Reclassification of *Lactobacillus kefirgranum* Takizawa *et al.* 1994 as *Lactobacillus kefiranofaciens* subsp. *kefirgranum* subsp. nov. and emended description of *L. kefiranofaciens* Fujisawa *et al.* 1988

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Fourteen homofermentative lactic acid bacteria that were isolated from kefir grains and kefir fermented milks were assigned to either *Lactobacillus kefiranofaciens* or *Lactobacillus kefirgranum*, based on their characteristic morphotypes, phenotypic features and SDS-PAGE profiles of whole-cell proteins. Further genotypic analyses on representative strains from both taxa demonstrated that *L. kefiranofaciens* and *L. kefirgranum* share 100% 16S rDNA sequence similarity and belong phylogenetically to the *Lactobacillus acidophilus* species group. DNA–DNA binding values of >79% and analogous DNA G+C contents of 37–38 mol% showed that the strains studied belonged to one species: *L. kefirgranum* is a later synonym of *L. kefiranofaciens*. An emended description is proposed for *L. kefiranofaciens*. Due to the specific morphological and biochemical characteristics of these taxa in kefir grain formation, it is proposed that *L. kefirgranum* should be reclassified as *L. kefiranofaciens* subsp. *kefirgranum* subsp. nov.

Kefir is a traditional dairy product that originated from the Caucasus. It is obtained by fermentation of milk at room temperature, using kefir grains as starters. Kefir grains are a naturally immobilized starter; they are made of a complex association of micro-organisms that are more or less embedded in a biopolymer called kefiran. During the last 40 years, many studies have focused on analysis of kefir grain microflora. It contains Gram-positive homofermentative and heterofermentative lactic acid bacteria (lactobacilli, lactococci and leuconostocs), Gram-negative acetic acid bacteria (*Acetobacter*) and both lactose-fermenting (*Kluyveromyces marxianus* and *Kluyveromyces lactis*) and non-fermenting (mostly *Saccharomyces* species) yeasts (Hirota, 1987; Toba, 1987; Halle, 1992; Halle *et al.*, 1994). Four novel *Lactobacillus* species were identified in the kefir microflora: *Lactobacillus kefiri* (Kandler & Kunath, 1983), *Lactobacillus kefiranofaciens* (Fujisawa *et al.*, 1988) and, more recently, *Lactobacillus parakefiri* and *Lactobacillus kefirgranum* (Takizawa *et al.*, 1994). *L. kefiranofaciens* and

L. kefirgranum are both homofermentative lactobacilli that seem to be major microbial constituents of kefir grains. *L. kefiranofaciens* strains form very viscous colonies and are responsible for the formation of kefiran, one of the major polysaccharides that forms kefir grains (Mukai *et al.*, 1990; Yokoi *et al.*, 1991).

In the process of a biodiversity study of several kefir grains and kefir fermented milks, a large number of bacterial strains were isolated on MLR medium (milk-based agar) after anaerobic incubation at 30 °C. MLR medium was prepared by mixing 1 vol. 3% agar solution in water (autoclaved for 15 min at 120 °C and cooled to 60 °C) with 1 vol. UHT (ultra high temperature-treated) milk that contained 10 g filter-sterilized yeast extract l⁻¹ and 10 g filter-sterilized glucose l⁻¹ and was acidified to pH 5.4 with acetic acid. All strains were cultivated and maintained on MLR medium unless indicated otherwise. Bacteriological purity was checked by plating and examining living and Gram-stained cells. Fourteen isolates were selected for further research. Sources of the new isolates studied and the type strains of *L. kefirgranum* and *L. kefiranofaciens* are given in Table 1.

Growth characteristics and colony morphology were investigated after 7–14 days incubation at 25 or 30 °C

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The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *Lactobacillus kefiranofaciens* LMG 19149^T and R-14703 and *Lactobacillus kefirgranum* LMG 15132^T and R-12929 are AJ575259, AJ575260, AJ575261 and AJ575262, respectively.

Table 1. Strains studied

Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; EXT, Danone Vitapole collection of micro-organisms, Palaiseau, France; JCM, Japan Collection of Microorganisms, the Institute of Physical and Chemical Research (RIKEN), Saitama, Japan; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium; R, Research Collection Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium.

Species/strain	Source, place and year of isolation*
<i>L. kefirifaciens</i> :	
LMG 19149 ^T =JCM 6985 ^T =ATCC 43761 ^T	Kefir grains
R-12937=EXT2.139	Kefir grain 16, France, 1999
R-14240=EXT2.143	Kefir grain 15, France, 1999
R-14242=EXT2.145	Kefir grain 14, France, 1999
R-14243=EXT2.146	Kefir grain 18, France, 1999
R-14244=EXT2.147	Kefir grain 8, France, 1999
R-14245=EXT2.148	Kefir grain 3, France, 1999
R-14703=EXT2.187	Kefir grain 9, France, 1999
<i>L. kefirgranum</i> :	
LMG 15132 ^T =JCM 8572 ^T	Kefir grains, Copenhagen, Denmark
R-12929=EXT2.86	Kefir grain 16, France, 2000
R-13043=EXT2.93	Kefir grain 3, France, 2000
R-14331=EXT2.130	Kefir grain 15, France, 2000
R-14334=EXT2.133	Kefir grain 8, France, 2000
R-14340=EXT2.153	Kefir grain 14, France, 1999
R-14343=EXT2.156	Milk fermented with kefir grain 9, France, 1999
R-14344=EXT2.157	Canadian commercial kefir, France, 1999

*Kefir grains 3, 8, 9, 16 and 18 originated from different towns or villages of the Caucasus; grains 14 and 15 originated from the same village. They were used for home-made kefir and, from what the household could remember, they had been propagated constantly in the house and did not originate from a dairy factory.

under anaerobic conditions. Colonies of the 14 new isolates showed two very distinct morphological characteristics or morphotypes: seven strains (R-12929, R-13043, R-14331, R-14334, R-14340, R-14343 and R-14344) formed white, dry, compact, dull, bulging colonies, a morphotype similar to that of *L. kefirgranum* LMG 15132^T. The second group of seven strains (R-12937, R-14240, R-14242, R-14243, R-14244, R-14245 and R-14703) formed transparent, glossy, convex, extremely slimy colonies, similar to those of *L. kefirifaciens* LMG 19149^T. Production of large amounts of polysaccharides is typical of *L. kefirifaciens* and is suggested to be an important property in kefir grain formation (Takizawa *et al.*, 1998).

Carbohydrate fermentation tests were carried out by using API 50CH galleries, following the instructions of the manufacturer (bioMérieux). Strains were cultivated for 4 days at 25 °C under anaerobic conditions in MRSfpH5.4 [MRS medium (Difco), acidified to pH 5.4 with acetic acid and sterilized by filtration through 0.22 µm filters]. The same grouping of the 14 isolates was found as was obtained on the basis of morphological criteria (see above). Strains that were assigned morphologically to *L. kefirgranum* tested positive for hydrolysis of aesculin and acid production from salicin and trehalose, whereas

all strains with the *L. kefirifaciens* morphotype tested negative for these features. High overall correspondence was obtained with the original species descriptions (Takizawa *et al.*, 1994, 1998; Fujisawa *et al.*, 1988). In these studies, acid production from salicin and trehalose was positive for most strains of *L. kefirgranum* and negative for all *L. kefirifaciens* strains. Fermentation patterns of other carbohydrates did not provide additional distinguishing features between the taxa (see descriptions below).

All strains studied were investigated further by using PAGE of whole-cell proteins. Cultures were pre-cultivated for 24 h on MLR medium and were then transferred to MRSfpH5.4 for 24 h. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis were performed by using the Gelcompar software package, versions 3.1 and 4.0, respectively (Applied Maths). Duplicate protein extracts were prepared, in order to check reproducibility of the growth conditions and preparation of the extracts. The correlation level for duplicate protein patterns was $r > 0.94$. Whole-cell protein profiles of newly isolated strains were compared with patterns of the type strains of *L. kefirifaciens* and *L. kefirgranum* (Fig. 1). All

Fig. 1. Protein profiles and corresponding dendrogram derived from UPGMA linkage of the correlation coefficient *r* (expressed as a percentage value for convenience) of *L. kefiranofaciens* and *L. kefirgranum* strains.

strains that were assigned phenotypically to *L. kefiranofaciens* showed a very similar profile to that of the type strain, whereas strains with the *L. kefirgranum* phenotype could be differentiated visually from each other by a varying position of a dominant protein band with a molecular mass of 38–60 kDa (Fig. 1). The latter dense band largely influenced the numerical analysis; both taxa clustered separately and PAGE could be used as a tool for differentiation only after omitting this variable region from the cluster analysis. Strain-specific variable dense bands in *L. kefirgranum* may indicate the presence of a surface (S)-layer on the outside of the bacteria, as was demonstrated previously for other species of the *Lactobacillus acidophilus* group (Boot *et al.*, 1996).

The phylogenetic positions of two representative strains of each species, *L. kefirgranum* LMG 15132^T and R-12929 and *L. kefiranofaciens* LMG 19149^T and R-14703, were determined by 16S rDNA sequence analysis. Genomic DNA was prepared according to the protocol of Niemann *et al.* (1997). 16S rDNA was amplified by using oligonucleotide primers that were complementary to highly conserved regions of bacterial 16S rRNA genes. The forward primer was 5'-AGAGTTTGATCCTGGCTCAG-3' (which hybridizes at positions 8–27, according to the *Escherichia coli* numbering system) and the reverse primer was 5'-AAG-GAGGTGATCCAGCCGCA-3' (positions 1541–1522). PCR products were purified by using a QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were sequenced by using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit and an Applied Biosystems 377 DNA sequencer, using the protocols of the manufacturer (Applied Biosystems). The eight sequencing primers used were listed by Coenye *et al.* (1999). Sequence assembly was performed by using the program AutoAssembler (Applied Biosystems).

Determined 16S rRNA gene sequences (a continuous stretch of 1516 bp for all four strains tested) and sequences of strains that were retrieved from GenBank/EMBL were aligned and a phylogenetic tree was constructed by the neighbour-joining method, using the Bionumerics 3.50 software package (Applied Maths). Unknown bases were discarded from the analysis. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree by using 500 bootstrap resamplings of the data (Fig. 2). Comparison of the four complete sequences revealed 100 % similarity among all strains. Further comparison with sequences from GenBank/EMBL classified the strains in the *L. acidophilus* group (Schleifer & Ludwig, 1995), with *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus hamsteri*, *Lactobacillus amylovorus*, *Lactobacillus amylolyticus*, *Lactobacillus intestinalis* and *L. acidophilus* as nearest neighbours (95.6–97.7 % similarity).

DNA G+C contents were determined for *L. kefirgranum* LMG 15132^T and R-12929 and *L. kefiranofaciens* LMG 19149^T and R-14703. Strains were grown for 24 h on MLR medium at 28 °C under anaerobic conditions. High-molecular-mass native DNA was extracted from 0.75–1.25 g cells (wet weight) by using the protocol described by Pitcher *et al.* (1989) with the following modifications: cell pellets (LMG 19149^T and R-14703 received an additional washing step with 0.1 M NaOH) were washed with RS buffer (0.15 M NaCl, 10 mM EDTA, pH 8.0) and resuspended in a buffer (10 mM Tris/HCl, 300 mM EDTA, pH 8.0) that contained RNase (200 µg ml⁻¹; Sigma), mutanolysin (200 U ml⁻¹; Sigma) and lysozyme (25 mg ml⁻¹; Serva) and incubated for 1 h at 37 °C. At 15 min before the addition of GES reagent, proteinase K (200 µg ml⁻¹; Merck) was added to the mixture. DNA was dissolved in 0.1 × SSC (0.15 M NaCl, 0.015 M citric acid,

Fig. 2. Distance matrix tree showing the phylogenetic relationships of *L. kefiranofaciens*, *L. kefirgranum* and other reference species that belong to the *L. acidophilus* group, based on 16S rDNA sequence comparisons. *Lactobacillus brevis* was used as the outgroup and bootstrap (stability) values are indicated at branch-points (from 500 tree replications).

0.4 M NaOH, pH 7.0) to obtain a concentration of 0.3–0.8 mg ml⁻¹. For determination of the DNA G + C content, DNA was degraded enzymically into nucleosides and separated by HPLC, as described by Mesbah *et al.* (1989). The measured DNA G + C contents for *L. kefirgranum* LMG 15132^T and R-12929 were 38.2 and 38.1 mol%, respectively, which conforms to the range of 34.3–38.6 mol% reported for the species (the lowest aberrant value was found for only one strain; Takizawa *et al.*, 1994). For *L. kefiranofaciens* LMG 19149^T and R-14703, the measured DNA G + C contents were 37.6 and 37.3 mol%, respectively; these values are somewhat higher than the reported values of 34.3–35.4 mol% (Fujisawa *et al.*, 1988) and 36.0–36.8 mol% (Takizawa *et al.*, 1994, 1998). This variation may be explained by the different procedures that were used for analysis: HPLC (present study) versus the thermal melting-point (T_m) method (Marmur & Doty, 1962), which was used for data in the literature.

DNA–DNA hybridizations were performed between *L. kefirgranum* LMG 15132^T and R-12929 and *L. kefiranofaciens* LMG 19149^T and R-14703 (DNA was prepared as above). The microplate method was used, as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS7000 Bio Assay reader (Perkin Elmer) for fluorescence measurements. Biotinylated DNA was hybridized with unlabelled ssDNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 36.1 °C in a hybridization mixture that contained 2 × SSC, 5 × Denhardt's solution, 2.5 % dextran sulphate, 50 % formamide, 100 µg denaturated salmon sperm DNA ml⁻¹ and 1250 ng biotinylated probe DNA ml⁻¹. DNA relatedness values are mean percentages, based on at least two independent hybridization experiments. Reciprocal reactions were performed and variation was within the limit of this method (Goris *et al.*, 1998). Hybridization values between two representative strains of *L. kefirgranum*, LMG 15132^T and R-12929, yielded a binding value of 86 %.

Two *L. kefiranofaciens* strains, LMG 19149^T and R-14703, were also closely related, with a DNA binding value of 96 %. When determining DNA relatedness among strains of both species, high binding values (79–91 %) were obtained, indicating clearly that both taxa belong to the same genospecies. The latter results contradict those given by Takizawa *et al.* (1994), who reported a low homology of 30 % between the type strains of both taxa. Unfortunately, these authors performed only a single hybridization experiment between one strain of each species. In a later study by the same authors on the biodiversity of kefir grains (Takizawa *et al.*, 1998), the same DNA–DNA binding value of 30 % is given between the type strain of *L. kefiranofaciens* and 'isolate no. 41', which belonged to *L. kefirgranum*. We observed, however, that the DNA homology data given in the latter paper are a duplication of results given in the earlier paper (Takizawa *et al.*, 1994) and do not provide additional confirmation of low homology between *L. kefiranofaciens* and *L. kefirgranum*. Comparison of data from Takizawa *et al.* (1998) with their earlier paper (Takizawa *et al.*, 1994) is confusing, as the same data were reported without making reference to the original strain designations and without indication of the type strain of *L. kefirgranum*.

Morphological and phenotypic data from the present study confirm that we are working with the authentic type strains and well-characterized isolates of both *L. kefiranofaciens* and *L. kefirgranum*. Phylogenetic and genotypic data on the type and other representative strains indicate clearly that both taxa belong to the same genospecies and are synonyms. According to nomenclatural rules, the name of the earlier synonym, i.e. *L. kefiranofaciens*, is retained, whereas the name of the later synonym, i.e. *L. kefirgranum*, becomes its heterotypic synonym. Due to the very different morphological features of both taxa, linked with their suggested specific roles in kefir grain formation and the availability of a number of differentiating

phenotypic features, we propose that *L. kefirgranum* should be reclassified as a subspecies of *L. kefiranofaciens*. Below, we give an emended description of *L. kefiranofaciens* and propose a description of two novel subspecies, *L. kefiranofaciens* subsp. *kefiranofaciens* subsp. nov. and *L. kefiranofaciens* subsp. *kefirgranum* subsp. nov., based on data from the literature (Fujisawa *et al.*, 1988; Takizawa *et al.*, 1994, 1998) and from the present study.

Emended description of *Lactobacillus kefiranofaciens* Fujisawa *et al.* 1988

Lactobacillus kefiranofaciens (ke.fi.ra.no.fa'ci.ens. N.L. n. *kefiran* a polysaccharide of kefir grain, kefiran; L. v. *facio* produce; N.L. part. adj. *kefiranofaciens* kefiran-producing).

Gram-positive, non-motile, non-spore-forming rods that are generally 0.5–1.2 × 3.0–20 µm in size and occur singly, in pairs or occasionally in short chains. Colony morphology is subspecies-dependent (see below). There is weak or no growth at 15 °C and no growth at 45 °C. Facultatively anaerobic and produces DL-lactic acid homofermentatively. Catalase is not produced. Hydrolysis of aesculin is variable. Gas is not produced from glucose or gluconate. Arginine is not deaminated. Milk is curdled. Acid is produced from galactose, D-glucose, D-fructose and lactose, but not from adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, gluconate, 2-ketogluconate, 5-ketogluconate, methyl α-D-glucoside, glycerol, glycogen, inositol, D-lyxose, mannitol, D-mannose, methyl α-D-mannoside, melezitose, rhamnose, ribose, sorbitol, L-sorbose, starch, D-tagatose, xylitol, D-xylose, L-xylose or methyl β-xyloside. Acid production from amygdalin, arbutin, cellobiose, β-gentiobiose, N-acetylglucosamine, inulin, maltose, melibiose, D-raffinose, salicin, sucrose, trehalose and D-turanose is strain-dependent. DNA G+C content is 37.3–38.2 mol% (HPLC) or 34.3–38.6 mol% (*T_m*).

The type strain is LMG 19149^T (=JCM 6985^T=ATCC 43761^T). The habitat of the species is kefir grains.

Description of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* subsp. nov.

The description is as that for the species, with the following additional characteristics. Cells are capsulated. On modified KPL agar (pH 5.5) at 30 °C after 10 days, colonies are circular or irregular, 0.5–3.0 mm in diameter, convex, transparent to translucent, white, smooth to rough and ropy. On MLR medium under anaerobic conditions after 7–14 days incubation at 25 or 30 °C, colonies are transparent, glossy, convex and extremely slimy. Large amounts of polysaccharides are produced. No growth occurs at 15 °C. Hydrolysis of aesculin is negative. Acid is produced from sucrose, but not from amygdalin, arbutin, cellobiose, β-gentiobiose, inulin, salicin, trehalose or D-turanose. Acid production from N-acetylglucosamine, maltose, melibiose and D-raffinose is strain-dependent.

The type strain is LMG 19149^T (=JCM 6985^T=ATCC 43761^T).

Description of *Lactobacillus kefiranofaciens* subsp. *kefirgranum* subsp. nov.

Lactobacillus kefirgranum (ke.fir.gra'num. Turkish n. *kefir* Caucasian sour milk; L. n. *granum* grain; N.L. adj. *kefirgranum* kefir grain).

The description is as for the species, with the following additional characteristics. On R-CW agar (Kojima *et al.*, 1993) at 30 °C for 5 days, colonies are 0.5–3.0 mm in diameter, circular to irregular, convex, opaque, white and smooth to rough. On MLR medium under anaerobic conditions after 7–14 days incubation at 25 or 30 °C, colonies are white, dry, compact, dull and bulging. Flocculus or powdery sediment is formed in broth. Weak growth occurs at 15 °C. Hydrolysis of aesculin is positive. Acid is produced from maltose and melibiose and, for nearly all strains, also from D-raffinose, salicin, sucrose and trehalose. Acid production from amygdalin, arbutin, cellobiose, β-gentiobiose, N-acetylglucosamine, inulin and D-turanose is strain-dependent.

The type strain is LMG 15132^T (=JCM 8572^T).

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References

- Boot, H. J., Kolen, C. P. A. M., Pot, B., Kersters, K. & Pouwels, P. H. (1996). The presence of two S-layer-protein-encoding genes is conserved among species related to *Lactobacillus acidophilus*. *Microbiology* **142**, 2375–2384.
- Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J. R. W., Kersters, K. & Vandamme, P. (1999). Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. *Int J Syst Bacteriol* **49**, 405–413.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Fujisawa, T., Adachi, S., Toba, T., Arihara, K. & Mitsuoka, T. (1988). *Lactobacillus kefiranofaciens* sp. nov. isolated from kefir grains. *Int J Syst Bacteriol* **38**, 12–14.
- Goris, J., Suzuki, K., De Vos, P., Nakase, T. & Kersters, K. (1998). Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. *Can J Microbiol* **44**, 1148–1153.
- Halle, C. (1992). *Etude taxonomique de la microflore du kéfir lacté et mise en évidence d'une interaction entre les bactéries lactiques et les levures dans le lait*. PhD thesis, l'Université de Nantes (in French).

- Halle, C., Leroi, F., Dousset, X. & Pidoux, M. (1994). Les kéfirs: des associations bactéries-levures. In *Bactéries Lactiques*, vol. 2, pp. 169–182. Edited by H. de Roissart & F. M. Luquet. Uriage, France: Lorica (in French).
- Hirota, T. (1987). Microbiological studies on kefir grains. *Rep Res Lab Snow Brand Milk Prod Co* **84**, 67–128.
- Kandler, O. & Kunath, P. (1983). *Lactobacillus kefir* sp. nov., a component of the microflora of kefir. *Syst Appl Microbiol* **4**, 286–294.
- Kojima, S., Takizawa, S., Tamura, S., Fujinaga, S., Benno, Y. & Nakase, T. (1993). A new medium for the isolation of lactobacilli from kefir grains. *Biosci Biotechnol Biochem* **57**, 119–120.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Mukai, T., Toba, T., Itoh, T. & Adachi, S. (1990). Structural investigation of the capsular polysaccharide from *Lactobacillus kefiranoferiens* K1. *Carbohydr Res* **204**, 227–232.
- Niemann, S., Pühler, A., Tichy, H.-V., Simon, R. & Selbitschka, W. (1997). Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. *J Appl Microbiol* **82**, 477–484.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester, UK: Wiley.
- Schleifer, K. H. & Ludwig, W. (1995). Phylogeny of the genus *Lactobacillus* and related genera. *Syst Appl Microbiol* **18**, 461–467.
- Takizawa, S., Kojima, S., Tamura, S., Fujinaga, S., Benno, Y. & Nakase, T. (1994). *Lactobacillus kefirgranum* sp. nov. and *Lactobacillus parakefir* sp. nov., two new species from kefir grains. *Int J Syst Bacteriol* **44**, 435–439.
- Takizawa, S., Kojima, S., Tamura, S., Fujinaga, S., Benno, Y. & Nakase, T. (1998). The composition of the *Lactobacillus* flora in kefir grains. *Syst Appl Microbiol* **21**, 121–127.
- Toba, T. (1987). Fermented milks produced with mesophilic lactic acid bacteria. Kefir and Scandinavian ropy sour milk. *Jpn J Dairy Food Sci* **36**, 235–244.
- Yokoi, H., Watanabe, T., Fujii, Y., Mukai, T., Toba, T. & Adachi, S. (1991). Some taxonomical characteristics of encapsulated *Lactobacillus* sp. KPB-167B isolated from kefir grains and characterization of its extracellular polysaccharide. *Int J Food Microbiol* **13**, 257–264.