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# Identification and Characterization of *Leuconostoc gelidum*, Isolated from Kimchi, a Fermented Cabbage Product

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**We recently identified *Leuconostoc gelidum*, a typical psychrophile, as a microbial component from kimchi that has been laboratory-prepared and fermented at 20°C. However, it has been shown that the growth of leuconostocs in food products is highly influenced by fermenting temperature. To determine the distribution of *L. gelidum* species in kimchi fermented at a lower temperature, 8°C, we characterized a total of 64 dextran-forming strains isolated from kimchi using a polyphasic method including 16S rDNA sequencing and DNA-DNA hybridization. We found that 80% of the isolates were *L. gelidum*, which has been found mainly at chill-stored meat products. We also found that *L. gelidum* could be a dominant *Leuconostoc* species in so-called Kimjang kimchi, which is traditionally prepared at late fall to be preserved during winter in Korea. These results suggest that *L. gelidum* can be a predominant species in kimchi especially when fermented at low temperature.**

***Key words:*** *Leuconostoc gelidum,* lactic acid bacteria, kimchi

Leuconostocs are found mainly in vegetables and play important roles in vegetable fermentations, such as kimchi fermentation (6). In these vegetable fermentations, leuconostocs dominate the early stage of fermentation and produce lactic and acetic acid that inhibit harmful organisms and also produce important aroma and flavor (13).

Among the *Leuconosotoc* species, *L. mesenteroides* is well known to be one of dominant species in kimchi and some *Leuconostoc* species, including *L. lactis*, has also been isolated from kimchi (2). Unlike previous results, our recent findings showed that *L. citreum* could be a predominant species in kimchi when it is fermented at higher temperature (20°C) than normal (3). In addition, the results showed that *L. gelidum*, which have not been isolated from kimchi so far (8), could be a microbial component in kimchi.

We therefore hypothesized that *L. gelidum* may be a dominant species in kimchi when it is fermentation at a lower temperature, for instance 8°C, because *L. gelidum* strains mostly grow well at low temperature (11). To test this hypothesis, we examined the distribution of *L. gelidum* both in laboratory-prepared kimchi, fermented at 8°C, and in Kimjang kimchi, a Korean traditional kimchi especially prepared at late fall and stored during winter.

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## Materials and Methods

### *Sucrose-utilizing bacteria from kimchi*

Sixty-four strains were isolated randomly every three days during the early stage of kimchi fermentation (18 days) at 8°C and were plated directly on sucrose agar. Kimchi used in this study was prepared in this laboratory and was fermented at 8°C.

### *Classification of isolates by PCR*

To start the classification of 64 bacterial isolates, we first used a PCR-based identification method for *Leuconostoc* species that was previously developed in this laboratory (submitted). Three species-specific primer sets that target the 16S rDNA sequence were used to discriminate *L. citreum*, *L. gelidum* and *L. mesenteroides/L. pseudomesenteroides* strains from total isolates. PCR amplification with primers, specific to *L. gelidum* (Lgel-f: 5-TCG TAT CGC ATG ATAC AAG-3 & Lgel-r: 5-TAG ACG GTT CCC TCC TTAC-3) produced a 1290 bp band. The same PCR with *L. mesenteroides* primer set (Lmes-f: 5-AAC TTA GTG TCG CAT GAC-3 & Lmes-r: 5-AGT CGA GTT ACA GAC TAC AA-3) produced an 1150 bp band. PCR with *L. citreum* primer set (5-AAA ACT TAG TAT CGC ATG ATA TC-3 & 5-CTT AGA CGA CTC CCT CCCG-3) produced a 1298 bp band. These PCR amplifications did not produce any non-specific bands from other than target DNA.

### *Physiological and biochemical tests*

The strains were biochemically characterized using the

API CH50 strip and API CHL medium systems (API, bioMerieux) according to manufacturer's instructions (7). For comparison of sugar-usage patterns, we also used a modified standard method by Smibert and Krieg (12). Briefly, all tests were carried out in modified MRS medium containing 1.0% (w/v) sugar. Modified MRS medium was composed of all components of the original formula except casitone, beef extract, and glucose. Chlorophenol red (0.0015%) was added as a pH indicator. All test preparations were incubated at 25°C for 48 h before reading color changes.

***16S rRNA gene sequencing and DNA-DNA relatedness studies***

Isolation of the chromosomal DNA, 16S rDNA sequencing and DNA-DNA related studies were carried out as described previously (7).

***Laboratory kimchi preparation***

Kimchi was prepared as follows: Chinese cabbage (*Brassica pekinensis*) was chopped and treated with NaCl solution to a final 2.5%. The pretreated cabbage was blended with onion (3.0%), garlic (1.0%), and other ingredients including ginger. A 500 g of kimchi was incubated in a glass bottle at 8°C. Two replicate samples were used for microbial growth and chemical tests. Total acidity was examined by titerating a 10 ml sample with 0.1 N NaOH solution and by the following equation: total acidity (%) = (amount NaOH used (ml) × concentration of NaOH solution (N) × 9)/sample (ml).

***Bacterial isolation from Kimjang kimchi***

To isolate *Leuconosotoc* strains from Kimjang kimchi, we plated kimchi juice on sucrose plates and incubated at 8°C. Three types of Kimjang kimchi were used in this study: Kimjang kimchi A was in the very early phase of fermentation and was purchase from a hotel in Seoul (pH 5.0; total acidity 0.3%; 2 weeks after preparation at early November). Kimjang kimchi B was house-prepared and stored in a refrigerator that was specially designed for kimchi fermentation. Kimchi B had good sensory taste (pH 3.9; total acidity 0.9%; 1.5 month after preparation at early December). Kimjang kimchi C was also house-prepared but stored in an underground-pot that is a traditionally used for kimchi fermentation during winter in Korea. It also had good sensory taste (pH 3.8; total acidity 0.9%; 2 months after preparation in early December).

***Nucleotide sequence accession number***

The partial 16S rRNA sequence of *L. gelidum* DSM5578$^T$ (type strain) has been deposited in GenBank database under accession number AF175402.

## Results

***Isolation and characterization of dextran-forming strains from kimchi fermented at 8°C***

The aim of this study was to examine the distribution of *L. gelidum* in kimchi, fermented at a low temperature, 8°C. For this aim, we first prepared kimchi in this laboratory, fermented at 8°C, and isolated dextran-forming strains using sucrose plates during the early phase of fermentation. Fermentation of kimchi was slow at 8°C (Fig. 1). When pH levels dropped to 3.8 after 12 days of fermentation at 8°C, bacterial population levels increased to $10^9$ CFU/ml and total acidity increased to 0.9%. Even though the bacterial population did not increase, pH gradually dropped to 3.6 and total acidity reached 1.0%.

We randomly isolated a total of 64 strains at 8°C during fermentation of kimchi (Table 1). All isolates were gram-positive, catalase-negative and coccus-shaped bacteria. To examine the ratio of *L. gelidum* among isolates from kim-

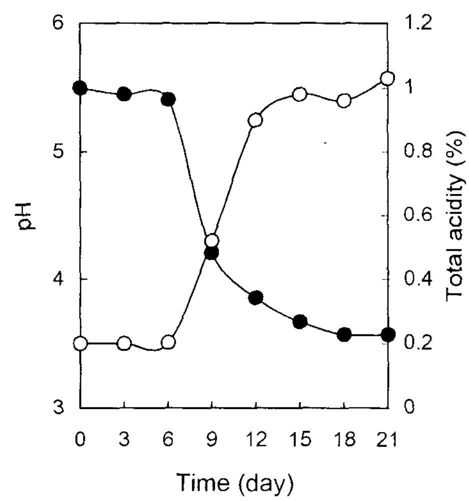


**Fig. 1.** Changes in pH and total acidity during kimchi fermentation, performed at 8°C: ●, pH; ○, total acidity. Data are average values of duplicate samples.

**Table 1.** *Leuconostoc* isolates from kimchi fermented at 8°C

| Time (Day) | Number of isolates[a] | | | |
|---|---|---|---|---|
| | *L. gelidum* | *L. mesenteroides* | *L. citreum* | Unknown |
| 0 | 14 | 3 | 1 | 5 |
| 3 | 7 | 2 | | |
| 6 | 6 | | | |
| 9 | 3 | 1 | | 1 |
| 12 | 4 | | | |
| 15 | 7 | | | |
| 18 | 9 | | | 1 |
| Total | 50 | 6 | 1 | 7 |

[a]Classification based on PCR that used species-specific primers targeted to the 16S rRNA gene. Some strains were used for further characterization.

**Table 2.** Characteristics of *Leuconostoc gelidum* strains and other isolates

| Characteristics | *L. gelidum* (n=50) | *L. mesenteroides* (n=6) | *L. citreum* (n=1) | *L. gelidum** | *L. mesenteroides** | *L. citreum** |
|---|---|---|---|---|---|---|
| Growth at 1°C | 49 | 0 | - | + | ND | - |
| Growth at 37°C | 8 | 50 | + | - | d | v |
| *Acid produced from*: | | | | | | |
| L-Arabinose | 98 | 83 | + | + | + | + |
| Cellobiose | 95 | 83 | + | + | d | + |
| Galactose | 78 | 100 | + | - | + | v |
| Lactose | 49[d] | 67[d] | - | - | d | - |
| Maltose | 98 | 100 | + | + | d | + |
| Melibiose | 100 | 83 | - | + | d | - |
| Ribose | 95 | 0 | v | - | + | - |
| Trehalose | 92 | 8 | + | + | + | + |
| Xylose | 98 | 67 | + | + | d | v |

Symbols: +, more than 90% of strains are positive; –, more than 90% of strains are negative; d, delayed reaction; v, variable; ND, not determined.
*Data taken from previous studies (6, 11).

chi fermented at 8°C, we next classified all isolates using a previously developed PCR-based identification method that uses species-specific primers (submitted). We found that 50 of 64 isolates belonged to *L. gelidum*, 6 isolates to *L. mesenteroides/L. pseudomesenteroides*, and 1 isolate to *L. citreum*. We were unable to identify the other 7 isolates. Analyzing the size of the PCR product further identified each isolate. PCR amplification produced a 1290 bp product from 50 isolates, an 1150 bp from 6 isolates and 1298 bp from 1 isolate (data not shown) and enabled us to classify them as *L. gelidum*, *L. mesenteroides/L. pseudomesenteroides* and *L. citreum*, respectively.

To confirm the PCR-based classification results, we tested the isolates with combined methods including phenotypic analysis, DNA-DNA relatedness test and 16S rDNA sequencing. A phenotypic analysis of all 50 isolates that classified as *L. gelidum* by a PCR-based method revealed a homogeneous group (Table 2) that could be divided into 4 subgroups. Strains of the *L. gelidum* group grew at 10°C and even at 1°C but not at 37°C. To further characterize these 50 isolates using DNA-DNA relatedness test and 16S rDNA sequencing, we chose 4 isolates from 4 subgroups of 50 *L. gelidum*-like isolates. We found that these 4 isolates belonged to the *L. gelidum* species, because DNA-DNA hybridization revealed more than 90% relatedness between 4 isolates and *L. gelidum* DSM 5578$^T$ (Table 3). 16S rDNA sequences of these 4 isolates showed over 99.8% identity (0-3 difference per 1505 nucleotides) to that of *L. gelidum* DSM 5578$^T$ (GenBank accession no. AF175402). Taken together, our results showed that 50 isolates that belonged to *L. gelidum* and this species, thriving through kimchi fermentation, was a dominant leuconostoc in a laboratory-prepared kimchi, fermented at 8°C.

**Table 3.** Mean levels of DNA relatedness (%) of isolated strains to *Leuconostoc* species

| Strain | Labeled strains | | |
|---|---|---|---|
| | DSM 5578 | KCTC 3526 | KCTC 3505 |
| BJ001 | 92 | 5 | 15 |
| BJ106 | 97 | 9 | 19 |
| BJ301 | 90 | 17 | 10 |
| BJ302 | 100 | 10 | 10 |
| *L. citreum* KCTC 3526$^T$ | 20 | 100 | ND |
| *L. gelidum* DSM 5578$^T$ | 100 | ND | ND |
| *L. mesenteroides* KCTC 3505$^T$ | 20 | ND | 100 |

For the *L. mesenteroides/L. pseudomesenteroides* group, we picked one strain from 6 *L. mesenteroides*-like isolates to determine its 16S rDNA sequence. We found that 16S rDNA sequence of this strain (1505 nucleotides) matched perfectly with that of the *L. mesenteroides* subsp *mesenteroides* type strain (M23035; 99.8% identity). We also identified an *L. citreum* isolate using its 16S rDNA sequence (100% identity with type strain).

***Detection of L. gelidum from Kimjang kimchi***

If *L. gelidum* is a dominant *Leuconostoc* species in laboratory-prepared kimchi that is fermented at 8°C, *L. gelidum* may be isolated from any kimchi that is fermented at a similar temperature. Kimjang kimchi is typically fermented at low temperatures. Therefore we tested for the existence of *L. gelidum* in three Kimjang kimchi using a PCR-base method. We found that *L. gelidum* was present. In addition, it was a dominant *Leuconostoc* species in Kimjang kimchi. All 10 isolates from Kimjang kimchi A, exhibited a 1290 bp PCR products (for *L. gelidum*) from species-specific PCR amplification (Fig. 2, lanes 5 & 6). Kimjang kimchi B contained both *L. gelidum* (1290 bp; Fig. 2, lane 7; 5 of 10 isolates) and *L. mesenteroides* (1150 bp; Fig. 2, lane 8; 5 of 10 isolates). However, *L. gelidum* was not isolated from Kimjang kimchi C, but *L. mesenteroides* was identified as a dominant species (Fig. 2, lanes 9 10; all 10 isolates). To provide an insight for the

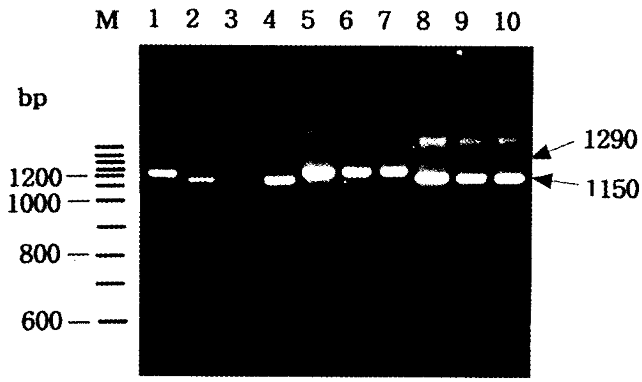


**FIg. 2.** PCR amplification of chromosomal DNA from Kimjang kimchi. Chromosomal DNA used from garlic (lanes 1 & 2), Chinese cabbage (3 & 4), Kimjang kimchi A (5 & 6), B (7 & 8), and C (9 & 10). DNA primer sets used are Lgel-f & Lgel-r (lanes 1, 3, 5, 6, 7) and Lmes-f & Lmes-r (lanes 2, 4, 8, 9, 10). Lane M, 100 bp ladder (BRL).

sources of these *Leuconostoc* species, we cultured the microflora of garlic or Chinese cabbage that were a vegetable component of kimchi. *L. gelidum* was found only in garlic (Fig. 2, lane 1). *L. mesenteroides* was found in both garlic and Chinese cabbage (Fig. 2, lane 2 & 4). These results indicate that cabbage and garlic may be original sources for *L. gelidum* or *L. mesenteroides* isolated from kimchi.

Taken together, our results indicate that *L. gelidum* can be a dominant *Leuconostoc* species in kimchi, when fermented at a low temperature.

## Discussion

In this study, we have shown that *Leuconostoc gelidum* is a dominant *Leuconostoc* species in kimchi, fermented at 8 °C. This result correlates with the fact that the natural habitat of leuconostocs is believed to be plants, including vegetable roots (10). However, this finding does not agree with previous results that *L. gelidum* has been isolated only from chill-stored meats (11). Based on these findings, it is possible to speculate that *L. gelidum* probably gained entry into raw meat from the pasture. This speculation is partly supported by the result that *L. gelidum* is isolated from garlic, an ingredient of kimchi (Fig. 2, lane 1). We conclude that *L. gelidum* from garlic may thrive in kimchi, when fermented at low temperatures. Similarly pasture-inhabiting *L. gelidum* may grow in raw meat. Evidence that leuconostocs are involved in silage fermentation (1), supports this notion. Our results also show that *L. mesenteroides* is isolated from both garlic and Chinese cabbage, implying that these strains may thrive in kimchi. These results suggest that the source of contamination of raw meats by *L. mesenteroides*, might be from the pasture (5).

The finding that *L. gelidum* and *L. mesenteroides* are isolated from garlic or Chinese cabbage (Fig. 2, lane 1-4) provides an insight in understanding the microbial composition in kimchi. Psychrophilic leuconostocs such as *L. gelidum* and most *L. mesenteroides* may dominate in kimchi, when fermented at temperatures, below 14°C (2, 9). However, at a higher fermenting temperature, other leuconosotocs such as *L. citreum* and some *L. mesenteroides*, may gain strength to outcompete the growth of the psychrophilic species, thus dominating in kimchi (3). On the basis of our finding and our recent evidence, we speculate that vegetable ingredients such as Chinese cabbage and garlic are the source of most *Leuconostoc* species found in kimchi.

Interestingly, to our knowledge *L. gelidum* has not been reported from kimchi or other vegetable products so far. This may be explained in two ways. First, *L. gelidum* isolates from kimchi might have been misclassified as *L. mesenteroides*, because discrimination of *L. gelidum* from *L. mesenteroides* is very difficult without a polyphasic method including 16S rDNA sequencing (4). In this study, however, our identification methods, namely PCR-based identification (Table 1), a phenotypic test (Table 2), DNA-DNA hybridization (Table 3) and 16S rDNA sequencing were able to unequivocally discriminate *L. gelidum* among isolates from kimchi. Second, it is possible that most *L. gelidum* from kimchi did not survive during plating on MRS or sucrose plates at high temperatures, such as at 30 or 37°C, due to their psychrophilic characteristics. However, this possibility is not likely because nearly all *L. gelidum* strains isolated in this study did not grow at 37 °C, but most did grow at 30°C.

In conclusion, this study shows that *L. gelidum* is found as a dominant *Leuconostoc* species in kimchi, fermented at 8°C, and thus may play important roles in kimchi fermentation at low temperatures.

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