Lactobacillus durianis Leisner *et al.* 2002 is a later heterotypic synonym of *Lactobacillus vaccinostercus* Kozaki and Okada 1983

Franco Dellaglio,^{1†} Marc Vancanneyt,^{2†} Akihito Endo,^{3†} Peter Vandamme,⁴ Giovanna E. Felis,^{1‡} Anna Castioni,¹ Junji Fujimoto,⁵ Koichi Watanabe⁵ and Sanae Okada³

Correspondence

Franco Dellaglio
franco.dellaglio@univr.it

¹Dipartimento Scientifico e Tecnologico, Università degli Studi di Verona, Strada le Grazie, 15, I-37134 Verona, Italy

^{2,4}BCCM/LMG Bacteria Collection² and Laboratory of Microbiology⁴, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

³NODAI Culture Collection Center, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, Tokyo 156-8502, Japan

⁵Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186-8650, Japan

The taxonomic status of the species *Lactobacillus durianis* and *Lactobacillus vaccinostercus* is briefly summarized and experimental evidence concerning their similarity is presented. Highly similar 16S rRNA gene sequences (99.8 % similarity over 1523 bp), partial *recA* gene sequences (99.5 % similarity over 600 bp) and partial *hsp60* gene sequences (99.1 % similarity over 924 bp) suggest that the two species are closely related. Moreover, a high DNA–DNA binding level (87 %) and similar genomic DNA G + C contents (41–44 mol% for both species) as well as similar biochemical characteristics support the evidence that they constitute a single species. Consequently, according to Rules 38 and 42 of the *Bacteriological Code*, the name *Lactobacillus vaccinostercus*, the oldest legitimate name, must be maintained and the name *Lactobacillus durianis* should be considered a later heterotypic synonym.

Lactobacillus durianis was described by Leisner *et al.* (2002) on the basis of the characteristics of three strains isolated from tempoyak, a Malaysian acid-fermented condiment. These strains were Gram-positive and obligately heterofermentative and produced DL-lactic acid and acetic acid. Growth at 15 °C but not at 45 °C, failure to produce ammonia from arginine and the fermentation pattern of arabinose (+), melibiose (–), ribose (+), sucrose (–) and xylose (+) were key characteristics for the differentiation of

L. durianis from other obligately heterofermentative species. The DNA G + C content was estimated as 43.2–43.3 mol%.

Lactobacillus vaccinostercus was described by Okada *et al.* (1979), but the name was not included in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980). Valid publication of the name was achieved in 1983 (Kozaki & Okada, 1983). Okada *et al.* (1979) studied seven strains isolated from dried cow dung. *L. vaccinostercus* strains are obligate heterofermenters, with meso-diaminopimelic acid in the cell-wall peptidoglycan. Cell morphology, growth temperature and fermentation abilities seem to be very similar to those of the species *L. durianis*. *L. vaccinostercus* strains grow at pH 4.5–7.5. The DNA G + C content was reported as 36 mol% in the original description (Okada *et al.*, 1979).

Two independent observations, the high sequence similarity of the 16S rRNA gene sequences of *L. durianis* and *L. vaccinostercus*, reported by Hammes & Hertel (2003), and the high genomic similarity of 10 *Lactobacillus* strains isolated from goishicha and awabancha, Japanese fermented tea leaves, with the type strains of both *L. durianis*

†These authors contributed equally to this work.

‡Present address: Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale Clinica, Viale San Pietro 43/b, I-07100 Sassari, Italy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *L. vaccinostercus* NRIC 1075^T and strains YIT 10271, YIT 10279, YIT 10292, YIT 10296, YIT 10300, YIT 10308, YIT 10316, YIT 10321, YIT 10323 and FG2-3 are AB212087, AB218793–AB218801 and AB212088, respectively; those for the partial *recA* gene sequences of *L. durianis* CCUG 45405^T and *L. vaccinostercus* LMG 9215^T are AJ621636 and AJ621678 and those for the partial *hsp60* gene sequences of *L. durianis* CCUG 45405^T and *L. vaccinostercus* LMG 9215^T are AJ621721 and AJ621724.

and *L. vaccinostercus* (see below), suggested that the two aforementioned species might constitute a single taxon. The present paper reports data concerning these two observations and proposes the reclassification of *L. durianis* as a later heterotypic synonym of *L. vaccinostercus*.

L. durianis LMG 19193^T and *L. vaccinostercus* strains LMG 9215^T and NRIC 1075^T were grown at 30 °C in XYP broth, composed of (l⁻¹) 10 g D-xylose, 10 g yeast extract, 5.0 g polypeptone and 2.0 g sodium acetate (pH 6.8). Chromosomal DNA was extracted following the procedure of Marmur (1961), unless indicated otherwise.

Phylogenetic analysis was performed by comparing 16S rRNA gene sequences of the two taxa. For *L. durianis* LMG 19193^T, the sequence was available at EMBL (accession no. AJ315640). Two 16S rRNA gene sequences were determined for the type strain of *L. vaccinostercus*: the sequence for NRIC 1075^T was obtained as described by Endo & Okada (2005) and sequence for LMG 9215^T was obtained as described by Felis *et al.* (2005). Since the two *L. vaccinostercus* sequences were not identical (3 mismatches), questions arose about the authenticity of the investigated subcultures of the type strain of the latter species.

The authenticity of the two subcultures of the *L. vaccinostercus* type strain, LMG 9215^T and NRIC 1075^T, was checked by RAPD-PCR analyses using four different primers, Coc1 (5'-AGCAGCGTGG-3') (Cocconcelli *et al.*, 1995), M13 (5'-GAGGGTGGCGGTTCT-3') (Stenlid *et al.*, 1994), 1254 (5'-CCGCAGCCAA-3') and D8635 (5'-GAGC-GGCCAAAGGGAGCAGAC-3') (Akopyanz *et al.*, 1992), which revealed that the two strains are identical and authentic (data not shown). In the same analyses, the *L. durianis* type strain was also included, and its genotypic profile was similar to but distinguishable from that of the *L. vaccinostercus* type strain (data not shown).

After verification of the authenticity of the studied *L. vaccinostercus* type strain and the observation of different 16S rRNA gene sequences, the presence of different ribosomal operons was investigated. Amplification products of the 16S rRNA gene from the two genomic DNA preparations (from the NRIC and LMG subcultures of the type strain) were obtained with primers reported by Felis *et al.* (2005) and cloned in the pGEM-T vector (Promega). Nine clones were analysed for each *Escherichia coli* transformation. Colony PCR was performed with primers T7 and SP6 and amplification products were digested with *Hinf*I enzyme, following standard laboratory procedures. Analysis of these RFLP profiles revealed the presence of at least three different patterns, suggesting that at least three operons with different sequences were present in the initial amplification product obtained from each genomic DNA. Once this intrastrain heterogeneity of ribosomal operons had been determined, it was possible to determine the relationship with the sequence available for *L. durianis*: comparative analysis revealed 99.8% sequence identity (3 positions in 1523 bp).

16S rRNA gene sequences of 10 strains isolated from Japanese fermented tea leaves, goishicha and awabancha, were determined as described by Endo & Okada (2005). The sequences of these isolates were almost identical to one another, and they showed high sequence similarity to the type strains of *L. vaccinostercus* and *L. durianis* (99.9 and 99.7%, respectively).

Sequence comparison of other housekeeping genes between the type strains of *L. durianis* and *L. vaccinostercus* was achieved by amplification and sequencing of the *recA* and *hsp60* genes. *recA* gene sequences were obtained using the degenerate primers recEXT-f (5'-GGCTATGAAACA-AATTGAAAAACAATWYGGNAARGG-3') and recEXT1-r (5'-TGTTTAAACGGTGGAGCAACTTTRTTYTTNAC-3') and the reaction mixture reported by Dellaglio *et al.* (2005). Amplification reactions were carried out with an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 2 min at 50 °C and 105 s at 72 °C and a final extension at 72 °C for 7 min. The amplifications products obtained were about 730 bp long, as expected. Sequencing reactions were performed at the Biomolecular Research Center (BMR), University of Padua (Italy), with the primers used for amplification. The sequences obtained (600 bp), representing about half of the total gene length in comparison with the *recA* genes of *Lactobacillus plantarum* WCSF1 (NT01LP2030, following the TIGR locus annotation, available at <http://www.tigr.org>) and *Lactobacillus johnsonii* (NT01LJ0701), were compared, and only three nucleotides were found to be different.

Partial *hsp60* gene sequences were also obtained as described in Dellaglio *et al.* (2005), using the primers cpn-f (5'-CTTGGGCCCAAAAGGCMGNAAYGTNGT-3') and cpn-r (5'-CCAACCGTTCTTGCAATTTTTCNCKR-TCRAA-3'). Amplification products of the expected lengths, about 1000 bp, were obtained and sequenced with the primers applied in the PCR. Sequences of 924 nucleotides were compared (about 57% of the complete gene length, considering the complete gene sequences NT01LP0651 and NT01LJ0492 found in the genome sequences of *L. plantarum* WCSF1 and *L. johnsonii* NCC 533, respectively), and only eight nucleotides were found to be different.

Conclusive evidence that the two taxa represent a single species was provided by performing DNA-DNA hybridizations between *L. vaccinostercus* LMG 9215^T and *L. durianis* LMG 19193^T. Cells were cultivated in MRS broth at 30 °C for 24 h under aerobic conditions and DNA was extracted from 0.75–1.25 g (wet weight) by using the protocol described by Gevers *et al.* (2001), using a combination of glass beads and enzymes, but with some modifications [volumes were increased tenfold for application on a large scale; vortexing of SDS-treated cells with beads was done for 30 s; after addition of 16.5 ml TE buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) and 5 ml 5 M NaCl and gentle shaking, the suspension was incubated at 65 °C for 10 min]. Subsequent chloroform/isoamyl alcohol extraction, precipitation, spooling of DNA on a glass rod, washing with

ethanol and RNase treatment were performed as described by Marmur (1961). For DNA–DNA hybridizations, 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 single-stranded DNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 37 °C in hybridization mixture (2 × SSC, 5 × Denhardt's solution, 2·5 % dextran sulphate, 50 % formamide, 100 µg denatured salmon sperm DNA ml⁻¹, 1·25 µg biotinylated probe DNA ml⁻¹). Results showed that *L. vaccinostercus* LMG 9215^T and *L. durianis* LMG 19193^T shared 87 % total DNA relatedness, thus confirming that the two type strains belong to the same species, according to the current delineation of bacterial species (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002).

Further proof of the infraspecific relationship between *L. vaccinostercus* NRIC 1075^T and *L. durianis* NRIC 0624^T was obtained when hybridizing DNA from the 10 strains isolated from Japanese fermented tea leaves with DNA from the two type strains. A different procedure was followed: extraction and isolation of bacterial DNAs were performed by the method of Marmur (1961) as modified by Ezaki *et al.* (1983). DNA–DNA hybridizations were carried out by the microdilution well technique by using photobiotin for labelling of DNA (Ezaki *et al.*, 1989). All 10 isolates showed high levels of DNA–DNA relatedness to *L. vaccinostercus* NRIC 1075^T and *L. durianis* NRIC 0624^T (ranging from 90 to 100 % and 80 to 100 %, respectively). Moreover, *L. vaccinostercus* NRIC 1075^T showed a high level of DNA–DNA relatedness to *L. durianis* NRIC 0624^T by this technique (95 %).

Hammes & Hertel (2003) reported that the very different reported genomic DNA G + C contents did not support the reclassification of *L. durianis* as a synonym of *L. vaccinostercus*. In order to clarify these data, the DNA G + C contents of the two type strains were redetermined by enzymic degradation and HPLC separation with two different protocols. The first method was described by Mesbah *et al.* (1989) and, after enzymic degradation, the nucleotides obtained were separated using a Waters SymmetryShield C8 column maintained at a temperature of 37 °C. The solvent was 0·02 M (NH₄)H₂PO₄ (pH 4·0) with 1·5 % acetonitrile and non-methylated λ phage DNA (Sigma) was used as the calibration reference. The second protocol applied was that described by Tamaoka & Komagata (1984). Depending on the protocol applied, the G + C contents of the *L. durianis* and *L. vaccinostercus* type strains were 44 mol% (protocol of Mesbah *et al.*, 1989) or 41–42 mol% (protocol of Tamaoka & Komagata, 1984). The G + C contents obtained for the two type strains *L. vaccinostercus* LMG 9215^T and *L. durianis* LMG 19193^T are significantly different from the value reported in the species description of *L. vaccinostercus* (Okada *et al.*, 1979), 36 mol%, which probably resulted from technical limitations and has to be considered as incorrect.

Considering phenotypic traits, gas production from D-glucose and D-gluconate by *L. vaccinostercus* NRIC 1075^T, *L. durianis* NRIC 0624^T and the 10 isolates was determined by using Durham tubes and broth containing (l⁻¹) 10 g D-glucose or 10 g D-gluconate, 10 g yeast extract, 5 g polypeptone and 2 g sodium acetate under stationary conditions or anaerobic conditions in anaerobic jars (GasPak System; BBL). The presence of *meso*-diaminopimelic acid in the cell wall and acid formation from carbohydrates were determined as described previously (Endo & Okada, 2005). Of the 12 strains tested, 10 produced gas from D-glucose under anaerobic conditions after 5 days and under stationary conditions after 7 days. The exceptions, strains YIT 10279 and FG2-3, produced gas from D-glucose under anaerobic conditions after 5 days, but not under stationary conditions after 7 days. Therefore, gas production from D-glucose for *L. vaccinostercus* strains should be determined under anaerobic conditions. All the tested strains produced a large amount of gas from D-gluconate after 3 days under stationary and anaerobic conditions. Acids were produced rapidly from the pentoses L-arabinose, D-ribose and D-xylose and from D-gluconate, D-glucose, maltose, D-galactose (11 of 12 strains) and D-fructose (2 of 12 strains), but not from D-mannose, L-rhamnose, cellobiose, lactose, melibiose, sucrose, raffinose, D-salicin, D-trehalose, D-melezitose, D-mannitol or D-sorbitol. The strains tested had *meso*-diaminopimelic acid in their cell walls.

On the basis of the evidence presented, it is proposed that the two species, *L. durianis* and *L. vaccinostercus*, be united under the same name; according to the rules of priority (Rules 38 and 42 of the *Bacteriological Code*; Lapage *et al.*, 1992), the name *Lactobacillus vaccinostercus* should be retained, strains of *Lactobacillus durianis* should be reclassified as such and the name *Lactobacillus durianis* should be considered a later heterotypic synonym.

L. vaccinostercus belongs to the *Lactobacillus reuteri* phylogenetic group as delineated by Hammes & Hertel (2003). In particular, *Lactobacillus suebicus* shows a strong relatedness to *L. vaccinostercus* and *L. durianis* based on high 16S rRNA gene sequence identity (98·56 %; 22 differences in 1526 bp), similar fermentation profiles and similar DNA G + C content (40·4 ± 0·4 mol%). However, the low levels of DNA–DNA relatedness (17–18 %) observed between *L. suebicus* strains and the *L. vaccinostercus* type strain (Kleynmans *et al.*, 1989) confirm the distinctiveness of these two taxa.

Emended description of *Lactobacillus vaccinostercus* Kozaki and Okada 1983

Cells are Gram-positive, non-motile, non-spore-forming rods, occurring singly, in pairs or in short chains depending on the medium for growth. Growth temperature is between 20 and 40 °C; some strains may grow at 15 °C but none grow at 45 °C. Obligately heterofermentative; acid is always produced from arabinose, gluconate, glucose, maltose, ribose and xylose, while degradation of D-galactose and

D-fructose does not occur for all strains. DL-Lactic acid, ethanol and acetic acid are produced from both glucose and xylose. All strains produce gas from D-glucose under anaerobic conditions, but some strains do not produce gas under stationary conditions. All strains produce gas from D-gluconate under anaerobic and stationary conditions. Catalase-negative, nitrate is not reduced and ammonia is not produced from arginine. Cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The DNA G + C content is 41–44 mol%.

The type strain is NRIC 1075^T=LMG 9215^T=ATCC 33310^T=DSM 20634^T=NCIMB 11808^T.

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References

- Akopyanz, N., Bukanov, N. O., Westblom, T. U., Kresovich, S. & Berg, D. E. (1992). DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res* **20**, 5137–5142.
- Cocconcelli, P. S., Porro, D., Galandini, S. & Senini, L. (1995). Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. *Lett Appl Microbiol* **21**, 376–379.
- Dellaglio, F., Felis, G. E., Castioni, A., Torriani, S. & Germond, J.-E. (2005). *Lactobacillus delbrueckii* subsp. *indicus* subsp. nov., isolated from Indian dairy products. *Int J Syst Evol Microbiol* **55**, 401–404.
- Endo, A. & Okada, S. (2005). *Lactobacillus satsumensis* sp. nov. isolated from mashes of shochu, a traditional Japanese distilled spirit made from fermented rice and other starchy materials. *Int J Syst Evol Microbiol* **55**, 83–85.
- Ezaki, T., Yamamoto, N., Ninomiya, K., Suzuki, S. & Yabuuchi, E. (1983). Transfer of *Peptococcus indolicus*, *Peptococcus asaccharolyticus*, *Peptococcus prevotii*, and *Peptococcus magnus* to the genus *Peptostreptococcus* and proposal of *Peptostreptococcus tetradius* sp. nov. *Int J Syst Bacteriol* **33**, 683–698.
- 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.
- Felis, G. E., Torriani, S. & Dellaglio, F. (2005). Reclassification of *Pediococcus urinaequi* (ex Mees 1934) Garvie 1988 as *Aerococcus urinaequi* comb. nov. *Int J Syst Evol Microbiol* **55**, 1325–1327.
- Gevers, D., Huys, G. & Swings, J. (2001). Applicability of rep-PCR fingerprinting for differentiation of *Lactobacillus* species. *FEMS Microbiol Lett* **205**, 31–36.
- 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.
- Hammes, W. P. & Hertel, C. (2003). The genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes. An Evolving Electronic Resource for the Microbiological Community*, release 3.15. Edited by M. Dworkin and others. New York: Springer. <http://141.150.157.117:8080/prokPUB/index.htm>
- Kleynmans, U., Heinzl, H. & Hammes, W. P. (1989). *Lactobacillus suebicus* sp. nov., an obligately heterofermentative *Lactobacillus* species isolated from fruit mashes. *Syst Appl Microbiol* **11**, 267–271.
- Kozaki, M. & Okada, S. (1983). *Lactobacillus vaccinostrercus* sp. nov. In *Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the IJSB*, List no. 10. *Int J Syst Bacteriol* **33**, 438–440.
- Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seelinger, H. P. R. & Clark, W. A. (editors) (1992). *International Code of Nomenclature of Bacteria (1990 Revision)*. *Bacteriological Code*. Washington, DC: American Society for Microbiology.
- Leisner, J. J., Vancanneyt, M., Lefebvre, K., Vandemeulebroecke, K., Hoste, B., Eura Vilalta, N., Rusul, G. & Swings, J. (2002). *Lactobacillus durianis* sp. nov., isolated from an acid-fermented condiment (tempoyak) in Malaysia. *Int J Syst Evol Microbiol* **52**, 927–931.
- Marmur, J. (1961). A procedure for the isolation of DNA from microorganisms. *J Mol Biol* **3**, 208–218.
- 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.
- Okada, S., Suzuki, Y. & Kozaki, M. (1979). A new heterofermentative *Lactobacillus* species with *meso*-diaminopimelic acid in peptidoglycan, *Lactobacillus vaccinostrercus* Kozaki and Okada sp. nov. *J Gen Appl Microbiol* **25**, 215–221.
- Rosselló-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.
- Skerman, V. B. D., McGowan, V. & Sneath, P. H. A. (editors) (1980). Approved lists of bacterial names. *Int J Syst Bacteriol* **30**, 225–420.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.
- Stenlid, G., Karlsson, J. O. & Hogberg, N. (1994). Intraspecific genetic variation in *Heterobasidion annosum* revealed by amplification of minisatellite DNA. *Mycol Res* **98**, 57–63.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.