Lactobacillus nantensis sp. nov., isolated from French wheat sourdough

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A polyphasic taxonomic study of the bacterial flora isolated from traditional French wheat sourdough, using phenotypic characterization and phylogenetic as well as genetic methods, revealed a consistent group of isolates that could not be assigned to any recognized species. These results were confirmed by randomly amplified polymorphic DNA and amplified fragment length polymorphism fingerprinting analyses. Cells were Gram-positive, homofermentative rods. Comparative 16S rRNA gene sequence analysis of the representative strain LP33^T indicated that these strains belong to the genus *Lactobacillus* and that they formed a branch distinct from their closest relatives *Lactobacillus farciminis*, *Lactobacillus alimentarius*, *Lactobacillus paralimentarius* and *Lactobacillus mindensis*. DNA–DNA reassociation experiments with the three phylogenetically closest *Lactobacillus* species confirmed that LP33^T (= DSM 16982^T = CIP 108546^T = TMW 1.1265^T) represents the type strain of a novel species, for which the name *Lactobacillus nantensis* sp. nov. is proposed.

Sourdough fermentation is a process used to obtain bread dough from wheat or rye flour by the combined metabolic activity of lactic acid bacteria (LAB) and yeasts (Hammes & Gänzle, 1998). Because of the superior sensory quality and the prolonged shelf life of the resulting baked goods, sourdough processes have retained their importance in modern baking technology (Stolz *et al.*, 1996; Ottogalli *et al.*, 1996). Yeasts contribute predominantly to dough leavening, whereas LAB are responsible for acidification, aroma formation and sensorial as well as nutritional improvement of the fermented product (Vogel *et al.*, 1999; Böcker *et al.*, 1990). Knowledge regarding LAB diversity is thus essential in sourdough investigations.

Recent studies of the LAB microbiota of sourdoughs have employed traditional cultivation methods in combination with phenotypic (physiological and biochemical) and/or genotypic [e.g. randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analyses] identification methods (Galli *et al.*, 1988; Mäntynen *et al.*, 1999; Rocha & Malcata, 1999; Corsetti *et al.*, 2001; Bervas, 1991; Onno & Roussel, 1994; Gabriel *et al.*, 1999; Valcheva *et al.*, 2005). The majority of LAB isolated from sourdoughs belong to the genus *Lactobacillus*, and more than 50 *Lactobacillus* species have been isolated in relevant cell counts from sourdough. Sourdoughs prepared by traditional procedures, type I sourdough, are characterized by the association between homo- and heterofermentative lactobacilli (Vogel *et al.*, 1999). *Lactobacillus sanfranciscensis* is most frequently isolated and was thus designated as a key organism in type I sourdough. The homofermentative species *Lactobacillus plantarum* and *Lactobacillus alimentarius* are also usually isolated (Vogel *et al.*, 1999; Corsetti *et al.*, 2001; De Vuyst *et al.*, 2002; Valcheva *et al.*, 2005). Industrial fermentations are carried out at elevated temperature and over prolonged periods. In these fermentations, producing type II sourdough, *Lactobacillus reuteri*, *Lactobacillus fermentum*, *Lactobacillus acidophilus* and *Lactobacillus amylovorus*

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Abbreviations: AFLP, amplified fragment length polymorphism; LAB, lactic acid bacteria; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LP33^T is AY690834.

RAPD and AFLP patterns of *Lactobacillus nantensis* LP33^T and selected reference sourdough lactobacilli are shown in supplementary figures available in IJSEM Online.

are often isolated (Vogel *et al.*, 1999; Hammes & Gänzle, 1998; Meroth *et al.*, 2003). Recent aims to characterize sourdough microflora by using a polyphasic taxonomic approach have resulted in the description of several novel *Lactobacillus* species, namely *Lactobacillus frumenti* (Müller *et al.*, 2000), *L. mindensis* (Ehrmann *et al.*, 2003), *L. spicheri* (Meroth *et al.*, 2004), *L. hammesii* (Valcheva *et al.*, 2005), *L. zymae* and *L. acidifarinae* (Vancanneyt *et al.*, 2005) and *L. rossii* (Corsetti *et al.*, 2005).

In the present study, the LAB of a traditional French wheat sourdough were investigated. A preliminary screening based on observed phenotypic characteristics led to the isolation of 30 bacterial strains. Additional information regarding the taxonomic status at species and strain level was obtained by amplified-fragment length polymorphism (AFLP) and RAPD analyses. These methods allowed the identification to species level of almost all LAB involved in this sourdough fermentation. However, a consistent group of 14 strains could be clearly discriminated from recognized *Lactobacillus* species. On the basis of the phenotypic and genotypic results obtained from a representative strain of this group, LP33^T, a novel *Lactobacillus* species is described.

The traditional French wheat sourdough used for this study was a firm sourdough originating from initially spontaneous fermentation of flour and water (dough yield 160 kg dough per 100 kg flour) at 18–20 °C. It was maintained by back slopping (repeated cycles of re-inoculation of flour, water and 30 % inoculum from the previous fermentation) under the same conditions. Sourdough samples used for bread preparation were taken aseptically, stored at 4 °C and analysed immediately. The LAB population was enumerated as described by Valcheva *et al.* (2005). After 48–72 h incubation at 30 °C under anaerobic conditions, the number of colonies with similar morphology and the total number of LAB (as c.f.u. g⁻¹) were estimated. The distribution of various colony forms was recorded and the relative distribution of the various isolates was determined. Thirty colonies representing these different morphological forms were picked and purified on the same medium by successive subculturing at 30 °C. Pure isolates were stored in 20 % (w/v) glycerol stock cultures at -80 °C.

Lactobacillus reference strains used were *L. alimentarius* LMG 9187^T (=DSM 20249^T), *L. alimentarius* LMG 9188t2, *Lactobacillus brevis* DSM 20054^T, *L. farciminis* LMG 9200^T (=DSM 20184^T), *L. hammesii* DSM 16381^T, *Lactobacillus kimchii* DSM 13961^T, *L. kimchii* LMG 19822^T, *L. mindensis* DSM 14500^T (=LMG 21932^T), *L. paralimentarius* DSM 13238^T (=LMG 19152^T), *L. sanfranciscensis* DSM 20451^T, *L. spicheri* DSM 15429^T and *L. versmoldensis* LMG 21929^T (=DSM 14857^T). They were grown anaerobically on mMRS4 medium at the appropriate temperature (Stolz *et al.*, 1996). All tests were performed at 30 °C unless otherwise stated. Colony morphology, Gram staining and cellular morphology were determined on cells grown anaerobically on mMRS4 incubated for 2 days. All tests for biochemical

characterizations were carried out at least in duplicate, and as described by Valcheva *et al.* (2005).

DNA was isolated according to the method of Marmur (1961) with the modification described by Ehrmann *et al.* (2003). The isolated DNA was used for 16S rRNA gene sequence amplification and for RAPD analysis. The complete 16S rRNA gene was amplified using primers fD1 and rD1 as described by Weisburg *et al.* (1991). A clone library of the 16S rRNA gene amplified with primers fD1 and rD1 of strain LP33^T was constructed in *Escherichia coli* JM109 using the pDrive cloning kit (Qiagen) and the insert of positive clones was sequenced. Primers T7-pro and SP6 flanking the multiple cloning site of pDrive DNA were used for sequencing. Internal primers 609R, 612R, 607R, 606R and 607V were additionally used for sequence verification (Ehrmann *et al.*, 2003). The complete 16S rRNA gene sequence of LP33^T was included in the phylogenetic analysis. A phylogenetic tree was constructed according to the neighbour-joining method using BioNumerics software (Applied Maths). RAPD-PCR was carried out as described by Ehrmann *et al.* (2003). The AFLP patterns of the sourdough isolates were compared with those of the reference strains. AFLP analysis was performed according to a modification of the procedure described by Gevers *et al.* (2001). DNA–DNA relatedness analysis was carried out as described by De Ley *et al.* (1970), with the modifications described by Escara & Hutton (1980) and Huß *et al.* (1983), and was performed using the fluorometric method as described by Ezaki *et al.* (1989). AFLP and DNA–DNA hybridization experiments were carried out in duplicate by BCCMTM/LMG. Cell wall composition and DNA G + C content were determined by the DSMZ according to the methods of Schleifer & Kandler (1972) and Mesbah & Whitman (1989), respectively.

The LAB microflora of the sourdough described above was determined qualitatively and quantitatively. This sourdough had a pH of 3.65 and total titratable acidity (TTA) of 26.6 ml (10 g)⁻¹. The LAB population was 1.7 × 10⁹ c.f.u. g⁻¹. Thirty morphologically different colonies were isolated and were further characterized morphologically and physiologically. On this basis, the bacterial population was divided into three groups of Gram-positive, catalase-negative rods: (i) heterofermentative with growth at 15 °C but not at 45 °C, (ii) homofermentative with growth at 15 and 45 °C and (iii) homofermentative with growth at 15 °C but not at 45 °C. Additionally, these isolates were clustered based on RFLP analysis of 16S–23S rRNA intergenic spacer regions (data not shown). According to RFLP and phenotypic analysis, the *Lactobacillus* microflora of the sourdough was characterized as a consortium of 37 % *L. hammesii* (11 isolates), 17 % *L. plantarum* (five isolates) and 46 % unknown (14 isolates). One representative strain of each cluster was subsequently identified based on 16S rRNA gene sequencing, RAPD and AFLP analyses. The results confirmed the initially presumed phylogenetic status of the first two groups of isolates as representing *L. hammesii* and *L. plantarum*. RAPD and carbohydrate fermentation patterns of five selected

strains of the third group were identical, indicating that at least five of the 14 isolates represent the same species. Isolate LP33^T, representative of the third group, was not assignable to any recognized species and was therefore included in a polyphasic approach using RAPD and AFLP genotyping methods. Strategies combining different typing methods, e.g. SDS-PAGE of cellular proteins, RAPD-PCR and AFLP, have increasingly been used to identify closely related species and strains (Valcheva *et al.*, 2005; Gancheva *et al.*, 1999). RAPD-PCR of isolate LP33^T and 11 reference *Lactobacillus* species usually found in sourdough was performed. The dendrogram thus constructed clearly indicated the separate taxonomic position of strain LP33^T. Similarly, strain LP33^T was included in AFLP analysis. The AFLP dendrogram constructed placed strain LP33^T in a cluster together with *L. kimchii* LMG 19822^T, *L. paralimentarius* LMG 19152^T and seven isolates of *L. paralimentarius*. The RAPD and AFLP patterns are available as Supplementary Figs S1 and S2 in IJSEM Online.

The complete sequence (1561 bp) of the 16S rRNA gene sequence of LP33^T was determined. In a neighbour-joining dendrogram based on the sequence of LP33^T from this study and sequences from the GenBank database, the phylogenetic position of LP33^T was determined. LP33^T was placed within the *L. plantarum* group and was phylogenetically most closely related to *L. farciminis*, *L. alimentarius*, *L. paralimentarius*, *L. mindensis* and *L. versmoldensis* (Fig. 1). 16S rRNA gene sequence similarity between LP33^T and *L. farciminis* DSM 20184^T, *L. mindensis* DSM 14500^T and *L. paralimentarius* DSM 13238^T was 97.5, 97.4 and 96.7 %, respectively.

DNA–DNA hybridization analyses were performed, including the three most closely related species based on 16S rRNA gene sequence analysis. DNA–DNA relatedness values between LP33^T and *L. farciminis* DSM 20184^T, *L. mindensis* DSM 14500^T and *L. paralimentarius* DSM 13238^T were 27 ± 3, 27 ± 3 and 22 ± 2 %, respectively (average ± range of two values). These values are well below the threshold of 70 % suggested for species delineation (Stackebrandt & Goebel, 1994), indicating that strain LP33^T represents a

separate genomic species. The DNA G + C content of LP33^T was 38.6 mol%, which is within the range 32–55 mol% reported for *Lactobacillus* species (Kandler & Weiss, 1986). Analysis of the cell wall composition of strain LP33^T revealed the presence of lysine and aspartic acid, indicating A4α L-Lys–D-Asp peptidoglycan type.

Cells of strain LP33^T were Gram-positive, non-motile, lacked endospores and had irregular internal granulation as revealed by methylene blue staining. After incubation on MRS agar for 3 days, colonies were white, circular to slightly irregular, convex, with a smooth to rough surface and diameter of 0.8–1.5 mm. Strain LP33^T exhibited no oxidase or catalase activities. In mMRS4 at pH 6.5 and 30 °C, the specific growth rate of strain LP33^T was 0.48 ± 0.01 h⁻¹. The optimal temperature for growth was in the range 30–35 °C; the specific growth rate was only 46 and 44 % (100 % at 30 °C) at 20 and 40 °C, respectively. LP33^T grew well at initial pH values of 4.4–7.7; the specific growth rate was only 4 and 22 % (100 % at pH 6.5) at pH 3.0 and 9.2, respectively. LP33^T grew well at an NaCl content of up to 2 %; the specific growth rate was 73 and 56 % (100 % without NaCl) at 3 and 5 % NaCl, respectively. The carbohydrate fermentation patterns of strain LP33^T are given in Table 1.

On the basis of 16S rRNA gene sequence analysis, DNA–DNA reassociation values, RAPD and AFLP fingerprinting analyses as well as phenotypic characteristics, we propose that strain LP33^T be classified as the type strain of a novel *Lactobacillus* species, for which the name *Lactobacillus nantensis* is proposed.

Description of *Lactobacillus nantensis* sp. nov.

Lactobacillus nantensis (nan.ten'sis. M.L. masc. adj. *nantensis* pertaining to Nantes, from where the first strain of this species was isolated).

Cells are Gram-positive, 2–5 µm long and 1.0 µm wide, non-motile, non-spore-forming rods with irregular internal

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence analysis, giving the position of *Lactobacillus nantensis* sp. nov. DSM 16982^T (=LP33^T) among selected lactobacilli. The tree was generated by the neighbour-joining method based on a comparison of approximately 1450 nt. Bootstrap values based on 1000 replications are given at branching points. Bar, 1 % sequence divergence.

Table 1. Differential phenotypic characteristics of *Lactobacillus nantensis* sp. nov. LP33^T and closely related *Lactobacillus* species

Strains: 1, *L. nantensis* LP33^T; 2, *L. farciminis* DSM 20184^T (data from Reuter, 1983); 3, *L. alimentarius* DSM 20249^T (Reuter, 1983); 4, *L. paralimentarius* JCM 10415^T (Cai *et al.*, 1999); 5, *L. kimchii* JCM 10707^T (Yoon *et al.*, 2000); 6, *L. mindensis* DSM 14500^T (Ehrmann *et al.*, 2003). All strains are of L-Lys-D-Asp peptidoglycan type and DL-lactate configuration. +, Positive; −, negative; w, weakly positive; ND, no data.

Characteristic	1	2	3	4	5	6
DNA G + C content (mol%)	38.6	34–36	36–37	37.2–38	35	37.5
NH ₃ from arginine	−	−	−	ND	−	−
Growth at 15/45 °C	+/−	+/−	+/−	ND/−	+/−	+/−
Carbohydrate fermentations:						
L-Arabinose	−	−	−	−	+	−
D-Xylose	−	−	−	−	+	−
Ribose	w	−	+	+	−	−
Galactose	+	+	+	−	+	−
Mannitol	+	−	−	−	−	−
Methyl α-D-mannoside	+	−	−	−	−	−
Sorbitol	+	−	−	−	−	−
N-Acetylglucosamine	+	−	+	+	−	+
Amygdalin	+	−	+	+	−	w
Arbutin	+	−	+	+	−	−
Aesculin	+	+	+	+	+	−
Maltose	+	w	+	w	+	+
Lactose	+	+	−	−	−	−
Sucrose	+	+	+	+	+	−
Melibiose	+	−	−	−	−	−
Trehalose	+	w	+	+	+	−
Raffinose	+	w	−	−	−	−
Melezitose	−	−	−	−	+	−
β-Gentiobiose	+	−	−	+	+	−
D-Tagatose	+	−	−	−	−	−

granulation. They occur singly, in pairs or in chains. Colonies are small (1.5 mm), circular to slightly irregular, convex, with a smooth to rough surface and white when grown on MRS agar. Cells grow well in liquid and solid MRS under anaerobic conditions. The optimum growth temperature is 30 °C and the optimal initial pH is 4.4–7.7. Strain LP33^T is able to grow at up to 8 % NaCl. Cells are catalase- and oxidase-negative. Glucose is metabolized homofermentatively and lactate is the sole final product. Strain LP33^T produces L(+)- and D(−)-lactic acid. Acid is produced from glucose, fructose, mannose, galactose, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, β-gentiobiose, tagatose, mannitol, sorbitol, methyl α-D-mannoside, N-acetylglucosamine, amygdalin, arbutin, aesculin and salicin. Ribose is weakly fermented. Ammonia is not produced from arginine. Citrate is utilized weakly in the absence of glucose.

The type strain is LP33^T (= DSM 16982^T = CIP 108546^T = TMW 1.1265^T).

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