***Lactobacillus frumenti* sp. nov., a new lactic acid bacterium isolated from rye-bran fermentations with a long fermentation period**

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Within the framework of the characterization of the microflora of an industrial sourdough fermentation, strains of *Lactobacillus amylovorus*, *Lactobacillus pontis* and two other strains were isolated which could not be associated with a valid species. These latter strains were Gram-positive, catalase-negative, non-spore-forming, non-motile rods that could be clearly differentiated from known species by 16S rDNA sequence analysis. For further characterization, the morphological, physiological (sugar fermentation, formation of DL-lactate, hydrolysis of arginine, growth temperature, CO₂ production) and chemotaxonomic (G+C content, cell wall composition, SDS-PAGE of whole-cell proteins) properties were determined. Fitting of the complete 16S rDNA sequence into alignments of such sequences, together with the subsequent phylogenetic calculations, allowed the reconstruction of a phylogenetic tree. These data showed that the two strains were phylogenetically related but formed an independent cluster distinct from their closest neighbours, *L. pontis*, *Lactobacillus panis*, *Lactobacillus oris*, *Lactobacillus vaginalis* and *Lactobacillus reuteri*. The results of DNA–DNA hybridization experiments indicated that the two isolates represent a new *Lactobacillus* species, for which the name *Lactobacillus frumenti* is proposed; the type strain of this species is DSM 13145^T (= LMG 19473^T).

Keywords: *Lactobacillus frumenti*, new species, sourdough, type II, fermentation

INTRODUCTION

Lactic acid bacteria (LAB), especially the genera *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus*, play an important role in fermentative food and feed production (Herrero *et al.*, 1996). In non-lactic fermentations, lactobacilli, in particular, contribute to a huge variety of spontaneously fermented indigenous foods and beverages as well as to biotechnological processes carried out under controlled conditions.

The fermentation of starchy substrates can be found in many countries. Numerous reports have elucidated the microbial populations involved in such fermentations, e.g. fermentation of sour cassava (Figueroa *et al.*,

1995; Giraud *et al.*, 1998; Morlon-Guyot *et al.*, 1998), ogi (Johansson *et al.*, 1995), pozol (Ampe *et al.*, 1998) and kishra (Hamad *et al.*, 1997). In Europe, cereal fermentations are mainly applied to the brewing industry, providing sourmashes, and to baking, in which sourdough plays an important role in the preparation of bread dough to improve dough machinability, breadcrumb structure, keeping properties and flavour (Salovaara, 1998). The microbial ecology of such cereal fermentations is determined by several exogenous and endogenous parameters, as reported by Vogel *et al.* (1996). Whilst a wide variety of LAB, e.g. *Lactobacillus brevis*, *Lactobacillus alimentarius*, *Lactobacillus farciminis*, *Weissella confusa* or *Enterococcus faecium*, can be found in spontaneous homemade sourdough fermentations, *Lactobacillus sanfranciscensis*, *Lactobacillus pontis* and *Lactobacillus panis* are endemic in cereal fermentations, because their competitive metabolism has adapted to this environment. Böcker *et al.* (1995) introduced a classification of industrial sourdough fermentations which

Abbreviations: chrDNA, chromosomal DNA; LAB, lactic acid bacteria; RAPD, randomly amplified polymorphic DNA.

The EMBL accession number for the 16S rDNA sequence of *Lactobacillus frumenti* strain DSM 13145^T is AJ250074.

takes the kind of propagation and the manner of preparation into consideration, resulting in typical bacterial communities. Whilst *L. sanfranciscensis* constitutes a stable element (Böcker *et al.*, 1990) in the so-called type I doughs, micro-organisms found in liquid doughs with an extended fermentation period and higher temperatures (type II) belong to the species *L. pontis*, *L. panis*, *Lactobacillus reuteri*, *Lactobacillus fermentum* and *Lactobacillus amylovorus* (Vogel *et al.*, 1999).

In this paper we report the description of a new *Lactobacillus* species from an industrial type II rye-bran fermentation and for which we propose the name *Lactobacillus frumenti*.

METHODS

Strains, medium and culture conditions. Strains were isolated from an industrial rye-bran sourdough fermentation. For cultivation, the modified MRS medium (mMRS) described by Vogel *et al.* (1994) was prepared with an additional modification whereby 90% of the water was replaced with a rye-bran extract. For the preparation of 1 l of this extract, 40 g rye bran and 2 g malted wheat meal together with 0.8 g trypsin were incubated for 24 h at 50 °C. The liquid was separated by filtration. Strains of *L. frumenti* were incubated at 40 °C in line with the fermentation temperature of the sourdough from which they were isolated. The other strains were incubated at the temperature recommended by the respective strain collection. Solid media were incubated under a modified atmosphere (N₂:CO₂ 90%:10%, v/v). Cultures were maintained at -80 °C in glycerol (a pellet of 10 ml overnight culture in 500 µl fresh medium to 500 µl glycerol). The purity of the cultures was checked microscopically and by preparing streak cultures. The two isolates that are described here as the new species *L. frumenti* sp. nov. bear the numbers of the strain collection of the Technische Mikrobiologie Weihenstephan, i.e. TMW 1.655 and TMW 1.666. Strain TMW 1.666 was deposited as the type strain of the species as LMG 19473^T and DSM 13145^T, and will be referred to as the latter in this paper. The following type strains were used as reference organisms: *Lactobacillus vaginalis* DSM 5837^T, *L. pontis* DSM 8475^T, *L. panis* DSM 6035^T, *L. reuteri* DSM 20016^T, *Lactobacillus oris* DSM 4864^T, *Lactobacillus buchneri* DSM 20057^T.

Physiological characterization. Sugar-fermentation patterns were determined by using a microtitre plate assay. The reproducibility was verified by repeated analysis using cultures grown on mMRS. All sugar solutions (2% w/w) were sterilized by filtration through a 0.2 µm filter. Cells were suspended in a medium containing (per 250 ml) 5 g peptone from casein, 1 g meat extract, 3.5 g yeast extract, 125 mg cysteine, 250 µl Tween 80, 100 mg bromocresol purple, 2.5 g sodium acetate, 250 mg citric acid (ammonium salt), 62.5 mg KH₂PO₄, 125 mg Na₂HPO₄, 100 mg MgSO₄, 5 mg MnSO₄ and 5 mg FeSO₄. The solution was autoclaved for 15 min at 121 °C. A 2 ml aliquot of an overnight culture of each strain was centrifuged and washed three times with 2 ml PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ made to 1 l with dH₂O, pH 7.4). The cell concentration was adjusted with water to an OD₅₇₈ of 1; 45 µl of this cell suspension was added to 1155 µl test medium. From this cell suspension, aliquots of 40 µl were added to each of the wells. In the first well, sugar was replaced with sterile water. To avoid evaporation and to

guarantee anaerobic conditions, the wells were covered with paraffin. The change of the indicator from purple to yellow was documented after 24, 48 and 168 h. Only definitive changes were rated as positive results. To evaluate the system, additional experiments were carried out using the API 50 CH kit (bioMérieux).

The formation of the lactate isomers in the fermented broth was determined enzymically using the DL-lactate test kit (Boehringer Mannheim).

Arginine hydrolysis was determined according to the methods described by Sharpe (1979).

Morphological characteristics. Cell morphology was studied using phase-contrast microscopy. Gram-determinations were performed using the KOH method of Gregersen (1978).

Whole-cell protein analysis. For the preparation of cell extracts, 70–80 mg (wet weight) of bacterial cells was treated as described by Vogel *et al.* (1994). Electrophoresis was performed as described by Laemmli (1970), using a 12% (v/v) SDS-polyacrylamide separating gel. Gels were run in a Mini-Protein II electrophoresis cell (Bio-Rad), Coomassie-stained and dried on a vacuum gel dryer. The protein patterns were compared visually. The LMW electrophoresis calibration kit (Amersham Pharmacia Biotech) was used as a size marker.

DNA base composition. The G+C contents (mol%) of the DNA of strains TMW 1.655 and DSM 13145^T were determined using an HPLC analytical method. The experiments were performed by the DSMZ and were carried out using the protocol previously described by Tamaoka & Komagata (1984). Wild-type lambda phage DNA was used as the standard (Mesbah *et al.*, 1989). The G+C content (mol%) was determined according to Mesbah *et al.* (1989).

Cell wall. The peptidoglycan structure of the cell wall was determined by the DSMZ.

DNA isolation. DNA was isolated according to a protocol described by Marmur (1961), with some modifications. One hour before cells were harvested, penicillin G (Sigma) was added to inhibit the synthesis of cross-linkages of the cell wall and therefore to facilitate the lysis. A wet weight of 70 mg cells was used for the DNA isolation. After the protocol, lysis was completed within 45–90 min after the addition of lysozyme and mutanolysine. For some strains, more effective lysis was obtained by using an overnight lysis at 4 °C and a subsequent proteinase K treatment at 60 °C for 1 h, before continuation with the normal protocol. The purified and vacuum-dried DNA was dissolved in 2 × SSC (0.3 M NaCl, 0.03 M Na₃ citrate, 2H₂O, pH 7.0). This DNA preparation served for the DNA–DNA hybridization experiments as well as for 16S rDNA amplification. The purity of the culture subjected to DNA isolation was proved as described above before the addition of penicillin. Furthermore, DNA preparations were checked for their authenticity in a randomly amplified polymorphic DNA (RAPD)–PCR assay (Paramithiotis *et al.*, 2000), comparing the patterns with those of primary preparations.

DNA–DNA hybridization. The determination of DNA homology values was carried out by using chromosomal DNA (chrDNA) from strain DSM 13145^T and from *L. pontis* DSM 8475^T as probes. These chrDNA probes were labelled by nick translation using a kit system (Promega) incorporating biotin-21-dUTP (Clontech) for 2 h at 16 °C. The DNA (5 µg) of selected type strains was transferred by using a dot-blot block (Stratagene) on a positively charged nylon membrane (Boehringer Mannheim) and then fixed by incubation at 80 °C for 1 h. The DNA of *L. buchneri* DSM

Table 1. Physiological and biochemical characteristics of *L. frumenti* and selected reference organisms

–, Negative reaction; +, positive reaction; ND, not determined. All strains contain DL-lactate, produce CO₂ from glucose, and produce acid from glucose, maltose and sucrose.

Characteristic	<i>L. frumenti</i> DSM 13145 [†]	<i>L. frumenti</i> TMW 1.655	<i>L. panis</i> DSM 6035 ^{†*}	<i>L. pontis</i> DSM 8475 ^{††}	<i>L. vaginalis</i> DSM 5837 ^{†‡}	<i>L. oris</i> DSM 4864 [§]	<i>L. reuteri</i> DSM 20016 [¶]
NH ₃ from arginine	+	+	–	+	+	–	+
Growth at 15 °C/45 °C	+ / –	+ / –	+ / –	+ / +	+ / –	+ / –	+ / –
Peptidoglycan type	L-Lys-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp	Orn-D-Asp	Orn-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp
DNA G + C content (mol %)	43.3 ± 0.2 [¶]	44.4 ± 0.3 [¶]	48.3 ± 0.3 [¶]	53 – 56 [¶]	38 – 41 ^{**}	49 – 51 ^{**}	40 – 42 ^{**}
Acid production from:							
DL-Arabinose	+ / –	– / –	+ / –	– / –	–	+ / –	ND / +
Ribose	+	+	+	+	–	+	+
DL-Xylose	–	–	+	–	–	+ / –	– / ND
Galactose	+	+	+	–	+	+	+
Fructose	+	+	+	+	ND	+	+
Mannose	+	+	+	–	ND	+	–
Mannitol	+	+	–	–	–	–	–
Sorbitol	+	+	ND	–	–	–	–
N-Acetylglucosamine	+	+	–	–	–	–	–
Amygdalin	+	+	–	–	ND	+	–
Arbutin	+	+	–	–	–	+	–
Aesculin	+	+	+	–	–	–	–
Salicin	+	+	+	–	–	–	–
Cellobiose	+	+	–	–	–	+	–
Lactose	+	+	+	–	+	+	+
Melibiose	+	+	+	–	+	+	+
Trehalose	+	+	–	–	–	+	–
Melezitose	+	–	–	–	–	–	–
Raffinose	+	+	+	–	+	+	+
Gluconate	+	+	–	–	–	+	+

* Data are from Wiese *et al.* (1996).

† Data are from Vogel *et al.* (1994); acid production from sugars was tested in this study.

‡ Data are from Embley *et al.* (1989).

§ Data are from Farrow & Collins (1988).

|| Data are from Kandler *et al.* (1980) and Axelsson & Lindgren (1987).

¶ Determined by the HPLC method.

** Determined by the thermal denaturation method.

20054^T was included to check the sensitivity of the system for a more distantly related *Lactobacillus* species. The hybridization procedure was carried out as described by Ehrmann *et al.* (1994), except for the hybridization and washing steps, which were performed with alternative buffers described in the protocol of Engler-Blum *et al.* (1993). To determine the accessibility of the DNA, a parallel hybridization was carried out with a 5' biotin-labelled universal probe (612R, 5'-GTAAGGTTTNCGCGT-3') targeting the 16S rDNA. For the hybridization, 100 ng chrDNA probe and 20 pmol oligo probe (each per ml hybridization solution) were used. Hybridization was performed at 65 °C for the chrDNA probe and at 42 °C for the oligo probe. The detection system consisted of a combination of alkaline phosphatase and the substrate CDP-Star (Boehringer Mannheim). Membranes were exposed to a Kodak X-Omat film (Sigma-Aldrich). The spot intensity was calculated with IMAGE MASTER 2D Elite software (Amersham Pharmacia Biotech). Calculations of homology values were performed in principle as described by Liebl *et al.* (1991).

Additional DNA–DNA hybridization experiments for *L. frumenti* DSM 13145^T, *L. frumenti* TMW 1.655 and *L. vaginalis* DSM 5837^T were performed by the DSMZ according to the protocol of De Ley *et al.* (1970), but with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). The renaturation rates were calculated according to Jahnke (1992) with the program TRANSFER: BAS.

16S rDNA amplification and sequencing. PCR-mediated amplification of the complete 16S rDNA was carried out in a Gradient Master thermocycler (Eppendorf). All reagents were from Amersham Pharmacia Biotech, unless otherwise indicated. The amplification conditions were as follows: 1 µl genomic DNA, 10 µl 10 × reaction buffer, 200 nM each of the four deoxynucleotides, 1.5 U *Taq* polymerase, 20 pmol each primer (Interactiva) (616V, 5'-AGAGTTTGATYMTGGCTCAG-3'; 630R, 5'-CAKAAAGGAGGTGATCC-3') and dH₂O to a final volume of 100 µl. The amplification conditions were as follows: 94 °C for 2 min; 30 cycles of 94 °C for 45 s, 52 °C for 1 min, 72 °C for 30 s; 94 °C for 1 min, 72 °C for 4 min. PCR products were purified by the QIAquick PCR purification kit (Qiagen) and were eluted with 60 µl elution buffer. DNA sequences were determined by the chain-termination method (Sanger *et al.*, 1977) using the Thermo Sequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Sciences) and separated on an ALF express sequencer (Amersham Pharmacia Biotech) or, alternatively, using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin Elmer) on an ABI 373 stretch sequencing system by a commercial service (SequiServe, Germany). For sequencing, the amplification primer 616V together with the internal primers 609R [5'-ACT AC(CT) (AGC)GG GTA TCT AA(GT) CC-3'], 612R [5'-GTA AGG TT(CT) T(AGCT)C GCG T-3'], 607R [5'-ACG TGT GTA GCC C-3'], 606R [5'-

T(AG)A CGG (GC)C(AG) GTG TGT ACA-3'] and 607V [5'-GGG CTA CAC ACG TGC-3'] were used.

Phylogenetic analysis. The complete 16S rDNA sequences of *L. frumenti* DSM 13145^T and TMW 1.655 were fitted into alignments of approximately 16000 homologous full and partial primary structures available in public databases (Ludwig, 1995). Distance-matrix, maximum-parsimony and maximum-likelihood methods were applied for tree reconstructions as implemented in the ARB software package (W. Ludwig & O. Strunk; <http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/>). Different data-sets varying with respect to included outgroup reference sequences as well as alignment positions were analysed.

RESULTS

Colony and cell morphology

On mMRS, the colonies of *L. frumenti* DSM 13145^T were white with regular sharp edges and, after 3 d growth, they were 1 mm in diameter. The colonies of TMW 1.655 were of a similar shape but were a little smaller and appeared whitish and transparent.

The cells of strains TMW 1.655 and *L. frumenti* DSM 13145^T were non-spore-forming, non-motile rods that occurred singly or in pairs (seldom in chains). In liquid cultures of mMRS after 24 h at 40 °C, cells of TMW 1.655 had a tendency to form nest-shaped aggregations. Depending on the period of growth, the ends of the cells were bent like hooks. The cells of *L. frumenti* DSM 13145^T were more regular in shape than those of TMW 1.655. The size of a single cell was difficult to determine, because of the heterogeneity. The cell length was between 3 and 5 µm and the diameter was 0.3 µm. The KOH test indicated Gram-positive behaviour.

Physiological and biochemical properties

The sugar patterns and further physiological and biochemical characteristics of *L. frumenti* strains and the reference organisms are listed in Table 1. Melzitose, rhamnose, methyl α-D-mannoside and L-arabinose were fermented only by strain *L. frumenti* DSM 13145^T. 5-Ketogluconate was fermented only by strain TMW 1.655.

SDS-PAGE pattern

Both strains of *L. frumenti* were included in a comparison of the SDS-PAGE pattern of the whole-cell proteins together with those of the type strains of the closest phylogenetic neighbours (Fig. 1). The protein patterns of *L. frumenti* DSM 13145^T and TMW 1.655 were very similar to each other and they could be clearly discriminated from the others.

Phylogenetic position

For both strain TMW 1.655 and *L. frumenti* DSM 13145^T, 1561 bp of the 16S rDNA were sequenced. Fig. 2 shows a phylogenetic tree based on these sequence data and reflects the phylogenetic position of

Fig. 1. SDS-PAGE protein patterns. Lanes: 2, *L. frumenti* sp. strain DSM 13145^T; 3, strain TMW 1.655; 4, *L. pontis* DSM 8475^T; 5, *L. panis* DSM 6035^T; 6, *L. reuteri* DSM 20016^T; 7, *L. oris* DSM 4864^T; and 8, *L. vaginalis* DSM 5837^T. Lanes 1 and 9: molecular mass standards (94, 67, 43, 30, 20 kDa).

Fig. 2. Phylogenetic tree demonstrating the relationship between *L. frumenti* DSM 13145^T and the most closely related lactobacilli. The tree was constructed by using a maximum-likelihood approach and was based on a dataset that included only those positions present in at least 50% of all available *Lactobacillus* 16S rRNA sequences. Bar, 10% homology divergence.

L. frumenti. The percentage similarities between *L. frumenti* and the type strains of the other species were as follows: *L. vaginalis*, 97.7%; *L. panis*, 97.6%; *L. pontis*, 97.3%; *L. oris*, 96.8%; *L. reuteri*, 94.3%. The 16S rRNA sequence of *Lactobacillus sakei* was used as an outgroup rRNA reference.

Table 2. Percentage DNA–DNA hybridization results

Strain	<i>L. frumenti</i> DSM 13145 ^T	<i>L. pontis</i> DSM 8475 ^T	<i>L. vaginalis</i> DSM 5837 ^T
<i>L. pontis</i> DSM 8475 ^T	48	100	ND
<i>L. panis</i> DSM 6035 ^T	49	43	ND
<i>L. oris</i> DSM 4864 ^T	66	29	ND
<i>L. vaginalis</i> DSM 5837 ^T	74	21	ND
<i>L. reuteri</i> DSM 20016 ^T	49	20	ND
<i>L. buchneri</i> DSM 20057 ^T	27	12	ND
<i>L. frumenti</i> DSM 13145 ^T	100	46	41*
<i>L. frumenti</i> TMW 1.655	90	33	45*

* Data were obtained by the spectrophotometric method.

DNA homology

The DNA–DNA hybridization studies were performed with chrDNA from *L. frumenti* DSM 13145^T against the DNA of the relevant type strains exhibiting a strong phylogenetic relationship to *L. frumenti*. To evaluate the hybridization system for the calculation of DNA–DNA homology values, DNA from *L. pontis* DSM 8475^T served as a probe against the same strains. Because of the high homology values between the *L. frumenti* strains and *L. vaginalis*, both strains were included in an additional spectrophotometrical DNA–DNA hybridization experiment against the latter to verify their independent position as a new species (Table 2).

DISCUSSION

Reliable identification techniques are fundamental to the unequivocal description of natural fermentation communities. Although methods that target the phenotypes of bacteria lead to an understanding of the physiological properties, for an increasing number of species, identification remains ambiguous. The 16S rDNA molecule emerged as the main target for identification purposes, reflecting the natural relationships of prokaryotes, and has improved our knowledge of the generic and suprageneric relationships among LAB. Within the framework of microbial investigations of long-term rye-bran fermentations, we isolated *Lactobacillus* strains which were identified by comparative 16S rDNA sequence analysis. They were phylogenetically most closely related to *L. vaginalis*, but formed an independent cluster.

On the basis of comparative sequence analysis and phylogenetic calculations, Schleifer & Ludwig (1995) proposed a species-specific grouping of LAB. Thus, *L. frumenti* strains clustered in the *L. reuteri* subgroup of the *Lactobacillus casei*–*Pediococcus* group, comprising only obligate heterofermentative lactobacilli, namely *L. oris*, *L. panis*, *L. pontis*, *L. vaginalis*, *L. reuteri* and *L. fermentum*. Starting from these 16S rDNA data and comparative sequence analysis, a determination of

		193	208
<i>L. frumenti</i>	5'	U A G U A U A A A G A A G G C U 3'	
<i>L. vaginalis</i>	5'	. C . . U . C U . . U . 3'	
<i>L. oris</i>	5'	. C C A A G . . . U . 3'	
<i>L. panis</i>	5'	. C U . . U . 3'	
<i>L. pontis</i>	5'	. G A . U . C U 3'	
<i>L. reuteri</i>	5'	. U C . n G G U 3'	

Fig. 3. Diagnostic region 193–208 (Brosius *et al.*, 1981) of the 16S rRNA, which shows sufficient sequence variability to differentiate *L. frumenti* from other lactic acid bacteria. Dots indicate sequence identity to the *L. frumenti* sequence.

sufficient sequence variation in the V2 region (Neefs *et al.*, 1990) of the 16S rDNA was feasible, which offered the possibility of distinguishing *L. frumenti* from other even closely related species (Fig. 3). A PCR-based system that allows *L. frumenti* to be differentiated from these and other sourdough lactobacilli has already been described by Müller *et al.* (2000). This supports the hypothesis that strains DSM 13145^T and TMW 1 655 should be separated from other members of the *L. reuteri* group.

Despite the close phylogenetic relationships of species within the *L. reuteri* group, G+C DNA contents range between 38 and 54 mol%. The closest phylogenetic neighbour of *L. frumenti* (with a 16S rDNA similarity of 97.7%) is *L. vaginalis*. This relationship is confirmed by their similar G+C content (43.3 versus 41 mol%). The biggest difference in the G+C contents was shown with *L. pontis*. In this case, no correlation with 16S rRNA homology data can be deduced.

The relatedness of *L. frumenti* and *L. vaginalis* with respect to G+C content and 16S rRNA sequence data contrasts with the chemical composition of the cell walls of these micro-organisms. *L. frumenti* has a cell wall of the peptidoglycan type [A4α (L-Lys–D-Asp)], which constitutes the main feature of species of the Cb group (Hammes & Vogel, 1995). The cell wall of *L. vaginalis* is in the same group but lysine is replaced by ornithine, which can be seen as additional proof of the status of as *L. frumenti* as an independent species.

SDS-PAGE pattern analysis of whole-cell proteins has proven to be a reliable tool for the discrimination of even closely related species (Pot *et al.*, 1993). SDS-PAGE comparisons (in which the two strains of *L. frumenti* exhibited almost identical patterns) allowed *L. frumenti* DSM 13145^T and TMW 1.655 to be clearly differentiated from the species *L. pontis*, *L. panis*, *L. oris*, *L. vaginalis* and *L. reuteri*. This, again, can be seen as a further proof of the independent status of *L. frumenti* as a new species.

The determination of DNA reassociation values constitutes a meaningful method for unequivocal species description (Stackebrandt & Goebel, 1994). In particular, when closely related species ($\geq 97\%$ rRNA homology) are inspected, the resolution power of 16S rRNA sequences is limited (Fox *et al.*, 1992), but DNA similarity can range between 10 and 100% (Stackebrandt & Goebel, 1994). The threshold value for the phylogenetic definition of a species, as proposed by Wayne *et al.* (1987), should not exceed 70%. In our case, the DNA–DNA hybridization studies clearly adhered to this principle. For *L. vaginalis* and *L. pontis*, rRNA similarity values with respect to *L. frumenti* were 97.7 and 97.3%, respectively. However, DNA–DNA homology values between *L. frumenti* and *L. vaginalis* and between *L. frumenti* and *L. pontis* amounted to 74 and 48%, respectively. The spectrophotometric technique used as a reference method for the determination of homology values for the closest relative, *L. vaginalis*, produced lower values between *L. vaginalis* and TMW 1.655 or DSM 13145^T (41 and 45%, respectively). The homology values obtained by dot-blot hybridization of *L. frumenti* against the other species were confirmed by alternative hybridization with the DNA of *L. pontis*. The value for similarity to *L. frumenti* (46%) was almost identical to that determined by hybridization of *L. frumenti* against *L. pontis* (48%). Moreover, the similarity calculated between *L. pontis* and *L. panis* was identical (43%) to the value determined by the spectrophotometric technique (Wiese *et al.*, 1996). Small differences in the homology values (up to 8%) are possible between the strains of one species and other reference organisms (see Wiese *et al.*, 1996). Summarizing the DNA hybridization results (Table 2), all species could be clearly separated from *L. frumenti*, indicating its independent status as a species.

In view of the phylogenetic evidence (16S rDNA, DNA–DNA homology) presented, the protein-pattern differences measured by SDS-PAGE and the phenotypic distinctiveness of DSM 13145^T and TMW 1.655, it is proposed that these strains belong to a new species, *L. frumenti* sp. nov.

Description of *Lactobacillus frumenti* sp. nov. (Müller, Ehrmann and Vogel)

Lactobacillus frumenti (fru.men'ti. L. gen. n. *frumenti* from cereal).

The cells are Gram-positive, non-motile, non-spore-forming rods that occur singly or in pairs (seldom in chains). Growth was observed at temperatures up to 45 °C but not at 15 °C. They are facultatively anaerobic, catalase-negative and obligately heterofermentative. The L-isomer constitutes more than 85% of the total lactic acid content. Acid is produced from L-arabinose, ribose, galactose, glucose, fructose, mannose, mannitol, sorbitol, N-acetylglucosamine, amygdalin, arbutin, aesculin (hydrolysed), salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose and gluconate. Melezitose, rhamnose, methyl α -D-mannoside and L-arabinose were fermented only by strain DSM 13145^T. 5-Ketogluconate was fermented only by strain TMW 1.655. The type strain is *Lactobacillus frumenti* DSM 13145^T (= LMG 19473^T).

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