi and characterized by a sugar fermentation analysis. *Leuconostoc* strains (1B12, BD5H and CH5H) growing at 40°C were isolated and compared with *L. mesenteroides* subsp. *mesenteroides* KCTC 3722 grown at a mesophilic temperature (30°C). Among these, *L. mesenteroides* BD5H could grow at 42°C (pH 4.70±0.02) and could still grow at 43°C (pH 5.51±0.04), which indicates that is a thermoduric bacterial strain. In the fermentation of pentose, all strains had exactly the same patterns of fermentation, except *L. mesenteroides* CH5H, which could not ferment ribose (pH 5.92±0.04). These strains had significantly different abilities to ferment galactose, with only *L. mesenteroides* BD5H capable of active galactose fermentation (pH 4.94±0.03). All strains failed to ferment rhamnose compared to other hexoses ($p<0.001$). *L. mesenteroides* BD5H could ferment cellobiose (4.42±0.02), while the remaining strains could not. *L. mesenteroides* BD5H could ferment raffinose (pH 4.66±0.07), but not melezitose. These fermentation characteristics are typical of *L. mesenteroides*. Interestingly *L. mesenteroides* BD5H could ferment amygdalin (pH 4.82±0.04), while other strains could not ($p<0.001$). Furthermore *L. mesenteroides* BD5H could ferment salicin (pH 4.77±0.15), while the other strains could not. According to 16S rRNA sequence analysis using primers 785F and 907R, all tested strains were *L. mesenteroides*, with 99~100% identity. Significant differences were observed between these strains in their ability to ferment carbohydrates, which enabled better differentiation than that afforded by 16S rRNA sequence analysis. Carbohydrate fermentation analysis allowed for subspecies-level identification of *L. mesenteroides* isolated from Baechu and Chonggak kimchi.

Key words : 16S rRNA, Carbohydrate fermentation, Kimchi, *Leuconostoc mesenteroides*, Thermoduric

Introduction

Leuconostoc mesenteroides is one of the most valuable lactic acid bacteria (LAB). *L. mesenteroides* is an anaerobic, gram-positive bacterium that produces lactic acid as a major product during the fermentation of carbohydrates [1-3]. Most lactic acid bacteria are mesophiles, and can grow at temperatures ranging from 24°C to 36°C [4,5]. *Leuconostoc* species have complex nutritional requirements, and reside in many plants, dairy foodstuffs and various fermented products [2,6,7]. The genus *Leuconostoc* is engaged in many commercial industries for foodstuffs [8,9]. *L. mesenteroides*, *L. gasicomitatum*, *L. dextranicum*, *L. cremoris*, *L. citreum*, *L. paramesenteroides* and *L. lactis* were isolated from fermented foods, and were evaluated on the basis of their physio-biochemical characteristics [3,10-13]. Among them, *L. mesenteroides* subsp. *mesenteroides* is frequently found in Korean kimchi and other fermented vegetables [10,14-16]. These bacterial species have not been studied to the same extent as other LAB such as *Lac-*

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tobacillus species [17]. 16S ribosomal RNA (rRNA) sequence analysis allows us to trace the ancient origins of bacteria, and to reveal the phylogenetic relationships between bacteria [18]. 16S rRNA plays an important role in protein synthesis by initiating mRNA synthesis at the 3′ terminus of the rRNA, and specific regions of the 16S rRNA in ribosomes interact directly with the anticodon regions of tRNA [19]. The popularity of 16S rRNA sequence analysis has been steadily increasing ever since Yang and Woese [20] applied comparative analysis of 16S rRNA sequences to clarify the phylogenetic relationships of *Leuconostoc* species [6]. In the present study, we characterized *L. mesenteroides* BD5H in order to identify its application in food industries, particularly concerning the high temperature fermentation ability of LAB. We examined the essential physio-biochemical nature of the isolated *Leuconostoc* strains by a carbohydrate fermentation test to distinguish *L. mesenteroides* subspecies. In addition, we applied 16S rRNA sequence analysis to further identify the isolated *Leuconostoc* strains at a molecular level by DNA fingerprinting.

Materials and Methods

1. Strains of lactic acid bacteria

Strains of *L. mesenteroides* used in the present study are listed in Table 1. *L. mesenteroides* subsp. *mesenteroides* KCTC 3722 was obtained from the Korean Collection for Type Cultures (KCTC), Biological Resources Center, Daejeon, Korea. Cultures of the bacterial strains were activated in a lactobacilli MRS (MB cells, Seoul, Korea) and stored in a solution containing 20% glycerol (Sigma, St. Louis, USA) in a freezer (LG, Korea) until reactivation.

2. Isolation of *L. mesenteroides* strains

L. mesenteroides strains were isolated from Chonggak and Baechu kimchi broth fermented in a commercial refrigerator (Samsung, Korea), and named 1B12, BD5H and CH5H. Briefly, an equal volume of the cooled kimchi broth was blended with 6% NaCl solution. After finishing serial dilution (10^{-1}∼10^{-10}), the kimchi broth was spread onto the prepared MRS plates containing 2% Bacto agar (Difco, MD, USA) for colony enumeration. After successive purifying the cultures, colonies were incubated in a Sanyo incubator (Model MIR-153, Gunma, Japan) at 26°C for 72 hours. For determination of cell growth kinetics, isolated LAB strains were incubated in the same conditions and collected after 3 days. The pH of culture media was measured with a pH meter (Mettler, Model 225, Schwerzenbach, Switzerland) after centrifugation of cultured solution at 2,500 × g, and 5°C for 20 minutes.

Table 1. *Leuconostoc mesenteroides* strains that have been used in this study

Species and Strains	Sources
Leuconostoc mesenteroides subsp. *mesenteroides* KCTC 3722	Silage
Leuconostoc mesenteroides 1B12	Chonggak kimchi
Leuconostoc mesenteroides BD5H	Baechu kimchi
Leuconostoc mesenteroides CH5H	Chonggak kimchi

3. Enumeration of colony forming units

Counting the colony forming units (CFU) can determine the accurate and precise numbers of viable bacterial cells in culture [12]. *L. mesenteroides* strains were cultured for 48 hours in a Sanyo incubator (Model MIR-162, Gunma, Japan) at 26 °C. The cultures were diluted from 10^{-1}∼10^{-13} by successive 10-fold dilution. A 100-μL aliquot of each of the diluted cultures was spread onto a prepared MRS plate containing 2% Bacto agar (Maryland, USA). After 2 days of incubation at 26 °C, colonies were counted and the number of viable bacterial cells was calculated for each strain three times for reproducibility.

4. Growth kinetics at high temperatures

In order to investigate the growth kinetics of the isolates as a function of temperature, 10 mL sterilized MRS media (Difco, MD, USA) was prepared in 15-mL plastic tubes (Corning, NY, USA). An aliquot containing 1×10^{10} bacterial cells of each strain was inoculated in the prepared MRS media and incubated (Sanyo Model MIR-153, Gunma, Japan). Each strain was incubated at a different temperature, ranging from 36°C to 44°C that were high temperatures for growth of *Leuconostoc* species. Optimum growth temperature for mesophilic *Leuconostoc* species is ranged from 25°C to 30°C. After 48 hours cultivation, the culture tubes were centrifuged for 20 minutes at 2,500 × g, and 5°C. The pH of culture supernatant was measured by a pH meter (Mettler Model 225, Schwerzenbach, Switzerland).

5. Carbohydrate fermentation test

The fermentation characteristics of the isolated strains were

Table 2. Nucleotide sequences of primers for 16S rRNA sequencing

Name	Nucleotide sequences	Remarks
785F (18 mer)	5′-GGATTAGATACCCTGGTA-3′	Sequencing primer
907R (20 mer)	5′-CCGTCAATTCMTTTRAGTTT-3′	Sequencing primer
27F (20 mer)	5′-AGAGTTTGATCMTGGCTCAG-3′	PCR primer
1492R (22 mer)	5′-TACGGYTACCTTGTTACGACTT-3′	PCR primer

F: forward primer, R: reverse primer.

tested by assessing their ability to ferment 24 carbohydrate substrates [12], which were added to the prepared MRS media (without glucose). A 10% stock solution of each carbohydrates was blended with glucose-free media at a ratio of 1 : 10. Then, 30 μL of bacterial solution (1×10^9 cells) was inoculated into 5 mL of carbohydrate-containing media in a 15 mL centrifuge tube (Corning, NY, USA). After 72 hours incubation at strain-specific optimum temperatures (Sanyo MIR-153, Gunma, Japan), the pH of the culture media was measured with a pH meter (Mettler Model 225, Schwerzenbach, Switzerland) after centrifugation of cultured solution at 2,500 × g, and 5°C for 20 minutes.

6. Analysis of 16S rRNA sequences

Three strains of lactic acid bacteria designated 1B12, BD5H and CH5H were cultured in MRS media (Difco, MD, USA) prepared in 15 mL centrifuge tubes (Corning, NY, USA) and were incubated for 48 hours at 26°C. The cultured samples were centrifuged at 2,500 × g, and 5°C for 20 minutes. After centrifugation was done, the pellets were blended with 1 mL of 20% glycerol solution and stored in 1.5 mL E-tubes. The prepared samples were sent to Macrogen (Daejeon, Korea) for analyzing 16S rRNA sequencing using primers listed in Table 2. The bacterial genus and species were then determined by comparing the 16S rRNA sequences of these strains against the NCBI database using the BLAST algorithm (BLASTN 2.5.1+).

7. Statistical analysis

Values of the pH were measured 3 times to give a reliable average measure of pH. Data were reported as mean ± standard deviation (SD) of three replicates using statistical software SPSS Statistics 22 (IBM, USA). The data analysis was completed by ANOVA and Tukey's test or t-test. Differences were considered significant at p value less than 0.05.

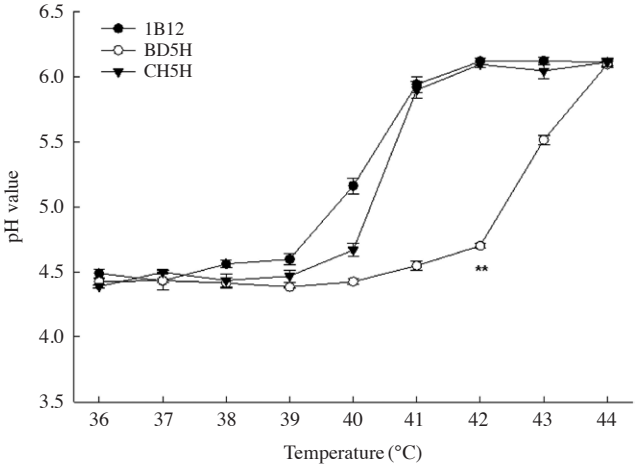

Fig. 1. Bacterial cell growth characteristics of the isolated strains of *Leuconostoc mesenteroides* as a function of temperature. Isolated strains were cultured at various temperatures ranging from 36°C to 44°C for 48 hours. Legend: 1B12 (●), BD5H (○), and CH5H (▼). Error bars indicate the standard error obtained in three independent experiments (**indicates significantly different from 41°C and 43°C in BD5H (○) by ANOVA and Tukey's test, $p < 0.01$).

Results and Discussion

1. Identifying thermoduric isolates

The growth of three *L. mesenteroides* isolates was precisely determined by measuring the pH of the culture media during incubation at temperatures ranging from 36°C to 44°C. A representative growth curve of each isolate is presented in Fig. 1. All tested strains were able to grow at increasing incubation temperatures ranging from 36°C to 40°C. Among the *Leuconostoc* strains, only BD5H (○) could grow continually at 42 °C (pH 4.70 ± 0.02), which is regarded as a high temperature for *Leuconostoc* species, and could also grow at 43°C (pH 5.51 ± 0.04). On the other hand, 1B12 (●) and CH5H (▼) could grow until 40°C (pH 5.16 ± 0.06 and 4.67 ± 0.05, respectively) and were unable to grow at 41°C and 42°C, with sharp incline pH 6.10 ± 0.03 at these temperatures. BD5H was therefore more tolerant of higher growth temperatures than

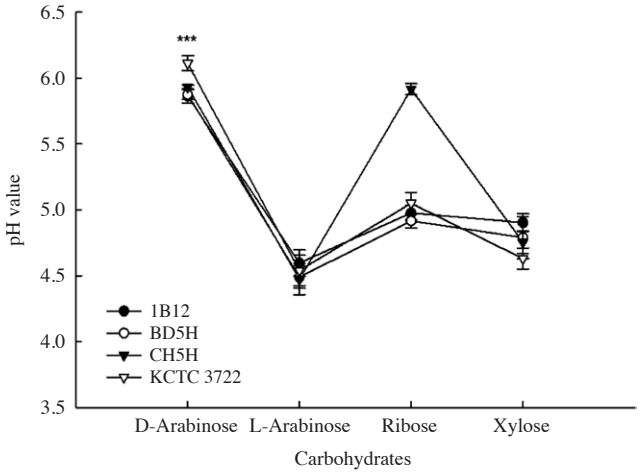

Fig. 2. Pentose fermentation of *Leuconostoc mesenteroides* isolated from Baechu and Chonggak kimchi. Legend: 1B12 (●), BD5H (○), CH5H (▼), and *L. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽). Error bars indicate the standard error obtained in three independent experiments (***indicates significantly different from the other groups in BD5H (○) by ANOVA and Tukey's test, $p<0.001$).

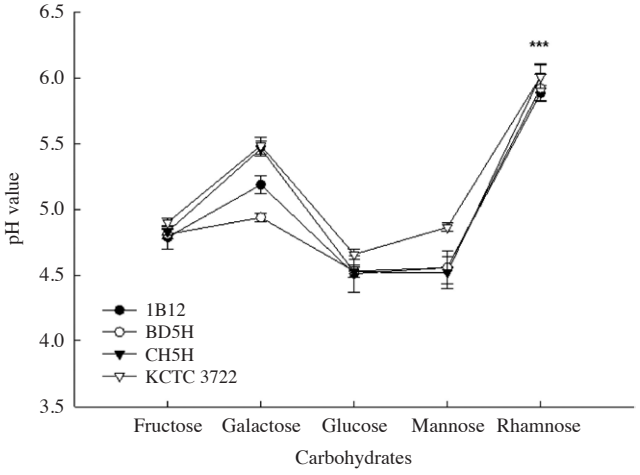

Fig. 3. Hexose fermentation of the *Leuconostoc mesenteroides* isolated from Baechu and Chonggak kimchi. Legend: 1B12 (●), BD5H (○), CH5H (▼), and *L. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽). Error bars indicate the standard error obtained in three independent experiments (***indicates significantly different from the other groups in BD5H (○) by ANOVA and Tukey's test, $p<0.001$).

1B12 and CH5H. The difference in growth between these strains at high temperatures was statistically significant. The strain BD5H, isolated from Baechu kimchi, can therefore be characterized as a thermoduric strain [21]. A standard strain, *L. mesenteroides* subsp. *mesenteroides* KCTC 3722 was characterized as a typical mesophilic *Leuconostoc* strain and could grow at temperature up to 30°C [23]. Since most *L. mesenteroides* have been isolated from kimchi that was fermented in a refrigerator at 4°C, it is surprising that BD5H could grow at high temperatures. Based on these results, we assume that BD5H can survive inside human bodies at 36∼37°C after ingestion. *L. mesenteroides* BD5H is therefore of interest to the food industry, as it is capable of surviving temperatures ranging from 14°C to 43°C (unpublished data), and can therefore survive food processing.

2. Fermentation of monosaccharides

Amongst the tested isolates, the fermentation tests with pentose and hexose revealed characteristic fermentation patterns (Figs. 2 and 3). During fermentation of pentose (Fig. 2), all strains had the same patterns of fermentation, except CH5H, which could not ferment ribose (pH 5.92 ± 0.04) or D-arabinose (pH 5.93 ± 0.02). For all tested strains, D-arabinose and L-arabinose were fermented at significantly different rates ($p<0.001$). The tested strains could ferment L-arabinose but they were unable to ferment D-arabinose. In the case of hexose fermentation (Fig. 3), the tested isolates had a significantly different ability to ferment galactose with BD5H demonstrating the fast fermentation rate (pH 4.94 ± 0.04). However, there were no significant difference observed in the fermentation of glucose and mannose, implying that these strains were all capable of utilizing these carbohydrates for their growth. Interestingly, these strains did not have ability to ferment rhamnose, which is regarded as a typical characteristic *Leuconostoc* and *Lactobacillus* [11,22-24].

3. Fermentation of disaccharides

All tested isolates were able to utilize disaccharides during bacterial growth including maltose, melibiose, sucrose, and trehalose as presented in Fig. 4. They could utilize maltose, melibiose, sucrose and trehalose very well (pH 4.3∼4.8). However, the tested strains grew poorly in the media containing lactose (pH 5.70 ± 0.15) which indicates that lactose was not fermented by *L. mesenteroides* in kimchi. That is a characteristic of LAB isolated from plants, while LAB isolated from animals can typically ferment lactose [22]. The tested isolates also differed in their ability to ferment cellobiose with only BD5H able to utilize cellobiose for growth, with its final pH reaching pH 4.42 ± 0.02. The tested disaccharides could therefore be divided into the following two groups based on the ability of the isolates to ferment them: fermentable disaccharides (maltose, melibiose, sucrose, and trehalose) and non-

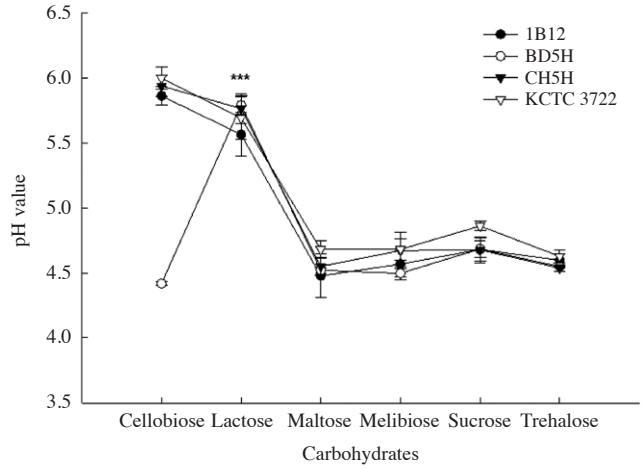

Fig. 4. Disaccharide fermentation of *Leuconostoc mesenteroides* isolated from Baechu and Chonggak kimchi. Legend: 1B12 (●), BD5H (○), CH5H (▼), and *L. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽). Error bars indicate the standard error obtained in three independent experiments (***indicates significantly different from the other groups in BD5H (○) by ANOVA and Tukey's test, $p<0.001$).

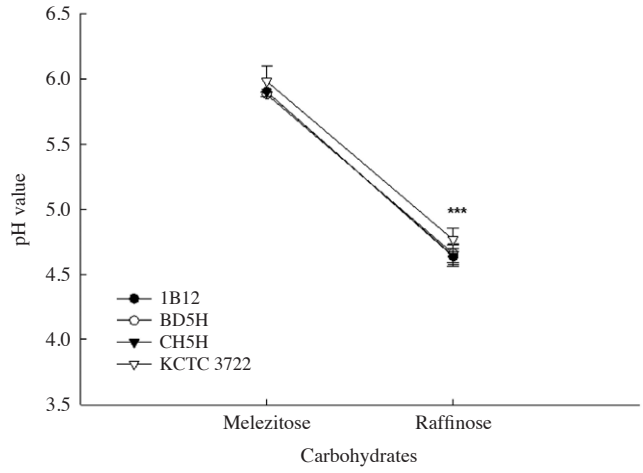

Fig. 5. Trisaccharide fermentation of *Leuconostoc mesenteroides* isolated from Baechu and Chonggak kimchi. Legend: 1B12 (●), BD5H (○), CH5H (▼), and *L. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽). Error bars indicate the standard error obtained in three independent experiments (***indicates significantly different from melezitose in BD5H (○) by t-test, $p<0.001$).

fermentable disaccharides (cellobiose and lactose). Out of the tested isolates, BD5H fermented cellobiose at a high rate than that of the other *L. mesenteroides* strains.

4. Fermentation of trisaccharides and complex sugars

Further strain characterization was possible following tests

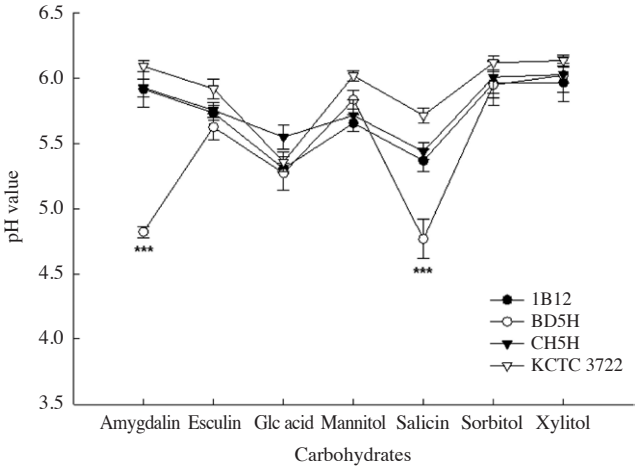

Fig. 6. Fermentation of carbohydrate derivatives in *Leuconostoc mesenteroides* isolated from Baechu and Chonggak kimchi. Legend: 1B12 (●), BD5H (○), CH5H (▼), and *L. mesenteroides* subsp. *mesenteroides* KCTC 3722 (▽). Glc acid indicates gluconic acid. Error bars indicate the standard error obtained in three independent experiments (***indicates significantly different from the other groups in BD5H (○) by ANOVA and Tukey's test, $p<0.001$).

for complex carbohydrates of trisaccharides (Fig. 5) and sugars derivatives (Fig. 6). All tested isolates fermented raffinose (pH 4.68 ± 0.10) but were unable to ferment melezitose (pH 5.92 ± 0.07). This analysis revealed the typical trisaccharides fermentation characteristics of *L. mesenteroides*. While BD5H could ferment amygdalin (pH 4.82 ± 0.04) as showed in Fig. 6, other *Leuconostoc* strains were unable to ferment it. The pH of BD5H culture media following amygdalin fermentation was significantly different to that of the other tested sugar derivatives ($p<0.001$), with the exception of salicin. The tested *Leuconostoc* strains could be divided into three groups based on their fermentation of salicin. The pH of the BD5H culture media was the lowest following fermentation of salicin (pH 4.77 ± 0.15), while 1B12 and CH5H, which are mesophilic LAB, had a culture pH of 5.37 ± 0.09, and 5.43 ± 0.06, respectively. The strain KCTC 3722 had the highest pH culture media with salicin as a carbohydrate (pH 5.71 ± 0.06), and could not grow over 36°C [23]. The tested *L. mesenteroides* strains differ in their ability to ferment carbohydrates although they are all grouped into *L. mesenteroides*.

5. 16S rRNA sequence analysis

16S rRNA sequencing analysis is a useful method to distinguish bacterial species. Based on sequences derived from 16S rRNA regions amplified by primers (785F and 907R), all test-

Table 3. Summary of 16S rRNA sequence analysis for *Leuconostoc mesenteroides* strains that can grow over 40°C from kimchi

Strains	Description	Length	Start	End	Coverage	Match	Pct.(%)
1B12	*L. mesenteroides*	1493	9	1493	99	1484/1485	99
BD5H	*L. mesenteroides*	1486	5	1480	100	1475/1476	99
CH5H	*L. mesenteroides*	1493	9	1493	99	1485/1485	100

NBRC	1	GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCACAGCGAAAGGTGCTTGC	60
BD5H	5	GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCACAGCGAAAGGTGCTTGC	64
NBRC	61	ACCTTTCAAGTGAGTGGCGAACGGGTGAGTAACACGTGGACAACCTGCCTCAAGGCTGGG	120
BD5H	65	ACCTTTCAAGTGAGTGGCGAACGGGTGAGTAACACGTGGACAACCTGCCTCAAGGCTGGG	124
NBRC	121	GATAACATTTGGAAACAGATGCTAATACCGAATAAAACTTAGTGTCGCATGACA**C**AAAGT	180
BD5H	125	GATAACATTTGGAAACAGATGCTAATACCGAATAAAACTTAGTGTCGCATGACA**A**AAAGT	184
NBRC	181	TAAAAGGCGCTTCGGCGTCACCTAGAGATGGATCCGCGGTGCATTAGTTAGTTGGTGGGG	240
BD5H	185	TAAAAGGCGCTTCGGCGTCACCTAGAGATGGATCCGCGGTGCATTAGTTAGTTGGTGGGG	244
NBRC	241	TAAAGGCCTACCAAGACAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGG	300
BD5H	245	TAAAGGCCTACCAAGACAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGG	304
NBRC	301	ACTGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAATCTTCCACAATGGGCG	360
BD5H	305	ACTGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAATCTTCCACAATGGGCG	364
NBRC	361	AAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGCTTTCGGGTCGTAAAGCACTGTT	420
BD5H	365	AAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGCTTTCGGGTCGTAAAGCACTGTT	424

Fig. 7. *Leuconostoc mesenteroides* BD5H 16S rRNA alignment with *L. mesenteroides* subsp. *mesenteroides* NBRC 100496 16S rRNA gene (partial sequence alignment). A single nucleotide mutation from C → A was found in *L. mesenteroides* BD5H 16S rRNA at position +175 (marked in bold). Nucleotide sequence of *L. mesenteroides* subsp. *mesenteroides* NBRC 100496 was obtained from NCBI BLAST (access number AB681194.1). Gene sequence alignment was done with BLASTN 2.5.1+. Abbreviations: NBRC: *L. mesenteroides* subsp. *mesenteroides* NBRC 100496, BD5H: *L. mesenteroides* BD5H.

ed strains were *L. mesenteroides*, with similarity following BLAST analysis (Table 3).

In the case of BD5H, 16S rRNA sequencing analysis revealed only one nucleotide difference to *L. mesenteroides* subsp. *mesenteroides*, at position +175 out of the 420 amplified nucleotides (Fig. 7). Although 16S rRNA sequence analysis was unable to differentiate these strains, they had significant differences in their abilities to ferment carbohydrate. The results of the carbohydrate fermentation test revealed distinguishing characteristics between these strains that enable to the subspecies-level identification of these strains isolated from Baechu and Chonggak kimchi. We can therefore conclude that the carbohydrate fermentation test is useful orthologous method for classifying lactic acid bacteria to subspecies-level, along with 16S rRNA sequencing analysis.

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