Sporolactobacillus vineae sp. nov., a spore-forming lactic acid bacterium isolated from vineyard soil

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Two spore-forming, facultatively anaerobic, lactic acid bacteria, strains SL153^T and SL1153, were isolated from vineyard soil in Korea. Cells of both strains were slightly curved, Gram-positive, motile rods that measured between 1 and 4 µm in length and were approximately 0.5 µm in diameter. Strains SL153^T and SL1153 fermented glucose, fructose, mannose and sorbitol, but were negative for nitrate reduction, catalase and oxidase. The predominant cellular fatty acids of the two isolates were iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. *meso*-Diaminopimelic acid, glucose, mannose and galactose were determined in their whole-cell hydrolysates. 16S rRNA gene sequences from the two strains were almost identical (99.9%) and they could be placed in the genus *Sporolactobacillus* according to phylogenetic analysis. The species most closely related to SL153^T were *Sporolactobacillus inulinus* and *Sporolactobacillus terrae* with 16S rRNA gene similarities of 95.7 and 95.5%, respectively, with the type strains. Levels of DNA–DNA relatedness between strain SL153^T and the type strains of *S. inulinus*, *S. terrae* and *Sporolactobacillus kofuensis* were 18.5, 18.0 and 17.0%, respectively. On the basis of the phylogenetic (16S rRNA gene), chemotaxonomic and phenotypic evidence given in this study, it is proposed that strains SL153^T and SL1153 should be assigned to the genus *Sporolactobacillus* as representatives of the novel species *Sporolactobacillus vineae* sp. nov. The type strain is SL153^T (=KCTC 5376^T=JCM 14637^T).

The genus *Sporolactobacillus* was defined by Kitahara & Suzuki (1963) to accommodate a catalase-negative, spore-forming, homofermentative, lactic acid-producing bacterial species, *Sporolactobacillus inulinus*, within the family *Lactobacillaceae*. Later, the genus *Sporolactobacillus* was transferred to the family *Bacillaceae* by Kitahara & Toyota (1972). More recently, in *Bergey's Manual of Systematic Bacteriology*, all members representing sporolactobacilli were grouped in the family '*Sporolactobacillaceae*' (Garrity & Holt, 2001). A comparison of physiological characteristics of species in the genus *Sporolactobacillus* and a spore-forming bacillus, *Bacillus coagulans*, revealed that members of the genus *Sporolactobacillus* lack catalase activity and require carbohydrate for growth (Yanagida *et al.*, 1987).

Six species in the genus *Sporolactobacillus* have been reported; they are from rather limited isolation sources. *S. inulinus* was isolated from chicken feed (Kitahara &

Suzuki, 1963) and the other species, *Sporolactobacillus kofuensis*, *S. lactosus*, *S. laevolacticus*, *S. nakayamae* and *S. terrae*, were isolated from the rhizosphere or soil around root hairs of a variety of wild plants collected in Japan and South-East Asia (Nakayama & Yanoshi, 1967a, b; Yanagida *et al.*, 1997). *S. laevolacticus* was reclassified from *Bacillus laevolacticus* Andersch *et al.* 1994 by Hatayama *et al.* (2006). Some evidence suggests that *Sporolactobacillus* species may be useful as a probiotic. *Sporolactobacillus* sp. L2407 presented antagonistic activity against *Bacillus* species (Holzapfel & Botha, 1988) and *S. inulinus* BCRC 14647 inhibits the growth of *Salmonella enteritidis* BCRC 10744 (Huang *et al.*, 2007).

In this study, the *Sporolactobacillus*-like strains, SL153^T and SL1153, which were isolated from a vineyard soil sample in Korea, are described. The micro-organisms were considered to be *Sporolactobacillus* on the basis of 16S rRNA gene sequence comparisons. Accordingly, the aim of this present work was to elucidate the taxonomic position of strains SL153^T and SL1153 by means of phenotypic, genetic and chemotaxonomic analyses.

Strains SL153^T and SL1153 were isolated following the methods of Yanagida *et al.* (1997). One gram of soil sample was mixed with 9 ml 0.75% NaCl solution. The mixed solution was heated for 30 min at 60 °C, after which one

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SL153^T and SL1153 are EF581819 and EF581818, respectively.

A phylogenetic tree based on 16S rRNA gene sequences using the neighbour-joining method showing the position of strains SL153^T and SL1153 and closely related strains of the family '*Sporolactobacillaceae*' is available as supplementary material with the online version of this paper.

drop of the suspension broth was spread onto GYP agar [2 % glucose, 1 % yeast extract, 1 % peptone, 1 % sodium acetate and 0.5 % (v/v) salt solution, which comprised 4 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.16 % $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.2 % $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.2 % NaCl; pH 6.8] plus 1 % CaCO_3 . The culture was incubated under anaerobic conditions at 30 °C for 2–3 days. After incubation, acid-producing bacteria were identified by the appearance of clear zones around colonies, selected and purified by repeated isolation.

Cells grown on GYP medium were used to determine the physiological characteristics of the isolates. Gram staining was performed using a Difco Gram stain set. Spore formation was determined by staining with malachite green. Morphology and cell size were examined using phase-contrast microscopy with a Nikon Optiphot-2 light microscope at 1500 magnification. For electron microscopy analysis, cells were negatively stained with 1 % (w/v) uranyl acetate according to the method described previously by Chang *et al.* (2002) and observed with a model H-7600 transmission electron microscope (Hitachi). Sugar fermentation patterns were determined using an API 50CH kit (bioMérieux). Catalase activity was determined by placing drops of 3 % (v/v) H_2O_2 on cultures growing on GYP and observing the production of oxygen bubbles. Oxidase activity was determined using an Oxy-swab (bioMérieux). For growth determination at various pH values (pH 4.0–9.0 in 1 unit increments), cells were inoculated in pH-adjusted media at 30 °C for 48 h and growth was measured by optical density at 595 nm (Bio-Rad). To determine optimum growth temperature and tolerance to NaCl, cells were cultured in GYP medium at temperatures of 15–60 °C and in NaCl concentrations of up to 7 % (w/v) for 2 days. Motility was observed in GYP medium with 0.4 % agar. The production of lactic acid was confirmed using a D-lactic acid/L-lactic acid kit (R-biopharm; Roche Diagnostics). Purified cell-wall preparations were obtained as described by Schleifer & Kandler (1972). The amino acid and sugar analysis of whole-cell hydrolysates were performed as described by Stanek & Roberts (1974) using TLC on cellulose plates with the solvent system of Rhuland *et al.* (1955).

Cells of the two isolates were motile, produced lactic acid, contained an oval spore and grew in GYP medium containing 7 % NaCl. The strains were negative for nitrate reduction, catalase and oxidase, but positive for acid production from glucose and fructose. Single cells of strain SL153^T were observed as slightly curved rods that measured between 1 and 4 µm in length and approximately 0.5 µm in diameter. Electron micrographs of thin sections of cells showed that the cytoplasmic membrane of strain SL153^T was surrounded by a multilayered cell envelope composed of a relatively thin electron-dense (peptidoglycan) layer and at least two additional exterior layers (data not shown). Physiological properties and sugar fermentation patterns of strains SL153^T and SL1153 are shown in Table 1. These physiological properties are consistent with previous descriptions of members of the

genus *Sporolactobacillus* (Nakayama & Yanoshi, 1967a, b; Yanagida *et al.*, 1997; Hatayama *et al.*, 2006).

The 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R, as described previously (Chang *et al.*, 2001). Phylogenetic relationships between strains SL153^T and SL1153 and other Gram-positive, spore-forming bacilli were inferred from a comparison of 16S rRNA gene sequences. Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967) and maximum-parsimony (Fitch, 1971) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining tree based on 1000 resamplings.

A preliminary comparison of the nucleotide sequences with sequences in GenBank and the Ribosomal Database Project (Maidak *et al.*, 2001) indicated that the isolates were closely related to members of the genus *Sporolactobacillus*. The 16S rRNA gene sequences of the test strains were aligned manually with representatives of the genus *Sporolactobacillus* and related taxa. Phylogenetic trees showing the relationship between isolates and representatives of the family ‘*Sporolactobacillaceae*’ are given in Fig. 1 and Supplementary Fig. S1 (available in IJSEM Online). The test strains formed a highly significant monophyletic clade with members of the genus *Sporolactobacillus*, which currently contains six species with validly published names. The *Sporolactobacillus* clade was also confirmed in other treeing algorithms supported by a highly significant bootstrap value of 100 %. On the basis of pairwise 16S rRNA gene sequence similarities, the closest relative to strain SL153^T was *S. inulinus* JCM 6014^T (95.7 %) followed by *S. terrae* JCM 3516^T (95.5 %) and *S. kofuensis* JCM 3419^T (95.2 %). The two sequences of SL153^T and SL1153 showed high similarity to each other (99.9 %) indicating that the two strains belong to the same species. It is evident from 16S rRNA gene sequence analysis that the two isolates represent a novel genomic species in the genus *Sporolactobacillus*, as none of the *Sporolactobacillus* species with validly published names showed more than 97 % 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994). Isolates SL153^T and SL1153 could also be clearly distinguished from the type species of the genus *Sporolactobacillus*.

The novel strains and some *Sporolactobacillus* reference strains were subjected to genomic relatedness analysis to confirm membership to the genus. DNA–DNA hybridization between strains SL153^T and SL1153 and their closest phylogenetic relatives based on 16S rRNA gene sequence similarity was determined by using the method described by Ezaki *et al.* (1989). Hybridization was carried out with photobiotin-labelled probes using a Fluoroskan Ascent Fluorescent plate reader (Thermo Life Science) with three

Table 1. Differential physiological characteristics of strains SL153^T and SL1153 and related species

Strains: 1, SL153^T and SL1153 (*S. vineae* sp. nov.); 2, *S. inulinus* JCM 6014^T; 3, *S. terrae* JCM 3516^T; 4, *S. nakayamae* subsp. *racemicus* JCM 3417^T; 5, *S. kofuensis* JCM 3419^T; 6, *S. nakayamae* subsp. *nakayamae* JCM 3514^T. +, Positive; –, negative; w, weakly positive. Cells of all strains are motile, produce lactic acid, have oval shaped spores and grow in 5 % NaCl. All strains are negative for catalase and oxidase, but positive for acid production from glucose, fructose, mannose and *N*-acetylglucosamine. For determination of acid production, cultures were incubated in GYP medium at 30 °C for 48 h.

Characteristic	1	2	3	4	5	6
Growth temperature range (°C)	25–40	25–40	25–45	15–40	25–40	25–40
Optimal temperature (°C)	37	37	30	30	37	37
Growth in 7 % NaCl	+	w	+	+	w	w
pH range for growth	6.0–7.0	6.0–7.0	6.0–8.0	5.0–8.0	6.0–7.0	6.0–8.0
Acid production from:						
Galactose	–	–	–	+	–	–
Sorbose	+	–	+	+	–	+
Mannitol	+	+	+	–	+	–
Sorbitol	+	+	–	–	–	–
Methyl α -D-glucoside	+	+	–	–	–	–
Amygdalin	–	–	–	w	–	–
Aesculin	–	–	–	+	–	–
Salicin	–	–	–	+	–	–
Cellobiose	–	–	–	+	–	–
Maltose	w	w	–	+	+	+
Melibiose	–	–	–	+	–	–
Sucrose	w	+	+	+	+	+
Trehalose	w	+	+	–	+	–
Inulin	–	+	–	–	–	–
Raffinose	–	+	–	+	–	+
Starch	–	–	–	–	+	–
Gentiobiose	–	–	–	+	–	–
Turanose	+	+	–	–	–	–
D-Tagatose	–	+	+	+	+	+

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences using the maximum-parsimony method showing the phylogenetic position of strain SL153^T and closely related strains of the family ‘*Sporolactobacillaceae*’. Bootstrap values (1000 replications) of 50 % or greater are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

replications for each sample at 45 °C. The genomic relatedness value between the two isolates was 81.9%. This value indicates that strains SL153^T and SL1153 represent the same species of *Sporolactobacillus*. The type strains of other *Sporolactobacillus* species were more distantly related to strain SL153^T. The genomic relatedness values of the type strains of *S. inulinus*, *S. terrae* and *S. kofuensis* were 18.5, 18.0 and 17.0 %, respectively. These values are below the 70 % threshold suggested for species delineation (Stackebrandt & Goebel, 1994). These low values indicate that strain SL153^T represents a novel species.

Cellular fatty acid compositions were determined for strains SL153^T and SL1153 grown on GYP agar for 2 days. Saponification, methylation and extraction were performed according to the standard protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed by GC (model 6890N and autosampler 7683; Agilent) and identified using the Microbial Identification Sherlock software package.

The major cellular fatty acids of all *Sporolactobacillus* strains are anteiso-C_{15:0} and anteiso-C_{17:0}, as reported in previous studies (Yanagida *et al.*, 1997). However, the predominant cellular fatty acids of the two isolates were iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0} (Table 2). Moreover, significant differences were found in the relative amounts of iso-C_{15:0} and anteiso-C_{15:0} fatty acids, which can be used to differentiate these isolates from other members of the genus.

In the whole-cell hydrolysate analysis, *meso*-diaminopimelic acid, glucose, mannose and galactose were determined in the isolates. The predominant amino acid and sugars determined in the cell wall components of the isolates correlated with those of the type strain of *S. inulinus*.

DNA was prepared according to Chang *et al.* (2002) and the DNA G+C value (mol%) was determined using the thermal denaturation method (Mandel & Marmur, 1968). The relative G+C contents were calculated by using standard DNA of *Escherichia coli*. The DNA G+C contents of strain SL153^T and SL1153 were 50.6 and 51.6 mol%,

respectively. These values were a little higher than those reported for *S. inulinus* (47.0–50.2 mol%), *S. terrae* (43–46 mol%) and *S. kofuensis* (43 mol%; Yanagida *et al.*, 1987, 1997), but the taxonomic position of the isolates still lies in the same genus, as determined by 16S rRNA gene sequence analysis. On the basis of molecular, chemical and phenotypic evidence presented in this study, it is proposed that strain SL153^T, isolated from vineyard soil, should be classified in the genus *Sporolactobacillus* as a representative of the novel species *Sporolactobacillus vineae* sp. nov.

Description of *Sporolactobacillus vineae* sp. nov.

Sporolactobacillus vineae (vin'e.ae. L. gen. n. *vineae* of a vineyard).

Cells are Gram-positive, catalase- and oxidase-negative, motile by peritrichous flagella, produce DL-lactic acid and have oval shaped spores. Nitrate reduction is negative. Colonies on GYP agar are round, smooth, ivory-white and approximately 1.5–3.0 mm in diameter. Single cells are slightly curved rods that measure between 1 and 4 µm in length and are approximately 0.5 µm in diameter. Growth occurs at temperatures between 25 and 40 °C in GYP medium (optimum 37 °C). Grows in the presence of up to 7 % NaCl and the optimal pH range for growth is 6.0–7.0. Facultatively anaerobic. Grows well on GYP agar when incubated under anaerobic conditions. Acid is produced from glucose, fructose, mannose, sorbose, mannitol, sorbitol, methyl α-D-glucoside, N-acetylglucosamine and turanose. Galactose, amygdalin, aesculin, salicin, cellobiose, melibiose, inulin, raffinose, starch, gentiobiose and D-tagatose are not fermented. Maltose, sucrose and trehalose are weakly fermented. The diagnostic amino acid is *meso*-diaminopimelic acid and the major cell wall sugars are glucose, mannose and galactose. Cells cultured in GYP medium contain iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0} as predominant cellular fatty acids. DNA G+C content is 50.6–51.6 mol%.

The type strain is SL153^T (=KCTC 5376^T=JCM 14637^T), isolated from vineyard soil. Strain SL1153, also isolated from vineyard soil, is a reference strain.

Table 2. Cellular fatty acid composition (%) of strains SL153^T and SL1153 and related species

Strains: 1, SL153^T; 2, SL1153; 3, *S. inulinus* JCM 6014^T; 4, *S. terrae* JCM 3516^T; 5, *S. nakayamae* subsp. *racemicus* JCM 3417^T; 6, *S. kofuensis* JCM 3419^T; 7, *S. nakayamae* subsp. *nakayamae* JCM 3514^T. tr, Trace amount (<1.0%); –, not detected.

Fatty acid	1	2	3	4	5	6	7
C _{14:0}	tr	tr	1.25	1.61	—	—	—
iso-C _{15:0}	13.28	12.57	7.9	6.62	7.71	7.61	3.13
anteiso-C _{15:0}	15.17	14.25	36.36	28.18	54.38	32.00	37.85
iso-C _{16:0}	4.68	5.92	3.33	4.39	3.74	4.81	2.03
C _{16:0}	5.37	4.97	4.48	8.81	5.87	6.03	6.97
iso-C _{17:0}	6.09	6.13	2.16	3.96	—	2.75	1.56
anteiso-C _{17:0}	53.79	55.50	44.52	46.44	28.29	46.81	48.46

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