**Lactobacillus formosensis** sp. nov., a lactic acid bacterium isolated from fermented soybean meal

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A Gram-reaction-positive, catalase-negative, facultatively anaerobic, rod-shaped lactic acid bacterium, designated strain S215ᵀ, was isolated from fermented soybean meal. The organism produced D-lactic acid from glucose without gas formation. 16S rRNA gene sequencing results showed that strain S215ᵀ had 98.74–99.60 % sequence similarity to the type strains of three species of the genus *Lactobacillus* (*Lactobacillus farcininis* BCRC 14043ᵀ, *Lactobacillus futsai* BCRC 80278ᵀ and *Lactobacillus crustorum* JCM 15951ᵀ). A comparison of two housekeeping genes, *rpoA* and *pheS*, revealed that strain S215ᵀ was well separated from the reference strains of species of the genus *Lactobacillus*. DNA–DNA hybridization results indicated that strain S215ᵀ had DNA related to the three type strains of species of the genus *Lactobacillus* (33–66 % relatedness). The DNA G + C content of strain S215ᵀ was 36.2 mol%. The cell walls contained peptidoglycan of the D-meso-diaminopimelic acid type and the major fatty acids were C₁₈ : 1⁰⁻⁹c, C₁₆ : ₀ and C₁₉ : ₀ cyclo ω₁₀c/C₁₉ : ₀cω6c. Phenotypic and genotypic features demonstrated that the isolate represents a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus formosensis* sp. nov. is proposed. The type strain is S215ᵀ (=NBRC 109509ᵀ=BCRC 80582ᵀ).

Soybean meal (SBM) is commonly used for poultry feed. The presence of anti-nutritional factors contained in soybeans has been recognized as the key factor that affects the nutritional value of SBM and other soy products (Dunsford et al., 1989; Batal & Parsons, 2003). Species of the genera *Lactobacillus*, *Aspergillus* and *Bacillus* are commonly used to reduce anti-nutritional substances in fermented soybean meal (Chen et al., 2010a, 2011; Feng et al., 2007; Kishida et al., 2000; Li et al., 2006). After investigating the nutritional quality of SBM fermented by species of the genera *Aspergillus* and *Lactobacillus*, a lactic acid bacteria (LAB) strain was isolated to study the LAB microflora. In the quality control step, several LAB strains showed different characteristics from the inoculated starter and thus were isolated for further study.

All isolates were identified based on their phenotypic and phylogenetic characteristics. Phylogenetic analyses, based on 16S rRNA gene sequences, initially placed strain S215ᵀ among the species of the genus *Lactobacillus*. In order to exactly identify strain S215ᵀ, analysis of two housekeeping genes, *rpoA* and *pheS* (Chao et al., 2012) and phenotypic analyses was performed. The purpose of the present study was to establish the taxonomic position of this bacterial strain.

In recent years, many novel species and subspecies of the genus *Lactobacillus* have been isolated from different fermented substances, such as fermented cocoa beans (De Bruyne et al., 2009), fermented cane molasses (Kitahara et al., 2010) and fermented rice grain (Tohno et al., 2013). In the present study, strain S215ᵀ was isolated following the procedure proposed by Chen et al. (2010b), using de Man, Rogosa and Sharpe agar (MRS; Difco Lactobacilli MRS Broth) at 37 °C for 48 h. In addition, the reference strains, *Lactobacillus farcininis* BCRC 14043ᵀ (Reuter, 1983), *Lactobacillus futsai* BCRC 80278ᵀ (Chao et al., 2012) and *Lactobacillus crustorum* JCM 15951ᵀ (Scheirlinck et al., 2007), isolated from sausage, fermented mustard (fu-tsai and suan-tsai) and Belgian wheat sourdoughs, respectively, were obtained from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan) and the Japan Collection of Microorganisms (JCM; Ibaraki Prefecture, Japan).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoA* and *pheS* gene sequences of strain S215ᵀ are AB794060, AB794061 and AB794062, respectively.

Two supplementary tables and seven supplementary figures are available with the online Supplementary Material.
Strain S215<sup>T</sup> is a Gram-reaction-positive and catalase-negative bacillus. Tests for phenotypic characteristics, such as isomers of lactic acid produced, lactic acid fermentation type, salt tolerance and growth temperature range, were carried out based on previously described procedures (Chen et al., 2006, 2010b and 2013). Tests for acid production from carbohydrates were performed using the API50 CHL fermentation kit (bioMérieux) according to the manufacturer’s instructions. The production of D- and L-lactic acid isomers from glucose by type strains in MRS broth was determined with a D- and L-lactic acid enzyme test kit (Roche) according to the manufacturer’s protocol. In addition, the enzymic activities of strain S215<sup>T</sup> and the reference strains of species of the genus *Lactobacillus* were assayed using the API ZYM system (bioMérieux). The tests were performed according to the manufacturer’s instructions. After incubation at 37 °C for 4 h, the reaction was terminated by the addition of one drop each of A and B API ZYM reagents and the results were examined. The presence of D-meso-diaminopimelic acid in the cell-wall peptidoglycan was investigated by the method of Marconí et al. (2000).

Amplification and sequencing of the 16S rRNA gene were carried out as described by Chen et al. (2006). Amplification and sequencing of the housekeeping genes *rpoA* and *pheS* were performed using primers *rpoA*-21-F (5′-ATGATYGARTTGTGGAAAAACC-3′), *rpoA*-23-R (5′-ACHGTRRTTRATDCDCDGCRG-3′), *pheS*-21-F (5′-CAYCCNGC-HCGYGAYATGC-3′) and *pheS*-22-R (5′-CCWARVCCRC-AARGCAARCC-3′), which have been designed and reported previously by Naser et al. (2005). PCR was carried out using a Genomics Taq gene amplification PCR kit (Genomics) and a Gene Amp PCR System 2720 (PerkinElmer) following the methods described by Chen et al. (2006) and Chao et al. (2012). DNA sequencing was performed using an ABI 3730 DNA Analyser (Applied Biosystems). Sequences were assessed for similarity by comparing the sequences obtained with those in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) using BLAST.

All sequences were aligned using the CLUSTAL W software (Thompson et al., 1997). Distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Then, phylogenetic trees were reconstructed using the neighbour-joining method (Saitou & Nei, 1987), with bootstrap analysis based on 1000 resamplings. The MEGA 5 package (Tamura et al., 2011) was used for all analyses.

Genomic DNA was extracted from cells grown in MRS broth for 24 h at 37 °C and purified using the Qiagen Blood and Cell Culture DNA kit. The DNA G+C content was determined using reversed-phase HPLC, as previously described (Tamaoka & Komagata, 1984; Wang et al., 2007). DNA–DNA relatedness values were determined using the fluorometric hybridization method in microdilution wells, as previously described (Ezaki et al., 1989; Goris et al., 1998; Wang et al., 2007).

Extraction of fatty acids and determination of the cellular fatty acid profile were performed by using the Sherlock Microbial Identification System (version 6.0), according to the instructions of the Microbial Identification System (MIDI).

Repetitive sequence-based (rep)-PCR fingerprint profile analysis was performed by using primers REP1-R (5′-IIICGICGICATCIGGC-3′) and REP2-I(5′-IIICGNCNGNC-ATCNNGGC-3′) described by Svec et al. (2005). PCR amplification was performed under the conditions described by Versalovic et al. (1994).

Nucleotide sequences of 16S rRNA (approximately 1533 nt), *rpoA* (approximately 749 nt) and *pheS* (approximately 497 nt) genes were determined. Phylogenetic analysis of the 16S rRNA gene sequences obtained in this study and from the GenBank indicated that strain S215<sup>T</sup> represented a member of the genus *Lactobacillus*. Strain S215<sup>T</sup> formed a monophyletic cluster with four type strains of species of the genus *Lactobacillus* supported by a bootstrap value of 100% (Fig. 1). Strain S215<sup>T</sup> showed 99.6% sequence similarity to *L. fuscitaii* BCRC 14043<sup>T</sup>, 98.88% similarity to *L. fuscitaii* BCRC 80278<sup>T</sup> and 98.74% similarity to *L. crustorum* JCM 15951<sup>T</sup>.

When comparing the *rpoA* gene sequences with those held in the GenBank database, strain S215<sup>T</sup> showed 98.39% similarity to *L. fuscitaii* BCRC 14043<sup>T</sup>, 97.81% similarity to *L. fuscitaii* BCRC 80278<sup>T</sup> and 94.67% similarity to *L. crustorum* JCM 15951<sup>T</sup>. The phylogenetic analysis of the *rpoA* gene is shown in Fig. 2.

A comparison of the *pheS* gene with that of type strains of other species of the genus *Lactobacillus* was also performed. The results indicated that strain S215<sup>T</sup> had 93.63% sequence similarity to *L. fuscitaii* BCRC 14043<sup>T</sup>, 92.67% similarity to *L. fuscitaii* BCRC 80278<sup>T</sup> and 86.48% similarity to *L. crustorum* JCM 15951<sup>T</sup>. The results of the phylogenetic analysis of the *pheS* gene are shown in Fig. 3.

The topology was also analysed using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony methods (Fitch, 1971, 1977). Bootstrap analysis was made with 1000 replicates. Similar results were obtained to those obtained using the neighbour-joining method (Figs 1–3 and Figs S1–S6 available with the online Supplementary Material).

The DNA G+C content of strain S215<sup>T</sup> was 36.2 mol%, which was different to those of the three reference species of the genus *Lactobacillus* (36.3–38.3 mol%) (Chao et al., 2012) (Table 1). DNA–DNA hybridization experiments were performed using DNA derived from strain S215<sup>T</sup>, *L. fuscitaii* BCRC 14043<sup>T</sup> (Reuter, 1983), *L. fuscitaii* BCRC 80278<sup>T</sup> (Chao et al., 2012) and *L. crustorum* JCM 15951<sup>T</sup> (Scheirlinck et al., 2007). DNA–DNA relatedness values of strain S215<sup>T</sup> with *L. fuscitaii* BCRC 14043<sup>T</sup>, *L. fuscitaii* BCRC 80278<sup>T</sup> and *L. crustorum* JCM 15951<sup>T</sup> were 46.1%, 66.0% and 33.0%, respectively (Table S1). The rep-PCR fingerprint profiles obtained also demonstrated the separation
of strain S215T from L. farciminis BCRC 14043T, L. futsaii BCRC 80278T and L. crustorum JCM 15951T (Fig. S7). These values are well below the threshold of 70% suggested for species delineation (Stackebrandt & Goebel, 1994), indicating that strain S215T represents a separate species.

In addition to phylogenetic analyses, a number of phenotypic tests were performed. Strain S215T showed homo lactic acid fermentation, production of L-lactic acid and growth in medium containing 12% (w/v) NaCl. The tolerance to 12% NaCl clearly differentiated strain S215T from L. futsaii BCRC 80278T and L. crustorum JCM 15951T (Table 1). Moreover, strain S215T grew well at 45°C but L. farciminis BCRC 14043T, L. futsaii BCRC 80278T and L. crustorum JCM 15951T did not. Acid production from carbohydrates was assessed using the API 50 CHL fermentation kit after 48 h of incubation; results differing from those for the three reference species of the genus Lactobacillus were observed (Table 1). Strain S215T produced D-lactic acid from glucose, which is similar to L. crustorum JCM 15951T but different from L. farciminis BCRC 14043T and L. futsaii BCRC 80278T (Table 1).

Enzyme activities evaluated by using the API ZYM system are shown in Table S2. Characteristics that differed between strain S215T and other species of the genus Lactobacillus (L. farciminis BCRC 14043T, L. futsaii BCRC 80278T and L. crustorum JCM 15951T) included activities of β-galactosidase and β-glucuronidase.

Extraction and determination of cellular fatty acids were performed using the Sherlock Microbial Identification System (version 6.0), according to the instructions of the Sherlock Microbial Identification System (version 6.0), according to the instructions of the

Fig. 1. Neighbour-joining tree of strain S215T and related species of the genus Lactobacillus based on 16S rRNA sequences. Bootstrap values are indicated at branch points based on 1000 replications. GenBank accession numbers are given in parentheses. 16S rRNA sequences of L. farciminis NBRC 107150T were obtained from the NITE Biological Resource Center (NBRC) database. Enterococcus faecium LMG 11423T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Fig. 2. Neighbour-joining tree of strain S215T and related species of the genus Lactobacillus based on rpoA sequences. Bootstrap values are indicated at branch points based on 1000 replications. Enterococcus faecium LMG 11423T was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.
Microbial Identification System (MIDI). The cellular fatty acids of strain S215T and related type strains are listed in Table 2. The major fatty acids were C₁₈:₁ω₉c (41.51 %), C₁₆:₀ (23.41 %), C₁₉:₀ cyclo₁₀/₁₀c/C₁₉:₁ω₆c (22.83 %), C₁₈:₁ω₇c (5.15 %) and C₁₈:₀ (2.26 %).

In addition to the characteristics described by Farrow et al. (1984), the following features were found. In the API ZYM strip, esterase (C₄), leucine aminopeptidase, valine aminopeptidase, cysteine aminopeptidase, acid phosphatase, phosphohydrolase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and glucosaminidase were positive, while alkaline phosphatase, esterase lipase (C₈), trypsin, α-chymotrypsin and α-galactosidase were negative.

Various phenotypic and chemotaxonomic characteristics of the novel strain differed from those of L. farciminis (Reuter, 1983), L. futsaii (Chao et al., 2012) and L. crustorum (Scheirlinck et al., 2007), which are the most

### Table 1. Differential characteristics of strain S215T and related strains of species of the genus Lactobacillus

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA G+C content (mol%)</td>
<td>36.2</td>
<td>38.3*</td>
<td>36.3*</td>
<td>37.2*</td>
</tr>
<tr>
<td>Lactic acid isomer</td>
<td>L</td>
<td>DL</td>
<td>L</td>
<td>D</td>
</tr>
<tr>
<td>Growth in 10% (w/v) NaCl</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Growth in 12% (w/v) NaCl</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 45°C</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Growth at pH 4.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arbutin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>+</td>
<td>W</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl α-D-glucoside</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Sucrose</td>
<td>+</td>
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<td>Tagatose</td>
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<td>Turanose</td>
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<td>+</td>
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<td>Enzymic activity from:</td>
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<tr>
<td>Esterase (C₄)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>β-Galactosidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*Data from Chao et al. (2012).

### Table 2. Cellular fatty acid composition of S215T and related strains of species of the genus Lactobacillus

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₄:₀</td>
<td>1.37</td>
<td>2.22</td>
<td>1.74</td>
<td>2.07</td>
</tr>
<tr>
<td>C₁₆:₀</td>
<td>23.41</td>
<td>30.38</td>
<td>28.22</td>
<td>30.84</td>
</tr>
<tr>
<td>C₁₈:₀</td>
<td>2.26</td>
<td>1.63</td>
<td>1.91</td>
<td>1.89</td>
</tr>
<tr>
<td>C₁₈:₁ω₉c</td>
<td>41.51</td>
<td>25.60</td>
<td>29.62</td>
<td>19.64</td>
</tr>
<tr>
<td>C₁₈:₁ω₇c</td>
<td>5.15</td>
<td>4.29</td>
<td>4.12</td>
<td>4.33</td>
</tr>
<tr>
<td>iso-C₁₉:₀</td>
<td>2.02</td>
<td>1.94</td>
<td>1.95</td>
<td>1.87</td>
</tr>
<tr>
<td>C₁₆:₁ω₇c/C₁₆:₁ω₆c</td>
<td>1.12</td>
<td>1.42</td>
<td>1.08</td>
<td>1.46</td>
</tr>
<tr>
<td>C₁₉:₀ cyclo₁₀c</td>
<td>22.83</td>
<td>30.74</td>
<td>30.12</td>
<td>36.46</td>
</tr>
</tbody>
</table>

Microbial Identification System (MIDI). The cellular fatty acids of strain S215T and related type strains are listed in Table 2. The major fatty acids were C₁₈:₁ω₉c (41.51 %), C₁₆:₀ (23.41 %), C₁₉:₀ cyclo₁₀c/C₁₉:₁ω₆c (22.83 %), C₁₈:₁ω₇c (5.15 %) and C₁₈:₀ (2.26 %).

In addition to the characteristics described by Farrow et al. (1984), the following features were found. In the API ZYM strip, esterase (C₄), leucine aminopeptidase, valine aminopeptidase, cysteine aminopeptidase, acid phosphatase, phosphohydrolase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and glucosaminidase were positive, while alkaline phosphatase, esterase lipase (C₈), trypsin, α-chymotrypsin and α-galactosidase were negative.

Various phenotypic and chemotaxonomic characteristics of the novel strain differed from those of L. farciminis (Reuter, 1983), L. futsaii (Chao et al., 2012) and L. crustorum (Scheirlinck et al., 2007), which are the most
closely related species phylogenetically (Tables 1 and 2). According to the data obtained, strain S215\(^T\) is genetically distinguishable from recognized species and thus represents a novel species, for which the name *Lactobacillus formosensis* sp. nov. is proposed.

**Description of *Lactobacillus formosensis* sp. nov.**

*Lactobacillus formosensis* [for.mos.en’sis. N.L. masc. adj. formosensis of or pertaining to Formosa (Taiwan)].

Cells are Gram-reaction-positive, catalase-negative, rod-shaped, facultatively anaerobic and grow well anaerobically on MRS agar at 37 °C. Utilizes D-glucose homofermentatively, does not produce gas from glucose and produces D-lactic acid from glucose. Grows at temperatures between 25 °C and 45 °C, but not at 10 °C and grows in 12 % NaCl and at pH 4.0. Acid is produced from D-glucose, D-fructose, methyl α-D-glucoside, trehalose, maltose, lactose, sucrose, N-acetylglucosamine, galactose, D-mannose, D-raffinose, amygdalin, arbutin, aesculin, salicin, cellobiose, gentiobiose and D-turanose. Acid is not produced from ribose, melibiose, D-xylose, L-xylene, D-arabinose, erythritol L-arabinose, adonitol, methyl β-D-xyllose, L-sorbose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, inulin, melezitose, D-raffinose, glycogen, xylitol, D-arabitol, L-arabitol, D-fucose or L-fucose. The cell wall contains peptidoglycan of the D-meso-diaminopimelic acid type. In API ZYM tests, positive for esterase (C4), leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, acid phosphatase, phosphohydrolase, β-galactosidase, β-glucuronidase, α-glucosidase and β-glucosidase and glucosaminidase. The major cellular fatty acids are C\(_{18:1}\)ω9c, C\(_{16:0}\) and C\(_{19:0}\) cyclo ω10c/C\(_{19:1}\)ω6c.

The type strain is S215\(^T\) (=NBRC 109509\(^T\) = BCRC 80582\(^T\)), which has a DNA G+C content of 36.2 mol%.

**Acknowledgements**

The authors thank the National Science Council of Taiwan for financially supporting this study under grant no. NSC 101-2313-B-005-009-MY3. The authors would also like to thank Mr. Pao-Cheng Chang, Yu-Hsuan Lin and Kun-hon Leong for excellent technical assistance.

**References**


