

Isolation of Dextran-producing *Leuconostoc lactis* from Kimchi

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Tentative identification of *Leuconostoc lactis* IH23 isolated from kimchi (a fermented vegetable product) has been described previously with 16S rDNA sequencing (Choi, I., M. Sc. Thesis Inha Univ. 1999). This strain produced the slime identified as dextran based on IR, ¹³C- and ¹H-NMR spectroscopic results. Further study proved that the isolate IH23 belongs to a homogeneous genetic group with *L. lactis* DSM 20202^T and *L. argentinum* DSM 8581^T. The results showed DNA-DNA homology of 99-100%, 16S rDNA gene sequence similarity (99.7%), and a phylogenetic relationship although *L. argentinum* DSM 8581^T had lower homology (80-91%). These data indicate that *L. argentinum* DSM 8581^T and the isolate IH23 belong to an identical species with *L. lactis* DSM 20202^T at the genetic level, although in carbohydrate fermentation, the isolate IH23 was most closely related to *L. argentinum* DSM 8581^T and quite different from *L. lactis* DSM 20202^T. Here we first report the isolation of consistent phenotypic variation in *Leuconostoc lactis*. We also emphasize that the nomenclature of subspecies needs to be differentiated into the three strains mentioned above in *Leuconostoc lactis*.

Key words: kimchi, lactic acid bacteria, *Leuconostoc lactis*, dextran

Isolation and identification of lactic acid bacteria (LAB) have long been attempted in kimchi, conventionally prepared and fermented with the mixture of salted Chinese cabbage and spices (sliced radish, red peppers, garlic, ginger, onions, green onions and soy beans or fish lysate as amino acid sources). Kimchi is fermented with the variety of vegetables and therefore it is expected that various LAB propagate in its habitat. In recent papers, LAB in kimchi were identified and differentiated rapidly by the Biolog system (Biolog Inc., USA). Here it is reported that *Leuconostoc* and *Lactobacillus* are the main genera associated with kimchi fermentation (14, 15). The former genus is generally found at less than 15°C and is comprised of isolated strains such as *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc citreum*, *Leuconostoc gelidum* and *Leuconostoc kimchii* (3, 12, 13). All species of the genus *Leuconostoc* isolated from kimchi have been found to produce slime so far, which is a dextran.

During isolation procedures, we have occasionally observed characteristic mucous colonies on sucrose plates for dextran formation. The colonies exhibiting the slime formation have white and irregular layers at the margins and transparent center, and therefore it is easy to distinguish them from the other colonies on the sucrose plates. This colony also has shown a stable phenotype whenever serial

transfers were performed. The colonies were named isolate IH23, which was tentatively identified as *Leuconostoc lactis* IH23 with 16S rDNA sequencing (3). However, the type culture of *Leuconostoc lactis* does not produce dextran (9, 10). Therefore, we tried to learn whether or not the slimy material is dextran, and our previous identification is correct.

In this study, biochemical characteristics, 16S rDNA sequence similarity, DNA-DNA hybridization, and phylogenetic relationship between the isolate IH23 and authentic species were determined to clarify the taxonomic relationship. On the basis of these results, the isolate IH23 was identified as dextran-producing *Leuconostoc lactis*.

Materials and Methods

Bacterial strains and culture conditions

Strain IH23 was isolated from kimchi. *Leuconostoc lactis* DSM 20202^T, *Leuconostoc lactis* KCTC 3528^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3505^T, and *Leuconostoc argentinum* DSM 8581^T were included as reference strains. All strains were routinely grown in MRS broth or agar medium at 25°C (11). Cultures were maintained in 20% glycerol solution at -70°C until they were needed. Dextran production was tested by growth on 5% (w/v) sucrose agar described by Garvie (9).

Biochemical characterization

Biochemical and physiological characteristics were deter-

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mined by ASM methods (21). Cultures were inoculated into 10 ml MRS broth. Carbohydrate fermentation characteristics were determined by using the API 50CHL system (API, BioMerieux) according to the manufacturer's instructions. The results were read after API galleries were incubated for 3 days at 25°C.

16S rDNA gene sequencing and DNA-DNA hybridization

Cells in the exponential growth phase were harvested from MRS broth incubated at 25°C. Isolation of the chromosomal DNA, 16S rRNA gene sequencing, and DNA-DNA hybridization were done by the methods described previously (13). Chromosomal DNA was isolated by a modification of the method of Varmanen *et al.* (25). PCR amplification of the 16S rDNA was performed in a 100 µl reaction mixture containing 0.5 µM of each primer, 0.2 mM of each dNTPs and 2.5 U *Taq* polymerase (Perkin-Elmer) by using the following program; 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 10 min. The primers used were 5'-GAGTTTGATCCTGGCTCAG-3' (*Escherichia coli* numbering system, positions 9-27) and 5'-AGAAAGGAGGTGATCCAGCC-3' (positions 1525-1544) (2, 23). PCR products were cloned into pGEM vector (Promega) and sequenced by using a dideoxy sequencing kit (Sequenase version 2.0, USB). DNA-DNA hybridization was performed with ECL direct nucleic acid labeling and detection kit according to the manufacturer's protocols (RPN 3000, Amersham). The signal intensities were determined by using an image analyzer (Pharmacia). The signal produced by self-hybridization was taken as 100% and percentage homology values were calculated for the triplicate samples.

Extraction and analysis of dextran from the isolate IH23

The isolate IH23 was cultured in sucrose broth for 2 days at 25°C, and culture broth was centrifuged at 6000 × g, 4°C for 10 min to separate cells from soluble slime. The supernatant was treated with 2 volumes of cold ethanol (Sigma) at 4°C overnight to precipitate the slime. The pellet was collected by centrifugation at 6000 × g, 4°C for 10 min, washed with distilled water, purified three times, and then dried at 80°C. A dextran (Sigma) was used as a standard to compare with the slime material. The spectra were recorded with Varian Mercury 300 NMR spectrometer and Mattson Genesis II FT-IR spectrophotometer. The purified slime and dextran were dissolved in deionized water to give 0.5% (w/v) solutions (17).

Phylogenetic analysis

The sequences obtained from the isolate IH23 were used for sequence database searches in GenBank. The sequences representing the best matches were retrieved and aligned with the newly determined sequence using the CLUSTAL X program (version 1.5). Gaps at the 5' and 3' ends of the

alignment were omitted from further analyses, and 1346 nucleotides were used.

A distance matrix was calculated with the DNADIST program of the PHYLIP package (7), using the Kimura two-parameter model, and a phylogenetic tree was constructed according to the neighbour-joining method (19) with the program NEIGHBOR (PHYLIP package). The stability of the grouping was estimated by bootstrapping (1000 replications) using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE of the PHYLIP package.

Nucleotide sequence accession numbers

A approximately 1497-bp sequences of the 16S ribosomal gene of *L. lactis* IH23 have been deposited in the GenBank data library with the accession number AY026048.

Results

Physiological and biochemical characterization

In contrast to *L. lactis* DSM 20202^T, *L. lactis* KCTC 3528^T, and *L. argentinum* DSM8581^T, dextran production from

Table 1. Main characteristics of *Leuconostoc lactis*, *Leuconostoc argentinum*, and isolate IH23

Characteristics	<i>L. lactis</i>			<i>L. argentinum</i>	
	1	DSM 20202 ^T	KCTC 3528 ^T	strain IH 23	DSM 8581 ^T
Mucoid colonies on 5% sucrose agar	-	-	-	+	-
Acid produced from :					
L-Arabinose	-	-	-	+	-
D-Xylose	-	-	-	+	-
D-Fructose	+	-	-	+	+
D-Galactose	+	+	+ ^w	+	+
Mannose	+	-	-	+	+
Cellobiose	-	-	-	+	-
Lactose	+	+ ^w	+	+	+
Maltose	+	+	+	+	+
Melibiose	+	-	-	+	+
Sucrose	+	+	+	+	+
Trehalose	-	-	-	+	-
Raffinose	-	-	-	+	-
Mannitol	-	-	-	-	-
Amygdalin	-	-	-	-	-
Esculin	-	-	-	-	-
Salicin	-	-	-	-	-
α-Methyl-D-glucoside	ND	-	-	+	-
N-Acetyl glucosamine	ND	-	-	+	+
D-Turanose	ND	-	-	+	-

Data for taxon 1 cited from Garvie (9).

Symbols: +, positive reaction; -, negative reaction; +^w, weak positive reaction; ND, not determined.

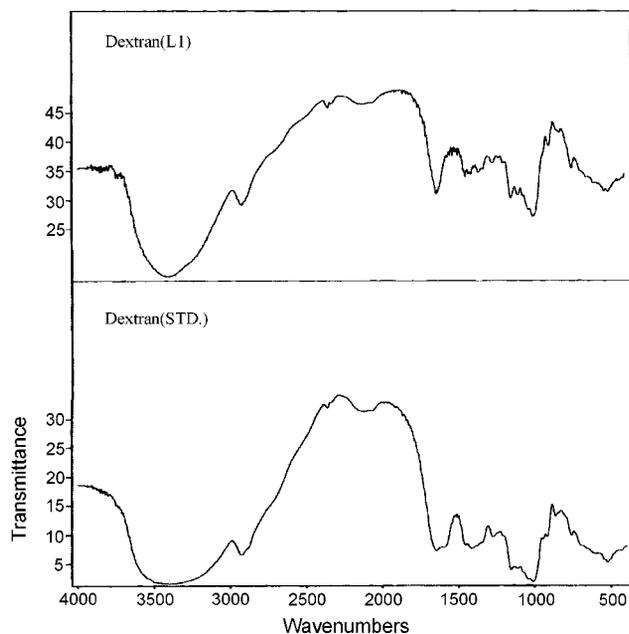


Fig. 1. IR spectra of slime substance from the isolate IH23 (L1), and standard dextran (STD).

sucrose occurred only in the cultures of isolate IH23. Other characteristics of relevant strains with isolate IH23 are summarized in Table 1. The following sugars were fermented by the isolate IH23: arabinose, xylose, fructose, glucose, galactose, mannose, cellobiose, lactose, maltose, melibiose, sucrose, trehalose, raffinose, α -methyl-D-glucoside, N-acetyl glucosamine and D-turanose. The isolate IH23 did not ferment mannitol, amygdalin, esculin, and salicin. Three strains commonly utilized lactose and galactose for fermentation. The isolate IH23 follows a very similar fermentation process as *L. argentinum* DSM 8581^T but different from *L. lactis* DSM 20202^T and KCTC 3528^T.

Dextran analysis

Qualitative studies were done with FT-IR for purity and the structure of polymer obtained from isolate IH23. Fig. 1 shows the peaks at 3410 cm^{-1} for -OH, at 2920 cm^{-1} for $-\text{CH}_2$, at 1600 cm^{-1} for $-\text{C}-\text{C}-$, at 1400 cm^{-1} for $-\text{CH}_2$ and -OH, and at 1000-1100 cm^{-1} for $-\text{C}-\text{O}-\text{C}-$. The spectra of standard dextran (Sigma) and the purified slime were perfectly identical. The slime was also confirmed by ^{13}C - and ^1H -NMR spectra (Fig. 2, 3). In ^1H -NMR, we observed 6 peaks at 3.15~5.8 ppm, binding to carbon on a lateral branch (Fig. 2) from both standard and sample L1. In ^{13}C -NMR, bridge carbon appeared at 93 ppm for both samples. The high similarity of these spectra demonstrated that the primary structure of the slime is identical to dextran.

DNA-DNA relatedness

For DNA-DNA hybridization against isolate IH23, three

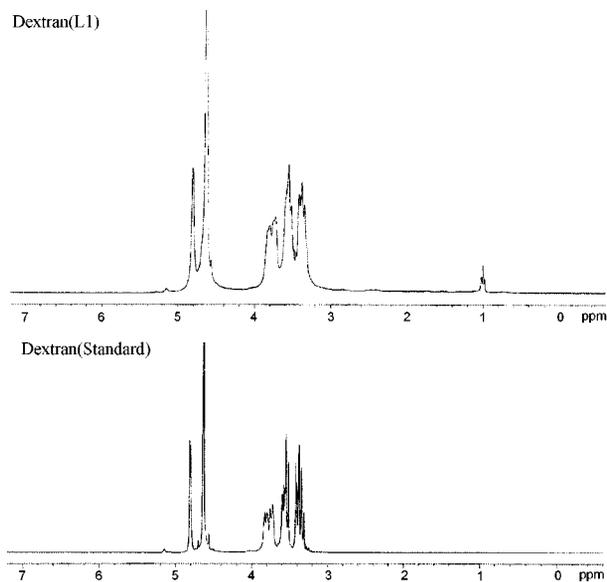


Fig. 2. Comparison of ^1H -NMR spectra of slime substance from IH23 (L1), and standard dextran (standard).

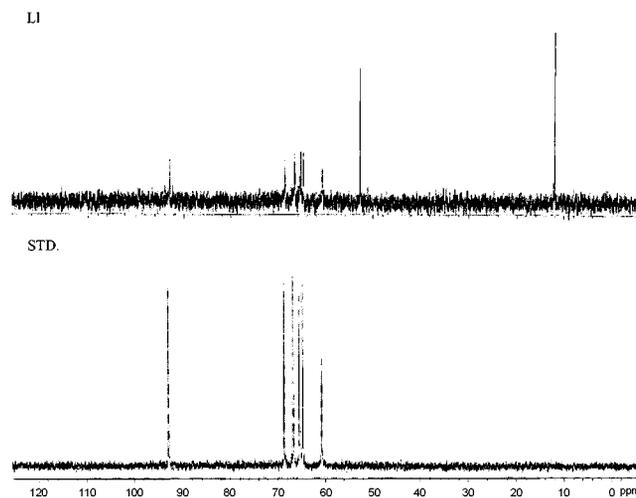


Fig. 3. Comparison of ^{13}C -NMR spectra of slime substance from IH23 (L1), and standard dextran (STD).

reference strains were chosen on the basis of phylogenetic group of genus *Leuconostoc*; *L. lactis* DSM 20202^T, *L. argentinum* DSM 8581^T and *L. mesenteroides* subsp. *mesenteroides* KCTC 3505^T. The results are listed in Table 2. Relative percentages of each species mentioned above were 99-100, 80-91 and 27~33%, respectively. Isolate IH23 was very similar to two species of *L. lactis* and *L. argentinum*, although *L. argentinum* has the value of a little lower homology.

Phylogenetic analysis and 16S rDNA similarity

The nearly complete sequence (1497 nucleotides) of IH23 was determined to confirm the phylogenetic position of isolate IH23. The phylogenetic position within genus *Leu-*

Table 2. DNA/DNA homologies between isolate IH23 and three reference strains of the genus *Leuconostoc*

<i>Leuconostoc</i> species	Reassociation with the isolate IH23(%)
<i>Leuconostoc lactis</i> DSM 20202 ^T	99-100
<i>Leuconostoc argentinum</i> DSM 8581 ^T	80-91
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> KCTC 3505 ^T	27-33

conostoc is depicted in Fig. 4, and its sequence similarities are given in Table 3. From the branching pattern of the tree, isolate IH23 is the closest relative to *L. lactis* DSM 20202^T and *L. argentinum* DSM 8581^T, and had 99.7% similarity of 16S rDNA sequences with those of *L. lactis* DSM 20202^T and *L. argentinum* DSM 8581^T.

Discussion

In 1960, Garvie first described *Leuconostoc lactis*, isolated in milk with no dextran production (9, 10). In Bergey's Manual (10), *Leuconostoc lactis* is the only species listed as a type strain. This strain is described as a dextran-nonformer on 5% (w/v) sucrose agar medium (9). Surprisingly, *Leuconostoc lactis*, as a dextran-former, has not been isolated and reported yet in the literature since the first isolation of 1960 by Garvie.

The isolation of any other strains producing dextran among this species has not been reported in the literature (6). In contrast, we isolated slimy colonies on sucrose agar media from kimchi and then tentatively identified them as *Leuconostoc lactis* IH23, by using sequence

Table 3. Percentage of 16S rDNA similarities between the isolate IH23 and other species in genus *Leuconostoc*

strain	similarity (%)
<i>L. argentinum</i> DSM 8581 ^T (AF175403)	99.7
<i>L. lactis</i> DSM 20202 ^T (M23031)	99.7
<i>L. citreum</i> strain IH22 (AF111949)	99.0
<i>L. mesenteroides</i> subsp. <i>mesenteroides</i> NRIC 1541 ^T (AB023243)	98.2
<i>L. mesenteroides</i> subsp. <i>cremoris</i> DSM 20346 ^T (M23034)	98.1
<i>L. pseudomesenteroides</i> NRIC 1777 ^T (AB023237)	97.9
<i>L. kimchii</i> strain IH25 ^T (AF173986)	97.8
<i>L. gelidum</i> DSM 5578 ^T (AF175402)	97.8
<i>L. carnosum</i> NCFB 2776 ^T (X95977)	97.2
<i>L. gasicomitatum</i> strain LMG 18811 ^T (AF231131)	97.1
<i>L. fallax</i> NRIC 0210 ^T (AB023239)	93.4

The numbers in parentheses are accession No. of GenBank.

matches between 16S rDNA sequences of the new isolate and those of the public data bases of GenBank.

Carbohydrate fermentation patterns of the isolate are similar to *L. argentinum* DSM 8581^T but different from *L. lactis* DSM 20202^T. Comparing our results with the others, fermentation patterns are also different among the same *Leuconostoc* species; therefore, acid production is not an absolute measure of grouping *Leuconostoc* (4, 5, 6, 20, 24). The comparative studies on FT-IR, ¹³C- and ¹H-NMR confirmed that the slimy substance was dextran. In diagnostic biochemical and physiological characteristics, the isolate forms typical and mucous colonies and may be easily distinguished from other *Leuconostoc* related species.

In our studies, all members within the genus *Leu-*

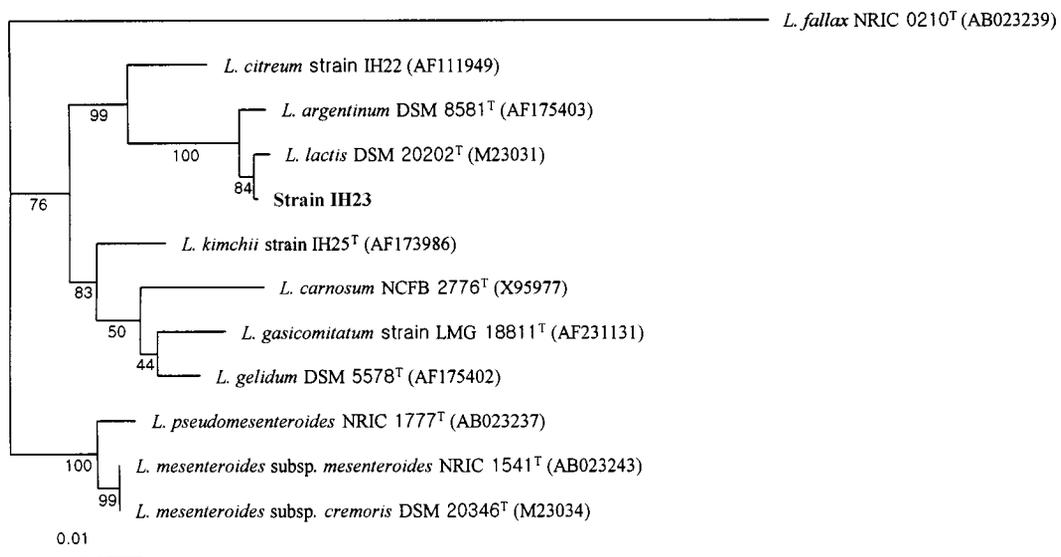


Fig. 4. Phylogenetic tree, based on 16S rDNA sequence analysis, showing the taxonomic position of isolate IH23 with members of *Leuconostoc*. The tree was rooted by using *L. fallax* as the outgroup. The tree was constructed using the DNADIST and neighbour-joining method. Bootstrap values were calculated from 1000 replications. Scale bar represents 1 nucleotide substitution per 100 nucleotides.

conostoc shows 97.1~99.7% 16S rDNA sequence homology with the isolate IH23 except for *L. fallax* with 93.4%. In particular, between *L. lactis* DSM 20202^T and *L. argentinum* DSM 8581^T 16S rDNA, sequence homology was 99.7%. In DNA-DNA hybridization, the isolate IH23 exhibited a high relatedness level of 99~100% with the type strain of *L. lactis* DSM 20202^T and lower relatedness level of 80-91% with *L. argentinum* DSM 8581^T. Comparing these results with the proposed criteria that a prokaryotic organism whose 16S rDNA sequence is more than 97% identical to any other sequence should be considered the same species, and also that hybridization values of above 70% have traditionally been taken as evidence for them being of the same species (16). The isolate was found to be genomically closer to *L. lactis* DSM 20202^T.

A phylogenetic tree was constructed for the detailed and confirmative identification, using the 16S rDNA sequence of 11 authentic species of the genus *Leuconostoc* deposited in GenBank, including the isolate IH23. The phylogenetic tree shows the isolate is also closely related to the same group as *L. lactis* DSM 20202^T and *L. argentinum* DSM 8581^T. This result, also discussed in Bjorkroth's paper, shows that *L. argentinum* is very difficult to differentiate from *L. lactis* LMG 7940 due to high similarities of ribopatterns, whole-cell protein profiles, API profiles and the highest 16S rDNA sequence homology (99.3%) (1). Based on the results, this isolate can be considered a variation of *Leuconostoc lactis*, and *L. argentinum* DSM 8581^T remains a doubtful species.

According to the division of species into two or more subspecies being defined by Staley and Krieg (22), it is necessary to give a new name to the isolate for differentiation from the type culture, for the isolate IH23 has an ability to produce dextran and form consistent phenotypic colonies (22). These characteristics can be benchmarks for a further classification of the species *L. lactis*. Another distinctive feature is that the isolate can ferment more diverse carbohydrates than those of the type strain. Interestingly, dextranase for the synthesis of dextran from sucrose has genes as large as 3050~4870 base pairs in *L. mesenteroides* subsp. *mesenteroides* strains (8, 18). Nothing is known of how the isolate IH23 acquired the gene for dextran-producing function. Further studies should be performed to search for the dextranase gene of the isolate IH23. Then we propose to establish the subspecies within the species *L. lactis*. Further discussion and information are necessary to include *L. argentinum* in the category of *L. lactis* type strain.

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