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

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A *Lactobacillus* strain, IMCC1736^T, 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 0604^T, with 97.9 % sequence similarity. In the present work, the taxonomic status of strain IMCC1736^T 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 IMCC1736^T 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 IMCC1736^T is Gram-positive, catalase-negative and microaerophilic. Cells are motile rods and show homofermentative metabolism. The name *Lactobacillus aquaticus* sp. nov. is proposed, with strain IMCC1736^T (=CECT 7355^T =DSM 21051^T) 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-Maya *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 IMCC1736^T, which was most closely related to *Lactobacillus satsumensis* NRIC 0604^T, with 97.9 % 16S rRNA gene sequence similarity, and placed it in the *Lactobacillus salivarius* phylogenetic group (Felis & Dellaglio, 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 & Dellaglio, 2007). In this study, an in-depth phylogenetic, genotypic and phenotypic characterization of strain IMCC1736^T and its closest relatives is reported. On the basis of these results, a novel species of the genus *Lactobacillus* is proposed.

Strain IMCC1736^T was isolated from the surface of a eutrophic freshwater pond in Korea. Reference strains [*Lactobacillus mali* strains DSM 20444^T, CECT 4149, Lb44 and Lb206, *L. satsumensis* strains DSM 16230^T, CECT 7371 and ENOLAB 4555, '*Lactobacillus uvarum*' strains CECT 7335 (the proposed type strain), 24 and 68 and *Lactobacillus vini* CECT 5924^T] and strain IMCC1736^T 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 GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC1736^T is DQ664203.

Phenotypic characteristics of strain IMCC1736^T and closely related strains are available as supplementary material with the online version of this paper.

The almost-complete 16S rRNA gene sequence of strain IMCC1736^T (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 IMCC1736^T 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 IMCC1736^T. The 16S rRNA gene sequence of the isolate showed 99.8 % sequence similarity with that of '*L. uvarum*' 8 (=CECT 7335), 97.5 % with *L. mali* DSM 20444^T and *L. satsumensis* NRIC 0604^T, 96.1 % with *L. vini* CECT 5924^T, 95.7 % with *Lactobacillus ghanensis* L489^T and 95.1 % with *Lactobacillus nagelii* NRIC 0559^T. In all of the phylogenetic trees (Fig. 1), strain IMCC1736^T 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 IMCC1736^T and '*L. uvarum*' strains CECT 7335, 24 and 68 and *L. mali* DSM 20444^T. 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 IMCC1736^T. These values were below 70 %, confirming that IMCC1736^T 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 IMCC1736^T 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 IMCC1736^T (63.23 % similarity).

The DNA G+C content of strain IMCC1736^T 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 IMCC1736^T contained *meso*-DAP.

Strain IMCC1736^T is a Gram-positive, catalase-negative and microaerophilic lactobacillus. Cells were motile when

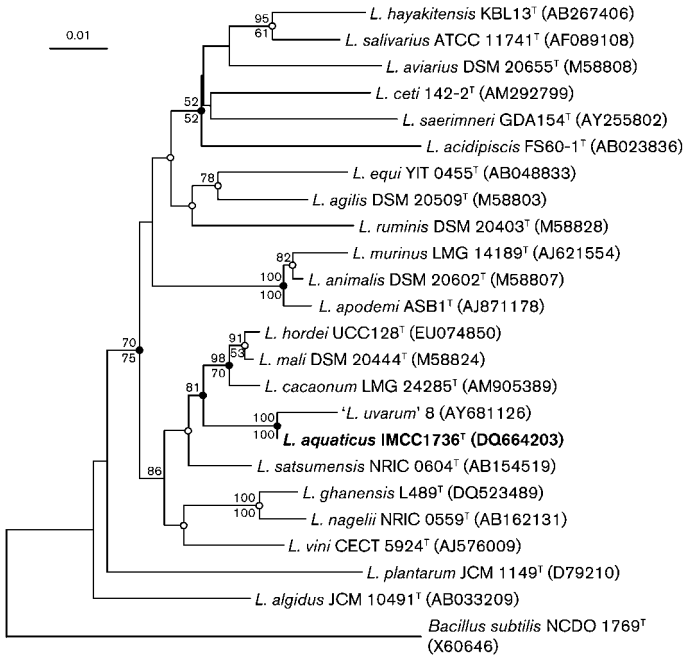

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain IMCC1736^T and other *Lactobacillus* species. Bootstrap values above 50 % from neighbour-joining (above nodes) and maximum-likelihood (below nodes) methods are shown. Filled and open circles 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 1769^T was used as an outgroup.

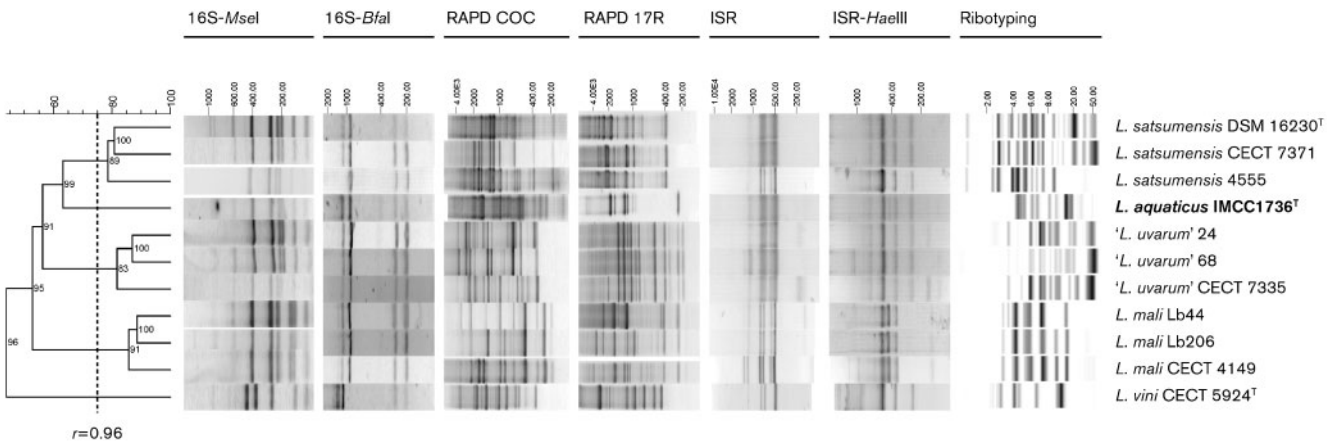

Fig. 2. Dendrogram derived from comparison of the combined 16S-ARDRA (*Mse*I and *Bfa*I), RAPD analysis (COC and 17R), ISR and ISR-ARDRA (*Hae*III) and ribotyping, obtained from strain IMCC1736^T and reference strains. Levels of similarity between patterns were calculated by using the similarity coefficient of each technique and the clustering is based on the UPGMA method. At similarities >75 %, five clusters could be delineated, corresponding to the five species analysed. The calculated global cophenetic correlation value for the global analysis was 96.

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^T 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^T 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^T 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^T and other members of the genus *Lactobacillus*, together with its phenotypic characteristics, strain IMCC1736^T 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*-acetylglucosamine, amygda-

Table 1. Differential phenotypic characteristics of strain IMCC1736^T and its closest phylogenetic neighbours

Strains: 1, *L. aquaticus* sp. nov. IMCC1736^T; 2, '*L. uvarum*' CECT 7335; 3, *L. mali* DSM 20444^T; 4, *L. satsumensis* DSM 16230^T; 5, *L. vini* CECT 5924^T; 6, *L. nagelii* CECT 5983^T. +, Positive; −, negative; W, weak reaction. Data were obtained in this study.

Characteristic	1	2	3	4	5	6
Growth in MRS with/at:						
5 % NaCl	−	+	−	+	+	+
pH 3.3	−	−	W	+	+	+
45 °C	W	−	−	W	+	−
Fermentation of:						
L-Arabinose	−	−	−	−	+	−
D-Galactose	−	−	−	+	−	+
L-Sorbose	−	−	−	+	−	+
L-Rhamnose	−	−	−	+	−	+
D-Mannitol	+	+	+	+	−	+
D-Sorbitol	−	−	+	+	−	+
Methyl α-D-mannoside	+	−	−	+	+	−
Methyl α-D-glucoside	+	+	−	+	−	+
Salicin	+	+	−	+	+	+
Cellobiose	+	−	+	−	+	+
Maltose	+	+	−	+	+	+
β-Gentiobiose	+	+	+	+	+	−
Turanose	−	+	−	+	−	−
D-Tagatose	−	−	−	+	−	−
Exopolysaccharide production from sucrose	+	+	+	+	−	+

lin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose and β-gentiobiose. Hydrolyses aesculin. Does not ferment glycerol, erythritol, D- or L-arabinose, ribose, D- or L-xylose, adonitol, methyl β-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate.

The type strain is IMCC1736^T (=CECT 7355^T =DSM 21051^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).

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