Lactobacillus aquaticus sp. nov., isolated from a Korean freshwater pond

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A Lactobacillus strain, IMCC1736ᵀ, was isolated recently from a Korean freshwater pond following an extensive study of the microbial community in this ecosystem. Its 16S rRNA gene was sequenced and phylogenetic analysis placed this strain within the Lactobacillus salivarius group, closely related to Lactobacillus satsumensis NRIC 0604ᵀ, with 97.9 % sequence similarity. In the present work, the taxonomic status of strain IMCC1736ᵀ has been re-evaluated. It was characterized phylogenetically, genotypically and phenotypically and, based on DNA–DNA hybridization values, this strain represents a novel Lactobacillus species. Strain IMCC1736ᵀ can be differentiated genotypically from its closest relatives by randomly amplified polymorphic DNA analysis and ribotyping patterns; phenotypically, it can be distinguished by its inability to grow in 5 % NaCl and at pH 3.3 and by certain carbohydrate fermentations. Strain IMCC1736ᵀ is Gram-positive, catalase-negative and microaerophilic. Cells are motile rods and show hemolysin fermentative metabolism. The name Lactobacillus aquaticus sp. nov. is proposed, with strain IMCC1736ᵀ (=CECT 7355ᵀ =DSM 21051ᵀ) as the type strain.

The genus Lactobacillus belongs to the lactic acid bacteria group and is a highly heterogeneous genus in terms of the metabolic pathways, DNA G + C contents and source of its members. Lactobacilli are almost ubiquitous; they are found in any environment where carbohydrates are available, although they are most frequently present in vegetable or animal food, fermented foods and beverages, mucosal membranes of humans and animals and plant material (Hammes & Hertel, 2006; Bernardeau et al., 2008). Although isolation of lactic acid bacteria from environmental samples is not common, some isolates have been reported from sewage (Weiss et al., 1981), soils (Chen et al., 2005; Yanagida et al., 2005), the deep subsea floor (Toffin et al., 2005) and coastal marsh sediments (Zamudio-Mayá et al., 2008); other isolates have been found associated with marine organisms (Ishikawa et al., 2003; Vela et al., 2008). Even though some lactobacilli have been isolated from aquatic sources (Yanagida et al., 2007), they have not been found in seawater.

Song et al. (2007), carrying out an extensive study of the microbial diversity in a Korean lake, isolated strain IMCC1736ᵀ, which was most closely related to Lactobacillus satsumensis NRIC 0604ᵀ, with 97.9 % 16S rRNA gene sequence similarity, and placed it in the Lactobacillus salivarius phylogenetic group (Felis & Dallaglio, 2007). In the past few years, some novel lactobacilli belonging to this group have been isolated from several sources, such as wild mouse faeces and wine (Osawa et al., 2006; Rodas et al., 2006). This heterogeneous genus is expanding constantly since novel species are being described continually and, thus, its phylogenetic structure is changing dramatically (Felis & Dallaglio, 2007). In this study, an in-depth phylogenetic, genotypic and phenotypic characterization of strain IMCC1736ᵀ and its closest relatives is reported. On the basis of these results, a novel species of the genus Lactobacillus is proposed.

Strain IMCC1736ᵀ was isolated from the surface of a eutrophic freshwater pond in Korea. Reference strains [Lactobacillus mali strains DSM 20444ᵀ, CECT 4149, Lb44 and Lb206, L. satsumensis strains DSM 16230ᵀ, CECT 7371 and ENOLAB 4555, Lactobacillus uvarum] strains CECT 7335 (the proposed type strain), 24 and 68 and Lactobacillus vini CECT 5924ᵀ] and strain IMCC1736ᵀ were grown in MRS broth (Scharlab) supplemented with 0.5 g l-cysteine hydrochloride l⁻¹ under the conditions described by Rodas et al. (2003).
The almost-complete 16S rRNA gene sequence of strain IMCC1736T (1528 bp) was obtained using the protocol described by Rodas et al. (2005) and, together with sequences from its nearest relatives, including the recently described 'L. uvarum' (Mañes-Lázaro et al., 2008), was subjected to phylogenetic analysis. The 16S rRNA gene sequence of strain IMCC1736T was aligned with those of members of the genus Lactobacillus using the ARB software package. Several reconstruction methods (neighbour-joining, maximum-parsimony and maximum-likelihood) were applied in the PAUP* 4.0 software package to infer the phylogeny of strain IMCC1736T. The 16S rRNA gene sequence of the isolate showed 99.8 % sequence similarity with that of 'L. uvarum' (CECT 7335), 97.5 % with L. mali DSM 20444T and L. satsumensis NRIC 0604T, 96.1 % with L. vini CECT 5924T, 95.7 % with Lactobacillus ghanensis L489T and 95.1 % with Lactobacillus nagelii NRIC 0559T. In all of the phylogenetic trees (Fig. 1), strain IMCC1736T formed a robust clade together with 'L. uvarum' 8. Because 16S rRNA gene sequence similarities between the isolate and strains of some species of the genus Lactobacillus were too high to define a novel species, DNA–DNA hybridization experiments were required.

DNA–DNA hybridization experiments were performed as described by Ziemke et al. (1998) between strain IMCC1736T and 'L. uvarum' strains CECT 7335, 24 and 68 and L. mali DSM 20444T. The results, expressed as mean percentages based on three independent hybridization experiments, were 41.74, 53.09, 43.29 and 37.5 %, respectively. Reciprocal hybridization experiments using genomic DNA of 'L. uvarum' CECT 7335 as template rendered a value of 41.49 % with strain IMCC1736T. These values were below 70 %, confirming that IMCC1736T is a member of a novel species (Stackebrandt & Goebel, 1994).

16S-ARDRA (amplified rDNA restriction analysis), ISR (internal spacer region) analysis, RAPD (randomly amplified polymorphic DNA) analysis and ribotyping were used to characterize this strain genotypically, as described previously (Rodas et al., 2003, 2005; Chenoll et al., 2006). Strain IMCC1736T could be differentiated from the reference strains by RAPD and ribotyping profiles, but not by the other techniques based on ribosomal gene analysis. As deduced from the dendrogram built with different fingerprinting analyses, five clusters could be delineated at >75 % similarity, corresponding to the five species analysed (Fig. 2). Genotypically, L. satsumensis was most closely related to strain IMCC1736T (63.23 % similarity).

The DNA G+C content of strain IMCC1736T was determined by hydrolysing the DNA enzymically and quantifying the nucleosides by HPLC as reported previously by Tamaoka & Komagata (1984) and Ziemke et al. (1998). The DNA G+C content was 39.15 ± 0.07 mol%, a value within the range (32–53 mol%) established for the genus Lactobacillus.

To test for the presence of meso-diaminopimelic acid (meso-DAP), whole cells were hydrolysed by incubating them for 15 h at 100 °C with 4 M HCl and the hydrolysates were subjected to TLC on cellulose plates using the solvent system of Rhuland et al. (1955). Results revealed that strain IMCC1736T contained meso-DAP.

Strain IMCC1736T is a Gram-positive, catalase-negative and microaerophilic lactobacillus. Cells were motile when

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain IMCC1736T and other Lactobacillus species. Bootstrap values above 50% from neighbour-joining (above nodes) and maximum-likelihood (below nodes) methods are shown. Filled and open circles respectively indicate nodes recovered reproducibly by all treeing methods or by two treeing methods, Bar, 0.01 substitutions per nucleotide position. The sequence from Bacillus subtilis NCDO 1769T was used as an outgroup.](image-url)
tested in MRS soft agar and when observed on wet mounts (Rodas et al., 2006). No gas was released from glucose and it did not ferment gluconate or pentoses; thus, it is considered to be obligately homofermentative. The ability of strain IMCC1736<sup>T</sup> and reference species to ferment carbohydrates was tested on API 50 CHL galleries (bioMérieux) according to the instructions of the manufacturer; detailed results are given in Supplementary Table S1 (available in IJSEM Online). Strain IMCC1736<sup>T</sup> differed from 'L. uvarum', its closest relative, in its inability to grow in 5 % NaCl and to ferment turanose (Table 1). In addition, strain IMCC1736<sup>T</sup> was able to grow at 45 °C and ferment methyl α-D-mannoside and cellobiose, whereas 'L. uvarum' was not.

In conclusion, in view of the low DNA–DNA relatedness between strain IMCC1736<sup>T</sup> and other members of the genus Lactobacillus, together with its phenotypic characteristics, strain IMCC1736<sup>T</sup> represents a novel species in the genus Lactobacillus, for which the name Lactobacillus aquaticus sp. nov. is proposed.

**Description of Lactobacillus aquaticus sp. nov.**

*Lactobacillus aquaticus* (a.qua’ti.cus. L. masc. adj. aquaticus from water, aquatic).

Gram-positive, motile, non-spore-forming rods, 1.01–1.38 μm wide and 1.74–3.84 μm long. Cells are found singly, in pairs and in short chains. Microaerophilic. Colonies on MRS agar after 4 days incubation at 28 °C are 1.5–2.0 mm in diameter, smooth, circular, regular and white. Catalase-negative. Growth occurs at 15–45 °C and pH 4.5–8.0, but not at pH 3.3 or in 5 or 10 % NaCl. Homofermentative. Ammonia is not produced from arginine and mannitol is not produced from fructose.

Exopolysaccharide is produced from sucrose. Ferments glucose, fructose, mannose, mannitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylgalactosamine, amygdalin.

**Table 1. Differential phenotypic characteristics of strain IMCC1736<sup>T</sup> and its closest phylogenetic neighbours**

<table>
<thead>
<tr>
<th>Characteristic</th>
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<tr>
<td>Growth in MRS with/at:</td>
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<td>5 % NaCl</td>
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<td>pH 3.3</td>
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<td>45 °C</td>
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<td>Fermentation of:</td>
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<td>L-Arabinose</td>
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<td>D-Galactose</td>
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<td>L-Sorbose</td>
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<td>D-Sorbitol</td>
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<td>Methyl α-D-mannoside</td>
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<td>Methyl α-D-glucoside</td>
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<td>Maltose</td>
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<td>β-Gentiobiose</td>
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<td>Turanose</td>
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<td>D-Tagatose</td>
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<td>Exopolysaccharide production from sucrose</td>
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lip, arbutin, salicin, cellobiose, maltose, sucrose, trehalose and β-gentiobiose. Hydrolyses aesculin. Does not ferment
glycerol, erythritol, D- or L-arabinose, ribose, D- or L-
xlylose, adonitol, methyl β-xyloside, galactose, L-sorbose,
rhamnose, dulcitol, inositol, sorbitol, lactose, melibiose,
inulin, melezitose, raffinose, starch, glycogen, xyitol,
turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-
arabitol, gluconate or 2- or 5-ketoglucuronate.
The type strain is IMCC1736\textsuperscript{T} (=CECT 7355\textsuperscript{T} =DSM 21051\textsuperscript{T}), isolated in 2005 (Song et al., 2007) from a
eutrophic freshwater pond. The DNA G+C content of the
type strain is 39.15±0.07 mol% (HPLC).

Acknowledgements
This work was supported partially by RM2007-00007-00-00 and by
the 21C Frontier programme of Microbial Genomics and
Applications from the MEST, Korea. We wish to thank Mercedes
Urdiani and Ramón Rosselló-Mora for kindly helping with the
hybridization and G+C analysis.

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