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

Martin R. A. Müller, Matthias A. Ehrmann and Rudi F. Vogel

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 D-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 13145T (≡ LMG 19473T).

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 farcininis, 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 13145T 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 Weißenstetten, 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 and DSM 13145, and will be referred to as the latter in this paper. The following type strains were used as reference organisms: *Lactobacillus vaginalis* DSM 5837, *L. pontis* DSM 8475, *L. panis* DSM 6035, *L. reuteri* DSM 20016, *Lactobacillus oris* DSM 4864, *Lactobacillus buchneri* DSM 20057.

**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 g 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 11 with DH₂O, pH 7.4). The cell concentration was adjusted with water to an OD₅₆₅ of 1.5, 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 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 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 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 Na₂ 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 and from *L. pontis* DSM 8475 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 2128
Table 1. Physiological and biochemical characteristics of *L. frumenti* and selected reference organisms

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>L. frumenti</em> DSM 13145&lt;sup&gt;†&lt;/sup&gt;</th>
<th><em>L. frumenti</em> TMW 1.655&lt;sup&gt;•&lt;/sup&gt;</th>
<th><em>L. panis</em> DSM 6035&lt;sup&gt;•&lt;/sup&gt;</th>
<th><em>L. pontis</em> DSM 8475&lt;sup&gt;†&lt;/sup&gt;</th>
<th><em>L. vaginalis</em> DSM 5837&lt;sup&gt;†&lt;/sup&gt;</th>
<th><em>L. oris</em> DSM 4864&lt;sup&gt;‡&lt;/sup&gt;</th>
<th><em>L. reuteri</em> DSM 20016&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt; from arginine</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>DNA G + C content (mol %)</td>
<td>45.3±0.2*</td>
<td>44.4±0.3*</td>
<td>48.3±0.3*</td>
<td>53.5–56.5*</td>
<td>38–41**</td>
<td>49–51**</td>
<td>40–42**</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Arabinoose</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arbutin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Aesculin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Melezitose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Glucuronate</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

* 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<sup>†</sup> 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 primer (612R, 5′-GTAAGGTTYTNCGCGT-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<sup>†</sup>, *L. frumenti* TMW 1.655 and *L. vaginalis* DSM 5837<sup>†</sup> 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 TRANSFR: 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 Tag polymerase, 20 pmol each primer (Interactiva) (616V, 5′-AGAGTTTGATYMTGGCT-CAG-3′; 630R, 5′-CAKAAAAAGGGTGATCC-3′) and dH<sub>2</sub>O 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 for 4 cycles of 1 min. The following cycles were used with the primers 609R [5′-ACT AC(CT) (AGC)GG GTA TCT AA(GT) CC-3′], 612R [5′-GTA AGG TT(CC) T(AGCT) C GCG T-3′], 607R [5′-ACG TGT GTA GCC C-3′], 597R [5′-
T(AG)A CGG (GC)(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 datasets 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 \(\mu\)m and the diameter was 0.3 \(\mu\)m. 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 \(\alpha\)-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 *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

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

* Data were obtained by the spectrophotometric method.

DNA homology

The DNA–DNA hybridization studies were performed with chrDNA from L. frumenti DSM 13145T 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 8475T 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. 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 sufficient sequence variation in the V2 region (Neeffs 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 13145T and TMW 1655 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 [A4(4)-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.

**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.
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 13145T 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 (≥ 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 13145T (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 13145T 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-sporulating 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. Melizitose, rhamnose, methyl β-D-mannoside and L-arabinose were fermented only by strain DSM 13145T. 5-Ketogluconate was fermented only by strain TMW 1.655. The type strain is Lactobacillus frumenti DSM 13145T (= LMG 19473T).

ACKNOWLEDGEMENTS

This work was supported by a grant from the European Union (FAIR project CT 96 1126) and by Ernst Böcker GmbH & Co. KG (Minden, Germany). We thank Dr Wolfgang Ludwig for the phylogenetic calculations and the reconstruction of the phylogenetic tree. We also wish to thank Jürgen Behr for performing all the physiological experiments.

REFERENCES


