Lactobacillus hammesii sp. nov., isolated from French sourdough

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Twenty morphologically different strains were chosen from French wheat sourdough isolates. Cells were Gram-positive, non-spore-forming, non-motile rods. The isolates were identified using amplified-fragment length polymorphism, randomly amplified polymorphic DNA and 16S rRNA gene sequence analysis. All isolates were members of the genus *Lactobacillus*. They were identified as representing *Lactobacillus plantarum*, *Lactobacillus paralimentarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus spicheri* and *Lactobacillus sakei*. However, two isolates (LP38^T and LP39) could be clearly discriminated from recognized *Lactobacillus* species on the basis of genotyping methods. 16S rRNA gene sequence similarity and DNA–DNA relatedness data indicate that the two strains belong to a novel *Lactobacillus* species, for which the name *Lactobacillus hammesii* is proposed. The type strain is LP38^T (= DSM 16381^T = CIP 108387^T = TMW 1.1236^T).

Lactic acid bacteria (LAB), especially the genera *Lactococcus*, *Lactobacillus* and *Leuconostoc*, are the most important group of micro-organisms involved in food fermentation (Hammes & Tichaczek, 1994; Herrero *et al.*, 1996). The microbial ecology of sourdough fermentation is determined by various exogenous and endogenous parameters (Vogel *et al.*, 1999). The sourdough microflora comprises LAB and yeasts existing in symbiotic relationships (Hammes & Gänzle, 1998). 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 (Böcker *et al.*, 1990; Hammes *et al.*, 1996).

France, as with many other countries, has a long tradition in bread making. Reports concerning the LAB biodiversity observed in natural French sourdough revealed a broad spectrum of different LAB, predominant among which are

members of the genus *Lactobacillus*, e.g. *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus sanfranciscensis* (Bervas, 1991; Infantes, 1992; Onno & Roussel, 1994; Gabriel *et al.*, 1999). For the development of stable starter cultures, a thorough analysis of the typical sourdough microflora is indispensable. In this study, the biodiversity of LAB in three fermented wheat sourdoughs from different regions of France was investigated. A preliminary screening based on the observed phenotypic characteristics led to the isolation of 20 bacterial strains. In order to obtain more information concerning their taxonomic status at the species and strain level, amplified-fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) and 16S rRNA gene sequence analyses were performed. These analyses allowed the identification of almost all LAB involved in these sourdough fermentations. However, based on phenotypic and genotypic results, two *Lactobacillus* strains were not assignable to any recognized species. Consequently, we describe and classify these strains as representing a novel *Lactobacillus* species, for which we propose the name *Lactobacillus hammesii* sp. nov.

The bacterial diversity of three French wheat sourdoughs was investigated at the LMAI – ENITIAA (Nantes, France). These were firm sourdoughs maintained by back slopping or *rafraîchi* in different specific conditions for each sourdough in terms of ratio (sourdough/dough), temperature

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Lactobacillus hammesii* LP38^T is AJ632219.

RAPD-PCR and AFLP patterns of the sourdough isolates and selected lactobacilli are available as supplementary figures in IJSEM Online.

(18–26 °C) and back slopping frequency. Twenty colonies were isolated on modified MRS agar (De Man *et al.*, 1960) supplemented with 1 % (w/v) maltose and 0.5 % (w/v) fresh yeast extract prepared according to the method of Kline & Sugihara (1971). LAB were grown anaerobically at 30 °C for 48 h. The distribution of various colony forms was recorded and distribution percentages of the various isolates were determined. Pure culture was obtained by successive subculturing on identical medium. For biochemical characterizations mMRS4 medium was used (Stolz *et al.*, 1996). *Lactobacillus* reference strains were obtained from the DSMZ or from the TMW culture collection at Lehrstuhl für Technische Mikrobiologie (Freising, Germany).

DNA was isolated according to the method of Marmur (1961) with the modifications described by Ehrmann *et al.* (2003). The isolated DNA was used for AFLP, RAPD and 16S rRNA gene sequence amplification. RAPD-PCR was carried out as described by Ehrmann *et al.* (2003) and AFLP analysis was performed according to Schmidt *et al.* (2003). Data analysis was performed using the BIONUMERICS software (Applied Maths). This program recorded the normalized electrophoretic patterns of the densitometric traces, grouped the isolates by the Pearson product-moment correlation coefficient and performed UPGMA (unweighted pair group method with arithmetic averages) cluster analysis of the bands. The complete 16S rRNA gene was amplified using primers 616V and 630R according to Ehrmann *et al.* (2003). A phylogenetic tree was constructed by the neighbour-joining method using BIONUMERICS software. The cell wall composition was determined at DSMZ according to Schleifer & Kandler (1972). DNA–DNA relatedness analyses were carried out as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980), and the G+C content of the DNA was determined according to Mesbah *et al.* (1989). All experiments were carried out in duplicate by DSMZ staff.

Gram staining, cell morphology and catalase activity were examined after 24 h of incubation on MRS agar. Sugar fermentation patterns were determined using the API 50CH kit (bioMérieux) over a period of 72 h. D/L-Lactate production was measured using an enzymic kit from Microzym (Diffchamb). To determine the temperature and pH growth optima as well as salt tolerance, cultures were grown on mMRS4 medium, harvested, washed with fresh medium, inoculated (1 %) and incubated at various temperatures (15, 20, 25, 30, 35, 40 and 45 °C) and pH values (3, 4, 4.5, 5, 5.5, 6, 7 and 8). Bacterial growth was monitored by measurement of the optical density at 590 nm. Production of ammonia from arginine was determined according to the method of Abo-Elnaga & Kandler (1965). Mannitol formation from fructose was demonstrated by HPLC as described by Müller *et al.* (2001). All tests for biochemical characterizations were carried out at least in duplicate.

The LAB microfloras of three wheat sourdoughs prepared by back slopping propagation from previous batches were characterized. The total LAB count in the studied sourdoughs ranged from 1.6×10^9 to 2.1×10^9 c.f.u. g⁻¹ and the final sourdough pH was between 4.0 and 4.2. Twenty morphologically different colonies were isolated from each sourdough and these were phenotypically characterized. All cells were Gram-positive, catalase-negative, facultatively anaerobic rods. The isolates were identified using AFLP, RAPD and 16S rRNA gene sequence analysis. All were members of the genus *Lactobacillus*. They were identified as *Lactobacillus plantarum*, *Lactobacillus paralimentarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus spicheri* and *Lactobacillus sakei*. However, two isolates, LP38^T and LP39, were clearly discriminated from recognized *Lactobacillus* species. The complete sequences (1559 bp) of the 16S rRNA genes of these two strains were determined. The two sequences showed high similarity (99.7 %), suggesting that the strains belong to the same species. In a neighbour-joining dendrogram (Fig. 1) based

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence analysis showing the position of *Lactobacillus hammesii* sp. nov. strains LP38^T and LP39 among selected lactobacilli. The tree was generated by the neighbour-joining method and *Lactobacillus delbrueckii* was used as the outgroup. Bootstrap values based on 1000 replications are given at nodes. Bar, 0.01 substitutions per nucleotide position.

on 16S rRNA gene sequences obtained from this study and from the GenBank database, strains LP38^T and LP39 clearly belong to the genus *Lactobacillus*, and are positioned close to *Lactobacillus brevis*, *Lactobacillus spicheri*, *Lactobacillus zymae* and *Lactobacillus acidifarinae*, all of which have been isolated from sourdoughs (Vancanneyt *et al.*, 2005).

DNA–DNA reassociation analyses were performed including the three most closely related strains based on 16S rRNA gene sequence analysis. DNA–DNA relatedness values of LP38^T to *Lactobacillus spicheri*, *Lactobacillus zymae* and *Lactobacillus brevis* were 64.7 ± 2.5, 43.3 ± 4.3 and 18.3 ± 8.1%, respectively. These values are below the threshold of 70% suggested for species delineation (Stackebrandt & Goebel, 1994), indicating that strain LP38^T represents a separate genomic species. The DNA G + C content of LP38^T is 52.6 mol%, which is within the range (32–55 mol%) reported for *Lactobacillus* (Kandler & Weiss, 1986). Analysis of the cell wall composition of strain LP38^T revealed the presence of lysine and aspartic acid, indicating an A4α L-Lys–D-Asp peptidoglycan type. Strain LP38^T accounted for about 30% (5 × 10⁸ to 6 × 10⁸ c.f.u.) of the total LAB in investigated sourdoughs, and the rest were strains of the species *Lactobacillus plantarum*, *Lactobacillus sanfranciscensis*, *Lactobacillus paralimentarius*, *Lactobacillus spicheri* and *Lactobacillus sakei*. Physiological properties and sugar fermentation patterns of strains LP38^T and LP39 are shown in Table 1.

Müller *et al.* (2001) reported the application of RAPD-PCR for the analysis of the microflora of an industrial sourdough. When the RAPD patterns of the 20 isolates were compared to those of reference lactobacilli usually found in sourdough, strain LP38^T showed no similarity to any other isolates or reference *Lactobacillus* strains (see Supplementary Fig. A in IJSEM Online), which confirmed the 16S rRNA gene sequence analysis results. Torriani *et al.* (2001) reported discrimination between the closely related species *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus* using RAPD-PCR and AFLP. Analogously to RAPD analysis, AFLP patterns of the sourdough isolates were compared with those of reference *Lactobacillus* strains. AFLP grouped the 20 sourdough isolates into five groups identical to those obtained by RAPD (Supplementary Fig. B).

The microflora of the investigated sourdoughs showed the typical composition frequently reported in French and Italian sourdoughs, *Lactobacillus plantarum*, *Lactobacillus sanfranciscensis*, *Lactobacillus paralimentarius* and *Lactobacillus sakei* (Bervas, 1991; Infantes, 1992; Onno & Roussel, 1994; Hammes *et al.*, 1996; Gobetti *et al.*, 1994). However, isolation and identification of nine isolates as *Lactobacillus spicheri*, which has been isolated as a novel species from rice sourdough (Meroth *et al.*, 2004), represents the first isolation of this species from a wheat sourdough. Strain LP38^T exhibited no similarity to any other sourdough isolates or *Lactobacillus* reference strains using RAPD or

Table 1. Differential phenotypic characteristics of *Lactobacillus hammesii* sp. nov. strains LP38^T and LP39 and closely related *Lactobacillus* species

+, Positive; –, negative; w, weakly positive; ND, not determined. All strains produce a mixture of D- and L-lactate. Data for reference strains were taken from Hammes & Vogel (1995) (*L. brevis*) and Meroth *et al.* (2004) (*L. spicheri*).

Characteristic	<i>L. hammesii</i> sp. nov.		<i>L. brevis</i>	<i>L. spicheri</i>
	LP38 ^T	LP39	DSM 20054 ^T	DSM 15429 ^T
G + C content (mol%)	52.6	ND	46	55
Peptidoglycan type	L-Lys–D-Asp	ND	L-Lys–D-Asp	L-Lys–D-Asp
NH ₃ from arginine	–	–	+	+
Growth at 15/45 °C	+ / –	ND	+ / –	+ / –
Acid production from:				
L-Arabinose	+	+	+	–
D-Xylose	+	+	–	+
Methyl β-xyloside	+	+	–	+
Galactose	+	w	w	–
Mannose	+	–	–	–
Mannitol	w	+	w	–
N-Acetylglucosamine	+	+	–	–
Aesculin	+	w	+	–
Cellobiose	+	–	–	–
Melibiose	–	–	+	–
Trehalose	+	–	–	–
Raffinose	–	–	+	–

AFLP, and analysis of the complete 16S rRNA gene sequence revealed that the bacterium is closely related to *Lactobacillus brevis* and *Lactobacillus spicheri*. On the basis of these results we propose that strains LP38^T and LP39 be classified in the genus *Lactobacillus* as *Lactobacillus hammesii* sp. nov.

Description of *Lactobacillus hammesii* sp. nov.

Lactobacillus hammesii (ham.me.si'i. N.L. gen. n. *hammesii* of Hammes, in honour of Walter P. Hammes, a German scientist who contributed to the microbiological and technological development of wheat and rye sourdough research).

Cells are Gram-positive, catalase-negative, non-motile, non-spore-forming straight rods that occur singly (0.5 × 2–4 µm), in pairs or occasionally in short chains. Colonies on MRS agar appear white and circular with a smooth surface and edges (1–1.5 mm in diameter after 2 days of growth). Cells grow well in liquid or solid MRS under aerobic conditions. Strain LP38^T grows at 15 °C but not at 45 °C. Optimum temperature for growth is 30–35 °C and the optimal initial pH is 4.7–7.2. Specific growth rate of strain LP38^T in mMRS4 at pH 6.2 and at 30 °C is 0.42 ± 0.01 h⁻¹. Strain LP38^T is able to grow well at up to 2% NaCl; at 6.6% NaCl the specific growth rate is 26% (100% without NaCl). Glucose is metabolized heterofermentatively. Glucose, maltose, arabinose, xylose, galactose, mannose, cellobiose and trehalose are fermented by strain LP38^T. Ammonia is not produced from arginine. Fructose is used either as an energy source or as an electron acceptor and is reduced to mannitol. Both strains LP38^T and LP39 produce lactate at a ratio of 45% L-lactate to 55% D-lactate. Strain LP38^T grows significantly better in media containing electron acceptors such as fructose in addition to a carbon source (maltose or glucose). HPLC analysis revealed that strain LP38^T produces lactic acid and ethanol from glucose or maltose and, in the presence of fructose, produces lactic acid and acetic acid. Peptidoglycan structure is A4α L-Lys-D-Asp type and the DNA G+C content is 52.6 mol%.

The type strain is LP38^T (= DSM 16381^T = CIP 108387^T = TMW 1.1236^T).

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