Lactobacillus namurensis sp. nov., isolated from a traditional Belgian sourdough

Ilse Scheirlinck,^{1,2} Roel Van der Meulen,³ Ann Van Schoor,^{1,2}
Ilse Cleenwerck,² Geert Huys,¹ Peter Vandamme,¹ Luc De Vuyst³
and Marc Vancanneyt²

Correspondence

Ilse Scheirlinck

Ilse.Scheirlinck@ugent.be

¹Laboratory of Microbiology, Department of Biochemistry, Physiology and Microbiology, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

²BCCM/LMG Bacteria Collection, Department of Biochemistry, Physiology and Microbiology, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

³Research Group of Industrial Microbiology and Food Biotechnology, Department of Applied Biological Sciences and Engineering, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

A biodiversity study on lactic acid bacteria (LAB) occurring in traditional Belgian sourdoughs resulted in the isolation of two *Lactobacillus* isolates, LMG 23583^T and LMG 23584, that could not be assigned to any recognized LAB species. The two isolates were initially investigated by means of phenylalanyl-tRNA synthase (*pheS*) gene sequence analysis and were found to occupy a separate position relative to recognized *Lactobacillus* species present in the *pheS* database. Subsequently, their phylogenetic affiliation was determined by 16S rRNA gene sequence analysis, indicating that the two isolates belong to the *Lactobacillus buchneri* species group with *Lactobacillus zymae*, *Lactobacillus acidifarinae* and *Lactobacillus spicheri* as closest relatives. Whole-cell protein analysis (SDS-PAGE) and amplified fragment length polymorphism fingerprinting of whole genomes confirmed their separate taxonomic status. DNA–DNA hybridization experiments, DNA G + C content, growth characteristics and biochemical features demonstrated that the two isolates represent a novel *Lactobacillus* species, for which the name *Lactobacillus namurensis* sp. nov. is proposed. The type strain is LMG 23583^T (= CCUG 52843^T).

Fermented foods produced from cereals, for example beer, spirits, sake, porridge and baked goods, have a long history of nutritional and economic importance. One of the ancient means of cereal fermentation is the traditional sourdough process (Vogel *et al.*, 1999). Sourdough is a mixture of flour and water that is fermented with lactic acid bacteria (LAB) and yeasts (Gänzle *et al.*, 1998; Vogel *et al.*, 1999). LAB predominate the microbial community in this complex ecosystem and are responsible for the acidification (mainly lactic acid) of the raw material and the production of acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides and several enzymes (Leroy & De Vuyst, 2004). Because of the broad metabolic potential of LAB, interest in them as starter cultures for the maintenance of food safety and quality as well as for the production of novel products

with improved nutritional properties is increasing. Owing to the large variety of cereals and fermentation conditions used, sourdoughs have been shown to harbour a large diversity of LAB species. In recent years, several novel *Lactobacillus* species isolated from sourdough have been described, including *Lactobacillus pontis* (Vogel *et al.*, 1994), *L. panis* (Wiese *et al.*, 1996), *L. paralimentarius* (Cai *et al.*, 1999), *L. frumenti* (Müller *et al.*, 2000), *L. mindensis* (Ehrmann *et al.*, 2003), *L. spicheri* (Meroth *et al.*, 2004), *L. rossiae* (Corsetti *et al.*, 2005), *L. hammesii* (Valcheva *et al.*, 2005), *L. zymae* (Vancanneyt *et al.*, 2005), *L. acidifarinae* (Vancanneyt *et al.*, 2005) and *L. nantensis* (Valcheva *et al.*, 2006).

During a study on the biodiversity of LAB in traditional Belgian sourdoughs, two *Lactobacillus* isolates could not be clearly placed within any recognized species of the genus by means of genotypic and phenotypic methods. In the present study, the taxonomic position of these two isolates in the genus *Lactobacillus* was determined.

The sourdough used in this study originated from a spontaneous fermentation of a mixture of wheat, rye and

Abbreviations: AFLP, amplified fragment length polymorphism; LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Lactobacillus namurensis* LMG 23583^T and LMG 23584 are AM259119 and AM259120, respectively.

A cluster analysis of protein profiles of the novel strains and related type strains is available as supplementary material in IJSEM Online.

spelt flour. The sourdough was maintained by back-slopping and was sampled during two investigations in August 2004 and November 2005 from a bakery in the province of Namur, Belgium. Samples were taken aseptically, stored at 4 °C and analysed within 24 h. Sourdough samples were suspended (1 : 10, w/v) and serially diluted in peptone-physiological solution [0.1 % (w/v) bacteriological peptone (L37; Oxoid) and 0.85 % (w/v) NaCl]. Isolates LMG 23583^T and LMG 23584 were incubated at 30 °C under aerobic conditions on MRS5 agar, containing 0.1 g cycloheximide l⁻¹ (Meroth *et al.*, 2003). The selected isolates were checked for bacteriological purity by successive plating on MRS5 agar (without cycloheximide) and stored in Microbank tubes (Pro-Lab Diagnostics) at -80 °C. Isolates LMG 23583^T and LMG 23584 respectively originated from the August 2004 and November 2005 sampling investigations. Gram-staining, cell morphology, catalase activity and all further experiments were performed by using cultures grown for 24 h on MRS5 medium at 30 °C under aerobic conditions, unless otherwise indicated. In the two sets of sourdough isolates obtained during the sampling investigations of August 2004 and November 2005, six (15 % of total) and three (30 % of total) isolates, respectively, were found to belong to the new taxon represented by isolates LMG 23583^T and LMG 23584. Other LAB species retrieved from the two sourdough samples included *L. paralimentarius*, *L. hammesii*, *Lactobacillus brevis* and *Lactobacillus plantarum*.

The taxonomic position of the sourdough LAB isolates LMG 23583^T and LMG 23584 was initially investigated using phenylalanyl-tRNA synthase (*pheS*) gene sequence analysis (Naser *et al.*, 2005). Analysis of *pheS* gene sequences has proved to be an excellent tool for identification of *Lactobacillus* isolates and delineation of novel taxa (S. M. Naser, P. S. R. Dawyndt, B. Hoste, D. Gevers, K. Vandemeulebroecke, I. Cleenwerck, M. Vancanneyt and J. Swings, unpublished; Vancanneyt *et al.*, 2006). Genomic DNA was extracted as described by Gevers *et al.* (2001). The primers for *pheS* sequencing were PheS-21-F and PheS-23-R, with amplification conditions and sequencing reactions as described by Naser *et al.* (2005). Sequences were imported into BioNumerics version 4.0 software (Applied Maths), aligned and compared by using the neighbour-joining method with available sequences of nearly all recognized *Lactobacillus* species. The two new sourdough isolates clustered together with a sequence similarity of 100 % and constituted a distinct branch in the *Lactobacillus buchneri* species group, showing sequence similarities below 85 % with the other members of the group (Fig. 1). Interspecies gaps within the genus based on *pheS* gene sequences normally exceed 7 % (S. M. Naser, P. S. R. Dawyndt, B. Hoste, D. Gevers, K. Vandemeulebroecke, I. Cleenwerck, M. Vancanneyt and J. Swings, unpublished), which suggested that the two isolates represent a novel *Lactobacillus* species.

The phylogenetic position of strains LMG 23583^T and LMG 23584 was further studied by 16S rRNA gene sequence analysis. Genomic DNA was extracted as described above.

Fig. 1. Neighbour-joining tree based on *pheS* gene sequence data showing the relationships between the sourdough isolates LMG 23583^T and LMG 23584 and related reference strains. *Lactobacillus sanfranciscensis* LMG 16002^T was used as the outgroup. Bootstrap percentages (based on 500 replications) ≥ 50 % are indicated at branch points. Bar, 10 % sequence divergence.

16S rRNA gene amplification, purification and sequencing were performed as described by Vancanneyt *et al.* (2006). The sequences obtained (continuous stretches of 1518 bp) were deposited in and aligned and clustered with sequences from the EMBL database. A phylogenetic tree was constructed with the neighbour-joining method by using the BioNumerics version 4.0 software package. Unknown bases were discarded for analysis. The statistical reliability of the tree was evaluated by bootstrap analysis of 500 replicates and the tree topology was also confirmed using maximum-parsimony and maximum-likelihood cluster analysis. The 16S rRNA gene sequences of strains LMG 23583^T and LMG 23584 were 100 % similar, and their phylogenetic position within the *L. buchneri* group was confirmed. Nearest neighbours to the two new strains were *L. spicheri* LMG 21871^T, *L. zymae* LMG 22198^T and *L. acidifarinae* LMG 22200^T, with 16S rRNA gene sequence similarities of 98.2, 98.7 and 98.7 %, respectively (Fig. 2).

The sourdough LAB isolates were also investigated using SDS-PAGE of cellular proteins. Extraction and gel electrophoresis were performed according to the method described by Pot *et al.* (1994) for Gram-positive bacteria. Densitometric digitization of patterns was performed by using an LKB 2202 Ultrascan Laser Densitometer (LKB). Normalization of densitometric traces was performed with GelCompar version 4.2 software (Applied Maths). Numerical analysis with profiles available in an extensive in-house database was performed by using the Pearson product moment correlation coefficient and the unweighted pair-group method using arithmetic averages (UPGMA) with BioNumerics version 4.0. Between the two isolates, minor differences were observed in the intensity of

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequence analysis showing the phylogenetic position of strains LMG 23583^T and LMG 23584 among related taxa. The tree was generated by the neighbour-joining method, and *Lactobacillus delbrueckii* subsp. *delbrueckii* DSM 20074^T was used as the outgroup. Bootstrap percentages (based on 500 replications) $\geq 50\%$ are indicated at branch points. Bar, 1% sequence divergence.

dominant bands situated in a molecular mass range of 30–40 kDa. Comparison of the protein profiles, omitting the latter zone, resulted in a distinct branch for isolates LMG 23583^T and LMG 23584 (see Supplementary Fig. S1 available in IJSEM Online).

To confirm the unique genotypic position of strains LMG 23583^T and LMG 23584 in the *L. buchneri* group and to verify strain-specific differences, amplified fragment length polymorphism (AFLP) fingerprinting of whole genomes was performed. Extraction and purification of total genomic DNA were done as described above. AFLP analysis was performed according to the protocol of Thompson *et al.* (2001) with several modifications. Total DNA was digested with *Eco*RI and *Taq*I restriction enzymes. Fragments were amplified using the primers E01 (5'-GACTGCGTAC-CAATTCA-3'), T01 (5'-CGATGAGTCCTGACCGAA-3'), E03 (5'-GACTGCGTACCAATTCG-3') and T03 (5'-CGATGAGTCCTGACCGAG-3'). The resulting electrophoretic profiles were normalized using GeneScan 3.1

software (Applied), and tables of peaks, containing fragments of 50–536 bp, were transferred into BioNumerics version 4.0. AFLP patterns were compared with profiles of LAB reference taxa using the Dice coefficient and UPGMA linkage. Cluster analysis of AFLP band patterns generated with primer pair E01/T01 (Fig. 3) confirmed the unique taxonomic position of strains LMG 23583^T and LMG 23584 and also indicated that they were genotypically closely related. Using three different primer pairs (E01/T01, E01/T03 and E03/T03), minor but reproducible band position variations in the AFLP patterns of the two isolates could be observed with the E01/T01 primer pair, suggesting that strains LMG 23583^T and LMG 23584 were genotypically slightly different (Fig. 3).

The G + C contents of the DNA of strains LMG 23583^T and LMG 23584 were determined. DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M (NH₄)H₂PO₄ (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 strains LMG 23583^T and LMG 23584 was 52 mol%, similar to the values determined for *L. spicheri* (55 mol%), *L. zymae* (53–54 mol%) and *L. acidifarinae* (51 mol%) (Meroth *et al.*, 2004; Vancanneyt *et al.*, 2005).

DNA–DNA hybridizations were performed between strain LMG 23583^T and the type strains of the three most closely related species (*L. acidifarinae*, *L. spicheri* and *L. zymae*) (Figs 1 and 2). DNA was extracted from 0.75–1.25 g (wet wt) cell mass using the protocol described by Gevers *et al.* (2001) with the following modifications. Volumes were increased tenfold for large-scale application. After the addition of 20% SDS and glass beads, cells were mixed for 30 s. Subsequently, 16.5 ml TE buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) and 5 ml 5 M NaCl was added, followed by 10 min incubation at 65 °C. Chloroform/isoamyl alcohol extraction and ethanol and RNase treatment were performed as described by Marmur (1961). DNA–DNA hybridizations were performed with biotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), by using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 44 °C in the presence of 50% formamide. Reciprocal experiments were performed for every pair of strains and the standard deviation ranged from 0.1 to 9.9%. Levels of DNA–DNA relatedness between strain LMG 23583^T and *L. spicheri* LMG 21871^T, *L. zymae* LMG 22198^T and *L. acidifarinae* LMG 22200^T were 34, 25 and 18%, respectively.

Fig. 3. Cluster analysis of digitized AFLP band patterns of strains LMG 23583^T and LMG 23584 and their closest phylogenetic relatives. The corresponding dendrogram was constructed from the UPGMA linkage of Dice coefficients.

These values are well below the threshold of 70 % suggested for species delineation (Stackebrandt & Goebel, 1994), indicating that strain LMG 23583^T and the genotypically highly related strain LMG 23584 represent a novel species in the genus *Lactobacillus*.

Growth characteristics of isolates LMG 23583^T and LMG 23584 were determined in MRS broth (pH 5.4) (de Man *et al.*, 1960). Growth was tested at 15 and 45 °C and in the presence of 5, 6 and 7 % NaCl. Aerobic and anaerobic growth and production of gas from 2 % glucose and 2 % gluconate in MRS broth (pH 5.4, without the addition of triammonium citrate) were also investigated. Arginine hydrolysis was tested in a medium containing 0.5 % tryptone, 0.5 % yeast extract, 0.3 % L-arginine, 0.05 % glucose and 0.2 % K₂HPO₄ (pH 7.0), with methyl orange as indicator. The isomeric type of lactate was determined enzymically (R-Biopharm). The carbohydrate fermentation patterns of the strains were determined with the API 50 CHL system (bioMérieux) following the manufacturer's instructions with strains cultivated at 37 °C. A detailed phenotypic description is given below and characteristics that differentiate strain LMG 23583^T from its closest relatives, *L. spicheri*, *L. zymae* and *L. acidifarinae*, are summarized in Table 1.

On the basis of the data presented, strains LMG 23583^T and LMG 23584 are considered to represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus namurensis* sp. nov. is proposed.

Table 1. Phenotypic characteristics of strain LMG 23583^T and type strains and reference strains of their closest relatives

Strains: 1, *L. spicheri* LMG 21871^T; 2, *L. zymae* LMG 22198^T; 3, *L. zymae* LMG 22199; 4, *L. acidifarinae* LMG 22200^T; 5, LMG 23583^T (an identical carbohydrate fermentation pattern was obtained for strain LMG 23584). +, Positive after 48 h incubation; (+), positive after 5 days incubation; –, negative. All strains were positive after 48 h incubation for ribose and were negative for 2-ketogluconate.

Production of acid from:	1	2	3	4	5
D-Arabitol	–	+	+	+	–
Galactose	+	+	–	+	+
Aesculin	–	–	–	–	+
5-Ketogluconate	+	–	–	–	–
Lactose	–	+	–	–	–
Mannitol	(+)	+	–	+	(+)
Melibiose	+	(+)	–	–	(+)
Methyl α-D-glucoside	(+)	–	–	–	–
D-Xylose	+	+	–	+	–
Methyl β-xyloside	–	–	–	–	+

Description of *Lactobacillus namurensis* sp. nov.

Lactobacillus namurensis (na.mur'en.sis. N.L. masc. adj. *namurensis* pertaining to the province of Namur, Belgium, from where the type strain was isolated).

Cells are Gram-positive, catalase-negative, non-motile, non-spore-forming rods. Cells occur singly or in pairs, and are 2–10 µm in length and 0.5–1 µm wide. After 2 days incubation on MRS5 agar, colonies are beige, circular with an irregular surface and approximately 0.5–1 mm in diameter. Cells grow well in liquid or solid MRS under aerobic or anaerobic conditions. Grows at 15 °C in the presence of 5, 6 and 7 % NaCl but not at 45 °C. Both D- and L-lactate are produced in equimolar amounts and glucose is metabolized heterofermentatively. Ammonium is produced from arginine. Gas is produced from glucose and gluconate. Produces acid from ribose, methyl β-D-xyloside, galactose, glucose, fructose, mannitol, N-acetylglucosamine, aesculin, maltose, melibiose and gluconate, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, adonitol, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, cellobiose, lactose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol or 2- or 5-ketogluconate. The DNA G + C content is 52 mol%.

The type strain, LMG 23583^T (=CCUG 52843^T), was isolated from an artisanal sourdough manufactured with wheat, rye and spelt flour in the province of Namur, Belgium.

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