Lactobacillus delbrueckii subsp. *indicus* subsp. nov., isolated from Indian dairy products

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Four strains isolated from Indian dairy products and initially identified as *Lactobacillus delbrueckii* could not be assigned to a definite subspecies because molecular identification and phenotypic traits did not agree with those of recognized subspecies of *L. delbrueckii*. Hybridization of total DNA (78–86 % against type strains of the other three subspecies), AFLP and RAPD-PCR fingerprints, phylogenetic analysis based on 16S rRNA gene sequences and sequence analysis of two coding genes (*recA* and *hsp60*), together with phenotypic profiles, indicated that the four strains form a coherent cluster and represent a novel subspecies, for which the name *Lactobacillus delbrueckii* subsp. *indicus* subsp. nov. is proposed. The type strain is NCC725^T (=LMG 22083^T = DSM 15996^T).

Lactobacillus delbrueckii is the type species of the genus *Lactobacillus* and comprises three subspecies, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Weiss *et al.*, 1983). Strains belonging to the three subspecies show a high degree of DNA–DNA relatedness but can be differentiated on the basis of different phenotypic traits (Weiss *et al.*, 1983; Kandler & Weiss, 1989).

In a previous study regarding the evolutionary structure of the species *L. delbrueckii* (Germond *et al.*, 2003), a number of lactobacilli originating from five dairy fermented products from India were analysed. Among those lactobacilli, four strains isolated from traditional Indian dairy fermented (type Dahi) products were identified as *L. delbrueckii* on the basis of molecular and phenotypic evidence, i.e. hybridization with a probe specific for *L. delbrueckii*, pY85 (Delley *et al.*, 1990), typing based on digested genomic DNA probed

with ribosomal or ISL7 sequences, specific PCR amplification and the exclusive production of D-lactate from lactose. However, they could not be assigned to one of the known subspecies of *L. delbrueckii* because they presented atypical characteristics such as the ability of *L. delbrueckii* subsp. *bulgaricus* to degrade lactose constitutively, but no amplification of the gene encoding β -galactosidase using specific primers for *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*.

We report here the polyphasic analysis performed to define the taxonomic position of the four strains and describe a novel subspecies for *L. delbrueckii*.

The bacterial strains NCC725^T, NCC665, NCC757 and NCC780 and the type strains of *L. delbrueckii* subsp. *delbrueckii* NCC621^T (=ATCC 9649^T = LMG 6412^T), *L. delbrueckii* subsp. *lactis* NCC946^T (=ATCC 12315^T = LMG 7942^T) and *L. delbrueckii* subsp. *bulgaricus* NCC641^T (=ATCC 11842^T = LMG 6901^T) were grown in de Man–Rogosa–Sharpe (MRS) or acidified MRS (pH 5.4) medium at 37 °C.

Sugar metabolism was evaluated using the API-50CH detection kit. All strains tested fermented glucose, fructose and mannose. The ability to metabolize lactose distinguished the four strains from *L. delbrueckii* subsp. *delbrueckii*. These four strains can also be differentiated from *L. delbrueckii* subsp. *lactis* because they did not ferment both maltose and trehalose. Three of the four strains (except NCC757) fermented sucrose and not *N*-acetylglucosamine in contrast to type strains of *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*, respectively.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Lactobacillus delbrueckii* subsp. *indicus* NCC725^T is AY421720; partial *recA* gene sequences of *L. delbrueckii* subsp. *delbrueckii* NCC621^T, AJ586863; *L. delbrueckii* subsp. *bulgaricus* NCC641^T, AJ586864; *L. delbrueckii* subsp. *lactis* NCC946^T, AJ586865; *L. delbrueckii* subsp. *indicus* NCC665, AJ586866; *L. delbrueckii* subsp. *indicus* NCC780, AJ586867; and partial *hsp60* gene sequences of *L. delbrueckii* subsp. *delbrueckii* NCC621^T, AJ586868; *L. delbrueckii* subsp. *bulgaricus* NCC641^T, AJ586869; *L. delbrueckii* subsp. *lactis* NCC946^T, AJ586870; *L. delbrueckii* subsp. *indicus* NCC725^T, AJ586872; *L. delbrueckii* subsp. *indicus* NCC665, AJ586871.

A partial phylogenetic tree, AFLP[®] and RAPD profiles, and distinguishing characteristics of the subspecies of *Lactobacillus delbrueckii* are available as supplementary material in IJSEM Online.

DNA for specific amplification and Southern blot analysis was extracted according to the methods of Marmur (1961) and Germond *et al.* (2003), respectively. Ribotyping and insertion sequence (IS) typing identification results showed a closer relationship of the four Indian strains to *L. delbrueckii* subsp. *lactis* than to the other two recognized subspecies (Germond *et al.*, 2003).

Subspecies-specific identification was performed by DNA amplification using a combination of primers designed on the proline iminopeptidase (*pepIP*) gene of *L. delbrueckii* subsp. *bulgaricus* (Torriani *et al.*, 1999): this test allows us to differentiate between *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* on the basis of the length of the amplification product, while *L. delbrueckii* subsp. *delbrueckii* should show no amplification. The four Indian strains were all negative for the assay (data not shown), even if DNA was present at the appropriate concentration and was able to be amplified, as verified by RAPD-PCR analyses.

16S rRNA gene amplifications and sequences were obtained as reported by Germond *et al.* (2003). Sequence alignments were obtained using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic analysis was conducted using MEGA version 2.1 (Kumar *et al.*, 2001): distance analysis (Tamura's three-parameter distance) and neighbour-joining clustering were applied, as well as the maximum-parsimony method using default options. An analysis of the robustness of the trees was obtained by a bootstrap approach with 1000 replicates. A maximum-likelihood analysis was also performed with the program DNAML of the PHYLIP software package (Felsenstein, 1993). Accession numbers of the sequences included in the analysis are shown in Fig. A, available as supplementary material in IJSEM Online. The phylogenetic trees obtained strongly confirm that the new isolates should be classified within *L. delbrueckii* owing to their very low sequence divergence (identity of 99%); the distance matrix tree (Fig. A) is available as supplementary material in IJSEM Online.

16S rRNA gene sequence similarity is not sufficient to guarantee species identity (Fox *et al.*, 1992). Protein-encoding genes are more variable; in particular, the *recA* gene has proved useful in the differentiation of closely related species with almost identical 16S rRNA gene sequences, including lactic acid bacteria species such as *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus* (Torriani *et al.*, 2001) or *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus zeae* (Felis *et al.*, 2001). Therefore, part of the *recA* gene was amplified using the degenerate primers recEXT-f (5'-GGCTAT-GAAACAAATTGAAAAACAATWYGGNAARGG-3') and recEXT1-r (5'-TGTTTAAACGGTGGAGCAACTTTRTTY-TTAC-3'). The reaction mixture (50 µl) contained 300 ng template DNA, 100 µM each dNTP, 1 µM each primer, 2 mM magnesium chloride, 5% (v/v) DMSO and 4 U *Taq* polymerase in a standard reaction buffer. After an initial denaturation of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C and a final extension at 72 °C

for 7 min were performed. Amplification products of the expected length of about 730 bp were obtained for all strains tested.

A partial sequence of the *hsp60* gene was also amplified using the degenerate primers cpn-f (5'-CTTGGGCCCCA-AAAGGCMGNAAYGTNGT-3') and cpn-r (5'-CCAAC-CGTTCTTGCAATTTTTCNCKRTCRAA-3'). The reaction mixture and amplification programme were the same as those reported above, except that an annealing temperature of 55 °C instead of 50 °C was used. Amplicons of the expected length of about 1000 bp were obtained for all the strains. Sequencing reactions were performed at the Biomolecular Research Centre (BMR), University of Padua, Italy. The partial *recA* and *hsp60* sequences obtained for the strains were aligned as described for 16S rRNA gene sequences and showed very low divergence (identity of 99%).

RAPD analysis was carried out using three different primers: Coc1 (5'-AGCAGCGTGG-3') (Cocconcelli *et al.*, 1995), M13 (5'-GAGGGTGGCGGTCT-3') (Stendid *et al.*, 1994) and D8635 (5'-GAGCGGCCAAAGGGAGCAGAC-3') (Akopyanz *et al.*, 1992), as described in the respective references except for the addition of 5% (v/v) DMSO in each reaction mixture. Pattern analyses of the amplification products, separated in agarose gels and stained with ethidium bromide, were performed with the GELCOMPAR software (Applied Maths) with the DICE coefficient and UPGMA as calculation and clustering options. AFLP[™] analysis of the four cultures was performed at the BCCM[™]/LMG Bacteria Collection (Laboratorium voor Microbiologie, Universiteit Gent, Belgium). Comparison of AFLP[™] (Fig. 1 and Fig. B, available as supplementary material in IJSEM Online) and RAPD profiles (Fig. C, available as supplementary material in IJSEM Online) demonstrated the genomic coherence of the four strains, their non-clonality and their close relationship with other strains of the species *L. delbrueckii*.

DNA-DNA hybridization testing was performed among the type strains of each subspecies at the BCCM[™]/LMG Bacteria Collection. Hybridization values above 70%, usually considered the threshold for species delineation, were obtained with the type strains of the other three subspecies. DNA-DNA relatedness values were calculated on a minimum of four hybridizations and reciprocal reactions were also performed. Results are as follows, with the difference between reciprocal values shown in parentheses: strain NCC725^T shared 78% (5), 79% (3) and 86% (15) relatedness with *L. delbrueckii* subsp. *delbrueckii* LMG 6412^T, *L. delbrueckii* subsp. *lactis* LMG 7942^T and *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T respectively. The level of hybridization between *L. delbrueckii* subsp. *delbrueckii* LMG 6412^T and *L. delbrueckii* subsp. *lactis* LMG 7942^T was 92% (6), and between *L. delbrueckii* subsp. *delbrueckii* LMG 6412^T and *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T was 82% (18); genomic similarity between *L. delbrueckii* subsp. *lactis* LMG 7942^T and *L.*

Fig. 1. Partial dendrogram of the cluster analysis of the AFLP[®] DNA fingerprints of the strains investigated and reference strains of the *L. delbrueckii* subspecies. A complete dendrogram including other strains and species of the *Lactobacillus acidophilus* complex is available as supplementary material in IJSEM Online (Fig. B).

delbrueckii subsp. *bulgaricus* LMG 6901^T was 74 % (11). With this technique, the mean standard deviation is estimated to be 14 units (Goris *et al.*, 1998).

These data demonstrated that the four strains belong to the same taxon, even though, owing to the standard error of the technique, the relative order of the relationships could not be evaluated.

DNA–DNA hybridization values, and 16S rRNA, *recA* and *hsp60* gene sequence analysis, confirm that the four strains constitute a homogeneous cluster belonging to the species *L. delbrueckii*. However, data obtained here and in Germond *et al.* (2003) suggest that they cannot be assigned to any of the three recognized subspecies of *L. delbrueckii*: (i) ARDRA (Germond *et al.*, 2003) and subspecies-specific PCR data support their differentiation from *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus*; (ii) phenotypic traits distinguish them from *L. delbrueckii* subsp. *delbrueckii* (lactose fermentation) and from *L. delbrueckii* subsp. *lactis* (maltose and trehalose metabolism) (see Table A, available as supplementary material in IJSEM Online); (iii) lactose degradation is constitutive in the four strains but is regulated in *L. delbrueckii* subsp. *lactis* (Lapierre *et al.*, 2002); and (iv) *Lactobacillus delbrueckii* subsp. *bulgaricus* is also able constitutively to degrade lactose but the four strains are negative for the specific PCR amplification for β -galactosidase (Germond *et al.*, 2003). This evidence suggests that the four strains possess a unique genetic equipment for lactose degradation, which differs from that of the other recognized subspecies. Characterization of this equipment is beyond the scope of the present study, but it does provide proof that the four strains represent a separate subspecies. Moreover, molecular analysis, such as AFLP[®] and RAPD-PCR, indicated that the four strains represent a genomic, coherent, non-clonal distinct group within the species *L. delbrueckii*. The data presented highlight several peculiarities to the four strains (probably a result of their geographical isolation) that fulfil the

requirements for the description of a new subspecies (Rosselló-Mora & Amann, 2001) within *L. delbrueckii*. This description may avoid future incorrect identification of ‘atypical’ isolates of *L. delbrueckii* species. The name *Lactobacillus delbrueckii* subsp. *indicus* subsp. nov. is proposed for these strains. Owing to the high overall similarity with the other strains of the species, an emended description of *L. delbrueckii* is not required, except that the species now comprises four subspecies.

Description of *Lactobacillus delbrueckii* subsp. *indicus* subsp. nov.

Lactobacillus delbrueckii subsp. *indicus* (in’ di.cus. L. masc. adj. *indicus* from India, referring to the geographical origin of the strains).

Cells are Gram-positive, catalase-negative rods with rounded ends, generally 0.5–0.8 by 2–9 μ m, occurring singly, in pairs and in short chains. Non-motile. Homofermentative, produces D-lactic acid exclusively. Growth occurs at 45 °C but not at 15 °C. Glucose, fructose, mannose and lactose are fermented by all strains, while fermentation of sucrose and N-acetylglucosamine is more variable. Glycerol, erythritol, arabinose, ribose, xylose, adonitol, methyl β -D-xyloside, galactose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, fucose, arabinol, gluconate, 2-ketogluconate and 5-ketogluconate are not fermented. Arginine is not deaminated. Growth is observed in MRS plus 2.5 % (w/v) NaCl, except for strain NCC780; none of the strains grows in MRS plus 5 % (w/v) NaCl. All the strains grow in MRS at pH 3, 4 and 5, but not at pH 7–8. Metachromatic granules are often observed after methylene blue staining.

The type strain, NCC725^T (= LMG 22083^T = DSM 15996^T),

was isolated from a traditional Indian dairy fermented (type Dahi) product.

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