Lactobacillus siliginis sp. nov., isolated from wheat sourdough in South Korea

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The taxonomic position of two lactic-acid-producing bacterial strains, isolated from wheat sourdough in South Korea, was studied using a polyphasic approach. Phylogenetic analysis on the basis of 16S rRNA gene sequences and biochemical and physiological characteristics indicated these two strains to be members of the genus Lactobacillus. They had high 16S rRNA gene sequence similarity (98.5%) with Lactobacillus rossiae DSM 15814T and very low (<94.0%) similarity with any other recognized species of the genus Lactobacillus. These two strains (designated M1-212T and M2-236) were heterofermentative, facultatively anaerobic, Gram-positive, non-spore-forming, non-motile, short rod-shaped bacteria. The optimum growth temperature for these strains was 30 °C (no growth at 15 or 45 °C) and they were able to tolerate 5% (w/v) NaCl. The G+C content of the genomic DNA of the two strains was 45.5 mol%, within the range of values reported for the genus Lactobacillus (32–53 mol%). The peptidoglycan was of the A3α (L-Lys–D-Glu–L-Ala) type. Physiological, biochemical and genotypic data, as well as results of DNA–DNA hybridization of the genomic DNA with one of the closest phylogenetic relatives, L. rossiae DSM 15814T, indicated that the strains represent a novel species of the genus Lactobacillus, for which the name Lactobacillus siliginis sp. nov. is proposed. The type strain is M1-212T (=KCTC 3985T = NBRC 101315T).

Wheat (Triticum aestivum) bread is a staple food in many countries. Moreover, it contributes to cultural and geographical identity. Artisanal bread production, which often employs sourdough processes or the use of pre-fermentations, provides a wide regional variety of breads and speciality products. Sourdough is a mixture of wheat or rye flour and water that is fermented with lactic acid bacteria (LAB) and yeasts (Ga¨ nzle et al., 1998; Vogel et al., 1999). LAB metabolism is responsible for the production of aromatic compounds (Damiani et al., 2005), for which the name Lactobacillus siliginis sp. nov. is proposed. The type strain is M1-212T (=KCTC 3985T = NBRC 101315T).

In this study, two novel strains of LAB were isolated from a wheat sourdough sample. These strains, designated M1-212T and M2-236, were initially characterized on the basis of 16S rRNA gene sequence analysis. Based on results from the API 50 CH test system and other chemotaxonomic and genotypic characteristics, it was shown that these two strains represent a novel species in the genus Lactobacillus.

A wheat-flour sample was taken from standard Korean wheat-flour No. 3, produced by DaeHan Flour Mills, Korea (http://www.dhfLOUR.co.kr). Ten grams of wheat-flour was kneaded with 6 ml autoclaved water and 1% NaCl (w/v) for 10 min on a clean bench. After 24 h incubation at 30 °C, a small portion of the sample was serially diluted and was applied to MRS agar and half-strength MRS agar, pH 5.6, at 30 °C. Bromocresol green (0-022 g l⁻¹; Shinyo Pure Chemicals) was used as a pH indicator. Cell numbers were measured by a plating method on MRS agar after incubation at 30 °C for 3–5 days. MRS agar was prepared with Lactobacilli MRS broth (50·0 g l⁻¹, w/v; Difco) and agar powder (15·0 g l⁻¹, w/v; Samchun). Total cell counts of

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains M1-212T and M2-236 are DQ168027 and DQ168028, respectively.

Figures showing cells of strain M1-212T and PCR-generated DNA fingerprints of strains M1-212T, M2-236 and L. rossiae DSM 15814T are available as supplementary material in IJSEM Online.
LAB were estimated at about $9.5 \times 10^6$–$3.05 \times 10^8$ cells g$^{-1}$. Of 20 selected strains, two, designated M1-212$^T$ and M2-236, appeared clearly after 72 h on MRS agar and half-strength MRS agar, respectively. All strains were maintained on MRS agar at pH 6.0 at 30°C and were preserved at −70°C in 20% (w/v) glycerol stocks. Strains M1-212$^T$ and M2-236 were further investigated for their taxonomic position because they had high 16S rRNA gene sequence similarity (98.5%) with \textit{L. rossiae} DSM 15814$^T$ but very low (<94.0%) similarity with other recognized species within the genus \textit{Lactobacillus}. Other strains were identified as representing \textit{Lactobacillus curvatus}, \textit{Lactobacillus sakei}, \textit{Leuconostoc argentinum}, \textit{Leuconostoc mesenteroides}, \textit{Pediococcus pentosaceus} and \textit{Weissella cibaria}, and comprised about 15, 21, 15, 21, 11 and 5%, respectively, of the total cell counts of LAB. They had high 16S rRNA gene sequence similarity (99.2–100%) with the type strains of the related genera. Novel strains were estimated to make up about 10% of the total cell counts of LAB.

The Gram reaction was performed by using the non-staining method as described by Buck (1982). Cell morphology was observed under a Nikon light microscope at ×1000, with cells grown for 3 days at 30°C on MRS agar. Catalase and oxidase tests were performed by the procedures outlined by Cappuccino & Sherman (2002). A fermentation profile was determined using the API 50 CH test system according to the manufacturer’s instructions (bioMérieux). Growth at 15 and 45°C was tested in MRS broth. CO$_2$ gas production was detected in sourdough bacteria broth (Kline & Sugihara, 1971) containing glucose in place of maltose and supplemented with 10% gelatin powder in test tubes sealed with 2% sterile molten agar. Arginine hydrolysis was determined according to the method of Rippka et al. (2000). Aesculin hydrolysis was determined on MRS agar by adding aesculin (1·0 g l$^{-1}$; BDH) and ferric ammonium citrate (0·5 g l$^{-1}$) after 7 days incubation. \textit{Lactobacillus ruminis} KCCT 3601 was used as a positive control. Growth at different temperatures and pH was assessed after 5 days incubation. Salt tolerance was tested in MRS broth supplemented with 1–10% (w/v) NaCl after 7 days incubation. Duplicate antibiotic sensitivity tests were performed using filter-paper discs containing the following: streptomycin (Mast Diagnostics), tetracycline, kanamycin, ampicillin (Sigma) and rifampicin, each at concentrations of 5, 10, 50 and 100 μg ml$^{-1}$. Discs were placed on MRS agar plates spread with the test strains and \textit{L. rossiae} DSM 15814$^T$ (reference strain) cultures and were then incubated at 30°C for 7 days. Almost all tests were performed with reference strain \textit{L. rossiae} DSM 15814$^T$. Physiological and biochemical characteristics of the novel strains and related type strains are summarized in Table 1.

Extraction of genomic DNA was performed using a commercial genomic DNA extraction kit (Core Biosystem) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim et al. (2005). The full 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed by using the CLUSTAL_X program (Thompson et al., 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1972) methods in MEGA 3 (Kumar et al., 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

For the measurement of G+C content of the chromosomal DNA, the genomic DNA of the novel strains was extracted and purified as described by Moore (1995), and was then enzymically degraded into nucleosides. G+C content was determined as described by Mesbah et al. (1989) using a reversed-phase HPLC. DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki et al. (1989), using an FLx 800 microplate fluorescence reader (Bio-Tek) for fluorescence measurements. The hybridization temperature was 50°C, and reciprocal experiments were performed for \textit{L. rossiae} DSM 15814$^T$, M1-212$^T$ and M2-236. DNA–DNA hybridization tests were performed only with \textit{L. rossiae} DSM 15814$^T$ because 16S rRNA gene sequence similarity of the two novel strains with \textit{L. rossiae} DSM 15814$^T$ was 98.5% but very low (<94.0%) with other recognized species of the genus \textit{Lactobacillus} (Wayne et al., 1987; Stackebrandt & Goebel, 1994).

Finally, to determine the relatedness between strains M1-212$^T$ and M2-236 and \textit{L. rossiae} DSM 15814$^T$, we conducted genetic fingerprinting experiments, using BOX-, REP- andERIC-PCR according to Wieser & Busse (2000).

Cells of strains M1-212$^T$ and M2-236 were Gram-positive, non-motile rods (0·3–0·5 x 1·0–1·6 μm), occurring singly and in pairs, in filament-like and even in cluster forms (see Supplementary Fig. S1 in IJSEM Online). Colonies grown on MRS agar plates (Difco) for 3 days were smooth, circular, white and 0·8–1·5 mm in diameter. Spores were not observed and cells were heat sensitive. On MRS agar, the optimal growth temperature for both strains was 30°C; both were able to grow at 37°C but not at 15 or 45°C. The pH range for growth was between pH 5·0 and 8·0 with an optimum at pH 5·5. Growth occurred in the absence of NaCl and in the presence of 5·0% (w/v) NaCl but not 10% (w/v) NaCl. The two strains were sensitive to 5 μg ampicillin ml$^{-1}$, but resistant to 100 μg tetracycline ml$^{-1}$, 100 μg streptomycin ml$^{-1}$, 100 μg kanamycin ml$^{-1}$ and 100 μg rifampicin ml$^{-1}$. The reference strain, \textit{L. rossiae} DSM 15814$^T$, showed the same results for the above antibiotics except that it showed sensitivity to 100 μg rifampicin ml$^{-1}$. Strains M1-212$^T$ and M2-236 produced CO$_2$ gas from glucose, and thus were obligately heterofermentative. Ammonia was produced from arginine. Aesculin was not hydrolysed. The two novel strains had DNA G+C contents of 45·5 mol%. These values are within the range reported
Table 1. Comparison of selected characteristics of strain M1-212<sup>T</sup> with related Lactobacillus species

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<td>CO₂ gas from glucose</td>
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<td>NH₃ from arginine</td>
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<td>Gluconate</td>
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<td>DNA G+C content (mol%)</td>
<td>45-5</td>
<td>44-6</td>
<td>43-2–43-3</td>
<td>41-42</td>
<td>40-40-7</td>
<td>36-38</td>
<td>38-41</td>
<td>44-47</td>
<td>52-54</td>
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*Gas is produced from gluconic acid but not from glucose.
†DAP, Diaminopimelic acid; Orn, ornithine.

for the genus Lactobacillus (32–53 mol%; Kandler & Weiss, 1986). The peptidoglycan structure was of the A3<sup>2</sup> (l-Lys–D-Glu–l-Ala) type. Physiological characteristics of strains M1-212<sup>T</sup> and M2-236 are summarized in the species description below, and comparison of selective characteristics with related type strains is given in Table 1.

The 16S rRNA gene sequence of M1-212<sup>T</sup> was a continuous stretch of 1310 bp. Sequence similarity calculations following a neighbour-joining analysis indicated that the closest relative of the two novel strains was L. rossiae DSM 15814<sup>T</sup> (98.5%), and low levels of similarity (<94.0%) were found with the type strains of other members of the genus Lactobacillus. A phylogenetic tree based on the neighbour-joining and maximum-parsimony methods is shown in Fig. 1. The phylogenetic tree showed that strains M1-212<sup>T</sup> and M2-236 formed a cluster at a level of 100% similarity, with 100% bootstrap support. The branch closest to the cluster containing strains M1-212<sup>T</sup> and M2-236 contained just one recognized Lactobacillus species, L. rossiae DSM 15814<sup>T</sup> (Corsetti et al., 2005).

Levels of DNA–DNA relatedness between strains M1-212<sup>T</sup> and M2-236 were 95–100%. Strains M1-212<sup>T</sup> and M2-236 showed low levels of DNA–DNA relatedness, 30 and 31%, respectively, with L. rossiae DSM 15814<sup>T</sup>. High levels of DNA–DNA relatedness between strains M1-212<sup>T</sup> and M2-236 and low levels with L. rossiae DSM 15814<sup>T</sup> clearly indicated that the two novel strains should be classified as representing a novel species (Stackebrandt & Goebel, 1994).

Strains M1-212<sup>T</sup> and M2-236, being closely related to species of the genus Lactobacillus, can produce acid from ribose, D-fructose, N-acetylglucosamine, maltose, melibiose, D-lyxose and gluconate (weakly for galactose and D-mannose), but could not produce acid from L-arabinose, D-xylene or D-fructose, which differentiates the two strains from L. rossiae DSM 15814<sup>T</sup>. In BOX-, REP- and ERIC-PCR
Fig. 1. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships between strains M1-212T and M2-236 and related species. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships between strains M1-212T and M2-236 and related species. The phylogenetic tree was constructed by using the neighbour-joining method and Jukes–Cantor evolutionary distance matrix data obtained from unambiguous aligned nucleotides. Filled circles indicate that the corresponding clades were also recovered in maximum-parsimony trees. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at branch points. Bar, 1 substitution per 100 nucleotide positions.

banding patterns (see Supplementary Fig. S2 in IJSEM Online), strains M1-212T and M2-236 showed identical band profiles, indicating that the two strains have the same mother cell, but these bands were clearly different from that of L. rossiae DSM 15814T.

Thus, 16S rRNA gene sequence similarity, DNA G+C content, Gram-positivity, catalase-negativity, the absence of spore formation and the production of acid from glucose clearly indicate that strains M1-212T and M2-236 belong to the genus Lactobacillus. But low DNA–DNA relatedness with L. rossiae DSM 15814T, phylogenetic analysis, BOX-, REP- and ERIC-PCR banding patterns, sugar-fermentation patterns and other physiological characteristics indicate that strain M1-212T is clearly representative of a novel species of the genus Lactobacillus, for which the name Lactobacillus siliginis sp. nov. is proposed.

Description of Lactobacillus siliginis sp. nov.

Lactobacillus siliginis (L. n. siligo -inis a kind of very white wheat, fine wheaten flour; L. gen. n. siliginis of flour, the source of the first strains).

Cells are Gram-positive, facultative anaerobes, rod-shaped, non-spore-forming, non-motile, 0.3–0.5 µm wide by 1.0–1.6 µm long. They occur singly and in pairs and make filament-like or cluster-form structures. Colonies grown on MRS agar at 30°C for 3 days are 0.8–1.5 mm in diameter, white, smooth and circular. The temperature range for growth is 20–37°C; no growth occurs at 15 or 45°C. The optimum temperature for growth is 30°C. The pH growth range is between pH 4.0 and 8.0 with an optimum at pH 5.0. Growth occurs in the absence of NaCl and in the presence of 5–10% (w/v) NaCl but not 10% (w/v) NaCl. Sensitive to 5 µg ampicillin ml⁻¹, but resistant to 100 µg tetracycline ml⁻¹, 100 µg streptomycin ml⁻¹, 100 µg kanamycin ml⁻¹ and 100 µg rifampicin ml⁻¹. Catalase-negative and oxidase-positive. It produces CO₂ from glucose, thus being obligately heterofermentative. It produces acid from ribose, D-fructose, N-acetyl-D-glucosamine, maltose, melibiose, D-lyxose and gluconate; weak production of galactose and D-mannose. Does not produce acid from L-arabinose, D-xyllose or D-fructose. The peptidoglycan structure is of the A3L type. Ammonia is produced from arginine. Aesculin is not hydrolysed. The DNA G+C content of the type strain is 44.5 mol%.

The type strain, M1-212T (=KCTC 3985T =NBRC 101315T), was isolated from wheat sourdough in Daejeon, South Korea.

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References


