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Lactobacillus cypricasei Lawson *et al.* 2001 is a later heterotypic synonym of *Lactobacillus acidipiscis* Tanasupawat *et al.* 2000

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The applicability of a multilocus sequence analysis (MLSA)-based identification system for lactobacilli was evaluated. Two housekeeping genes that code for the phenylalanyl-tRNA synthase α-subunit (*pheS*) and RNA polymerase α-subunit (*rpoA*) were sequenced and analysed for members of the *Lactobacillus salivarius* species group. The type strains of *Lactobacillus acidipiscis* and *Lactobacillus cypricasei* were investigated further using a third gene that encodes the α-subunit of ATP synthase (*atpA*). The MLSA data revealed close relatedness between *L. acidipiscis* and *L. cypricasei*, with 99·8–100 % *pheS*, *rpoA* and *atpA* gene sequence similarities. Comparison of the 16S rRNA gene sequences of the type strains of the two species confirmed the close relatedness (99·8 % gene sequence similarity) between the two taxa. Similar phenotypes and high DNA–DNA binding values in the range of 84 to 97·5 % confirmed that *L. acidipiscis* and *L. cypricasei* are synonymous species. On the basis of the present study, it is proposed that *Lactobacillus cypricasei* is a later heterotypic synonym of *Lactobacillus acidipiscis*.

Lactobacillus acidipiscis was described by Tanasupawat *et al.* (2000) based on 11 strains isolated from fermented fish (pla-ra and pla-chom) in Thailand. *L. acidipiscis* utilizes D-glucose homofermentatively and produces L-lactic acid from glucose without the production of gas. *L. acidipiscis* strains do not grow at pH 4·0 or 8·5 and grow in 10 % NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that *L. acidipiscis* strains are positioned in a monophyletic cluster consisting of *Lactobacillus salivarius*, *Lactobacillus aviarius*, *Lactobacillus ruminis*, *Lactobacillus agilis*, *Lactobacillus murinus*, *Lactobacillus animalis* and *Lactobacillus mali*. The DNA–DNA relatedness of *L. acidipiscis* strains and other related *Lactobacillus* species was found to be in the range of 3·6 to 26·7 % (Tanasupawat *et al.*, 2000). One year later, *Lactobacillus cypricasei* was described by Lawson *et al.* (2001) based on four strains isolated from Halloumi, a cheese produced in Cyprus. The strains were Gram-positive, non-spore-forming, facultatively anaerobic and catalase- and oxidase-negative. No growth was observed at 15 or 45 °C. Like *L. acidipiscis*, *L. cypricasei* strains formed a distinct branch within the *L. salivarius* species group, with *L. salivarius* and *L. aviarius* as the nearest neighbours. However, Lawson *et al.* (2001) did not include the related recognized species *L. acidipiscis* in comparisons with other species. In the present study, the relatedness between *L. acidipiscis* and *L. cypricasei* strains was investigated and revealed synonymy between the two taxa.

Two reference strains of *L. acidipiscis*, LMG 19820^T and LMG 23135, and four reference strains of *L. cypricasei*, LMG 21592^T, CCUG 42959, CCUG 42960 and CCUG 42962, were selected for further comparative study. *L. cypricasei* LMG 21592^T was cultivated and maintained on de Man, Rogosa and Sharpe medium (MRS; Difco). All other strains studied were cultivated and maintained on MRS (Oxoid) medium and incubated anaerobically at 37 °C, unless otherwise indicated.

The use of protein-coding gene sequence data for the determination of genomic relatedness at the bacterial species and genus levels has recently been advocated because of its ability to provide higher taxonomic resolution, since 16S rRNA

Abbreviation: MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the partial *pheS* gene sequences for strains LMG 19820^T, LMG 21592^T, LMG 23135, CCUG 42959, CCUG 42960, CCUG 42962, LMG 9843^T, LMG 14189^T, LMG 22087^T, LMG 21748^T, LMG 9186^T, LMG 10753^T, LMG 10756^T, LMG 21593^T, LMG 9477^T, LMG 6899^T and LMG 6903^T are AM087762, AM087687, AM168426–AM168429, AM087679, AM087760, AM087717, AM087740, AM087734, AM087737, AM087756, AM087708, AM087721, AM087746 and AM168425. Those for the *rpoA* partial gene sequences for strains LMG 19820^T, LMG 21592^T, LMG 23135, CCUG 42959, CCUG 42960, LMG 9186^T and LMG 14189^T are AM087849, AM087784, AM168431–AM168433, AM087831 and AM087801, respectively. Those for the *atpA* partial gene sequences for strains LMG 19820^T and LMG 21592^T are AM168424–AM168423, respectively.

gene sequence analysis may not be sufficiently specific to discriminate between closely related species (Chavagnat *et al.*, 2002; Stackebrandt *et al.*, 2002; Torriani *et al.*, 2001; Ventura *et al.*, 2003; Zeigler, 2003). Recently, the application of multilocus sequence analysis (MLSA) using the genes that code for the α-subunit of bacterial phenylalanyl-tRNA synthase (*pheS*), the α-subunit of RNA polymerase (*rpoA*) and the α-subunit of ATP synthase (*atpA*) provided a robust system for the identification of *Enterococcus* species (Naser *et al.*, 2005a, b). The MLSA-based identification system has proved to be valuable for the detection of synonymous species names within the genus *Enterococcus* (Naser *et al.*, 2006b). In addition, relatedness between strains of *Lactobacillus helveticus* and *Lactobacillus suntoryeus* was initially evaluated using the same MLSA loci. Naser *et al.* (2006a) demonstrated that *Lactobacillus suntoryeus* is a later synonym of *Lactobacillus helveticus*. The *pheS* and *rpoA* genes were subsequently used for the analysis of lactobacilli of the *L. salivarius* species group, including the two strains of *L. acidipiscis* and the four reference strains of *L. cypricasei*. The type strains of *L. acidipiscis* and *L. cypricasei* were further investigated using the *atpA* gene. The primer sequences, amplification conditions and sequencing reactions were as described by Naser *et al.* (2005a, b). Raw sequence data were transferred to GeneBuilder (Applied Maths) where consensus sequences were determined. Consensus sequences were imported into BioNumerics 4.0 software (Applied Maths). The determined partial *pheS* and *rpoA* gene sequences (453 and 402 bp, respectively) were compared for the two *L. acidipiscis* strains (LMG 19820^T and LMG 23135), four *L. cypricasei* strains (LMG 21592^T, CCUG 42959, CCUG 42960 and CCUG 42962) and other members of the *L. salivarius* species group. Comparison of the sequences of the strains of *L. cypricasei* and *L. acidipiscis* revealed *pheS* and *rpoA* gene sequence similarities in the range of 99·8 to 100 %. The *atpA* partial gene sequences of *L. cypricasei* LMG 21592^T (GenBank accession no. AM168423) and *L. acidipiscis* LMG 19820^T (AM168424) were also compared (976 bp) and showed high relatedness. Strains of the two species had a maximum of 78 % *pheS* gene sequence similarity with *L. mali* and *L. murinus*, 82 % *rpoA* gene sequence similarity with *L. agilis* and 80 % *atpA* gene sequence similarity with *L. murinus* (Fig. 1). Consequently, the MLSA data indicated that *L. acidipiscis* and *L. cypricasei* might represent a single species.

The phylogenetic relatedness between *L. acidipiscis* FS60-1^T and *L. cypricasei* CCUG 42961^T was investigated by comparing the available 16S rRNA gene sequences (*L. acidipiscis* GenBank accession no. AB023836, 1406 bp; *L. cypricasei* GenBank accession no. AJ251560, 1456 bp). The latter sequences and those of related species were retrieved from GenBank and aligned. A phylogenetic tree was constructed by the neighbour-joining method using BioNumerics software, version 4.0 (Applied Maths). Unknown bases were discarded for the analyses. Comparison of the sequences of the type strains of *L. cypricasei* and *L. acidipiscis* revealed 99·8 % 16S rRNA gene sequence similarity.

In the original description of *L. cypricasei*, the authors did not determine the DNA G+C content. In the present study, we investigated this characteristic for the type strain of *L. cypricasei*. Cells were cultivated in MRS broth at 37 °C for 24 h. DNA was extracted from 0·5–0·75 g (wet weight) by using the protocol described by Marmur (1961) with the following modifications: (i) cells were suspended overnight in Tris/HCl buffer that contained lysozyme (8 mg ml^{-1}) before the addition of SDS and (ii) lysed cells were treated with proteinase K (360 mg l^{-1}; Merck) at 37 °C for 2 h. For determination of the DNA G+C content, DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was then separated by HPLC using a SymmetryShield RP8 column (Waters) maintained at 37 °C. The solvent was 0·02 M $NH_4H_2PO_4$ (pH 4·0) with 1·5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference. The DNA G+C content of *L. cypricasei* LMG 21592^T was 40·1 mol%. In comparison, the DNA G+C content of *L. acidipiscis* LMG 19820^T was 38·7 mol%.

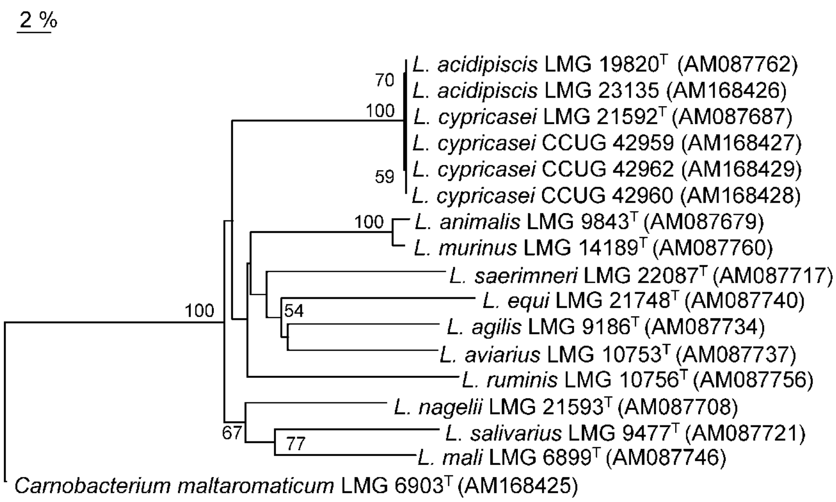

Fig. 1. Neighbour-joining tree based on the partial *pheS* gene sequences of *L. cypricasei* and *L. acidipiscis* strains. *Carnobacterium maltaromaticum* LMG 6903^T was included as an outgroup. Bootstrap percentages (⩾ 50 %) after 500 simulations are shown. Bar, 2 % sequence divergence.

Finally, DNA–DNA hybridizations were performed between *L. cypricasei* strains LMG 21592^T and CCUG 42960 and *L. acidipiscis* strains LMG 19820^T and LMG 23135. Genomic DNA was prepared according to the protocol of Pitcher *et al.* (1989) with the following modifications: the washed cell pellet was resuspended and lysed in a buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8·0) that contained RNase (200 μg ml^{-1}; Sigma), mutanolysin (100 U ml^{-1}; Sigma) and lysozyme (25 mg ml^{-1}; SERVA) for 1 h at 37 °C. The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998) using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with unlabelled ssDNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 36 °C in hybridization mixture (2 × SSC, 5 × Denhardt's solution, 2·5 % dextran sulfate, 50 % formamide, 100 μg denatured salmon sperm DNA ml^{-1}, 1250 ng biotinylated probe DNA ml^{-1}). Reciprocal reactions (e.g. A × B and B × A) were performed. The DNA–DNA binding values reported were the mean values of a minimum of four hybridization experiments, including the reciprocal reactions. *L. acidipiscis* strains LMG 19820^T and LMG 23135 and *L. cypricasei* strains LMG 21592^T and CCUG 42960 showed high DNA–DNA binding values in the range of 84 to 97·5 %, indicating clearly that the two taxa represent the same species. A DNA–DNA hybridization value of 93 % was found between the type strains of the two species.

On the basis of the evidence presented, it is concluded that *Lactobacillus cypricasei* is a later heterotypic synonym of *Lactobacillus acidipiscis*. It is proposed that the two species be united under the same name. As a rule of priority (Rules 38 and 42 of the Bacteriological Code; Lapage *et al.*, 1992), the name *Lactobacillus acidipiscis* should be retained and strains of *Lactobacillus cypricasei* should be reclassified as such. The description of *L. acidipiscis* remains essentially the same except for some strain-dependent reactions, such as the production of acid from mannitol and D-ribose and the production of arginine dihydrolase.

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