Lactobacillus paucivorans sp. nov., isolated from a brewery environment

Matthias A. Ehrmann,¹ Patrick Preissler,¹ Markus Danne²
and Rudi F. Vogel¹

Correspondence

Matthias A. Ehrmann
M.Ehrmann@wzw.tum.de

¹Lehrstuhl für Technische Mikrobiologie, Technische Universität München,
Weihenstephaner Steig 16, 85350 Freising, Germany

²Brauerei C. & A. Veltins GmbH & Co. KG, An der Streue, 59872 Meschede-Grevenstein, Germany

A Gram-stain-positive, catalase-negative and rod-shaped bacterium was isolated from a brewery environment. Its phylogenetic affiliation was determined by using 16S rRNA gene sequence analysis. It was found that strain TMW 1.1424^T belongs to the genus *Lactobacillus*, with the three nearest neighbours *Lactobacillus parabrevis* LMG 11984^T (97%), *Lactobacillus brevis* DSM 20054^T (95.9%) and *Lactobacillus hammesii* DSM 16381^T (96.2%). Comparative sequencing of additional phylogenetic marker genes *tuf* and *pheS* confirmed the 16S rRNA gene sequence tree topology. The DNA G+C content of strain TMW 1.1424^T is 46.6 mol%. Genomic DNA–DNA relatedness values with *L. brevis* DSM 20054^T, *L. parabrevis* LMG 11984^T and *L. hammesii* DSM 16381^T do not exceed 52.8%, revealing that the novel isolate represents a separate genomic species. The strain can be distinguished from other related species of the genus *Lactobacillus* by physiological and biochemical tests. Based on biochemical, physiological and phylogenetic data, it is proposed that the new isolate be classified as a novel species of the genus *Lactobacillus*, *Lactobacillus paucivorans* sp. nov. The type strain is TMW 1.1424^T (=DSM 22467^T =LMG 25291^T).

The genus *Lactobacillus* represents a large group within the Gram-positive bacteria with over 140 species with validly described names at the time of writing. The high diversity and species number results from association with a wide range of different environments. Lactobacilli can be found on plants, associated with humans or animals or in man-made habitats such as food and feed fermentations. Typically, these ecological niches are rich in nutrients, enabling high growth rates for many species that may be regarded as generalists. Other such niches are characterized by clearly defined conditions selective for only a few highly adapted species.

A unique environment for a small number of specialized species of the genus *Lactobacillus* is found in breweries. Besides raw materials and process equipment, they even occur in storage tanks as well as in the finished product, where growth conditions are limited by low oxygen

content, low pH and antibacterial effects of hop bitter acids and alcohol (Back, 2005; Suzuki *et al.*, 2006). Known species with significant beer-spoilage ability include *Lactobacillus brevis*, *Lactobacillus lindneri*, *Lactobacillus paracollinoides* and *Pediococcus damnosus*. Less common beer-spoiling bacteria are *Lactobacillus buchneri*, *L. casei*, *L. coryniformis*, *L. curvatus* and *L. plantarum* (Priest, 1996). Recently, few newly discovered species of beer-associated lactobacilli have been described. An obligate beer-spoiling species, '*Lactobacillus backi*', was typically found in lager, pils and wheat beers (Bohak *et al.*, 2006). The brewery environment offers niches allowing for adaptation of lactic acid bacteria to grow in beer (Behr & Vogel, 2009). During routine hygiene monitoring, we isolated a strain that was not allocable to any known species. In this study, we report on the taxonomy of the novel strain isolated from a storage tank. The isolate is designated strain TMW 1.1424^T.

For enrichment and isolation of strain TMW 1.1424^T we used 3-fold concentrated MRS-broth (Oxoid CM359) supplemented with 30 g D-fructose l⁻¹ and 1.5 g L-cysteine hydrochloride monohydrate l⁻¹. Pure cultures were routinely grown in modified MRS medium (mMRS) composed of 10 g tryptone l⁻¹, 2 g meat extract l⁻¹, 7 g yeast extract l⁻¹, 10 g fructose l⁻¹, 10 g xylose l⁻¹, 2 g sodium gluconate l⁻¹, 5 g sodium acetate trihydrate l⁻¹, 2 g citric acid (diammonium salt) l⁻¹, 2.5 g KH₂PO₄·3H₂O l⁻¹,

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, *tuf* and *pheS* gene sequences of strain TMW 1.1424^T are FN185731–FN185733, respectively. The accession numbers for the *tuf* gene sequences of *Lactobacillus parabrevis* 11948^T, *L. hammesii* LMG 23074^T, *L. nantensis* DSM 16982^T and *L. crustorum* LMG 23699^T are FN395008–FN395011, respectively.

Maximum-likelihood and maximum-parsimony trees derived from 16S rRNA gene sequence analysis are available with the online version of this paper.

0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ l^{-1} , 0.1 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ l^{-1} , 0.5 g cysteine hydrochloride l^{-1} , 0.05 g $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ l^{-1} and 1 g Tween 80 l^{-1} . The pH was adjusted to 5.4. Solid media contained agar (15 g l^{-1}).

Cell morphology and Gram staining of one- to two-day-old broth cultures of strain TMW 1.1428^T were examined by phase-contrast and normal light microscopy, respectively, at $\times 1200$ magnification. Cells were Gram-stain-positive, short, straight rods and sometimes slightly curved, occurring singly, in pairs or in short chains. Opaque colonies of varying size appeared in two forms: circular, convex and smooth edged, or roughly textured with fringed edges.

Physiology

Catalase activity was tested by addition of 8 M H_2O_2 to cells. Lack of bubble formation indicated that strain TMW 1.1428^T is catalase-negative. Ability to grow at different temperatures (15, 30, 37 and 45 °C) was determined in mMRS broth incubated under anaerobic atmosphere without agitation. Optimal growth was observed at 30 °C. To investigate the effect of NaCl on bacterial growth, cells were inoculated at 30 °C in mMRS broth supplemented with 4, 6, 8 and 10 % (w/v) NaCl in anaerobic atmosphere. Growth at 8 % NaCl was one quarter of growth at 5 % NaCl, and almost no growth was observed at 10 % NaCl. NH_3 production from arginine was not observed when determined as described by Bover-Cid & Holzapfel (1999). L-Ornithine and L-lysine were decarboxylated.

To discriminate homofermentative from heterofermentative pathway gas production from glucose, fructose, gluconate and xylose (20 g l^{-1}), mMRS broth with Durham tubes was used. Glucose was generally not fermented by strain TMW 1.1424^T. Fructose and xylose were fermented without gas production. Gluconate was not fermented.

The enantiomer of the lactic acid produced was determined enzymically (Boehringer Mannheim). D-Lactic acid and L-lactic acid are produced in a ratio of 4:1.

Carbohydrate fermentation of additional sugars was determined by using API 50 CHL strips with API 50 CHL medium (bioMérieux). Strain TMW 1.1424^T produced acid solely from D-fructose, D-ribose and D-xylose. Characteristics that differentiate strain TMW 1.1424^T from other members of the *L. brevis* clade of lactobacilli are summarized in Table 1.

Fructose was simultaneously used as carbon source as well as electron acceptor to produce mannitol. Mannitol formation was detected by HPLC using a Metacarb 87P column (Varian) with a refractive index detector as described by Korakli *et al.* (2000).

Resistance to isomerized hop extract is considered as a pivotal feature of beer-spoiling lactic acid bacteria (Simpson & Fernandez, 1992). As strain TMW 1.1424^T was isolated

Table 1. Physiological characteristics of the novel strain TMW 1.1424^T useful in differentiating it from its nearest phylogenetic relatives

Strains: 1, *L. paucivorans* sp. nov. TMW 1.1424^T; 2, *L. brevis* DSM 20054^T (data from Valcheva *et al.*, 2005); 3, *L. parabrevis* LMG 11984^T (Vancanneyt *et al.*, 2006); 4, *L. hammesii* DSM 16381^T (Valcheva *et al.*, 2005). All strains were negative for acid production from D-mannose, cellobiose, trehalose, raffinose and sucrose. All strains were positive for acid production from D-ribose. +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3	4
Acid produced from:				
L-Arabinose	−	+	+	+
Maltose	−	+	+	+
D-Fructose	+	+	+	+
Melibiose	−	+	−	−
Gluconate	−	+	+	w
D-Xylose	+	−	+	+
Hydrolysis of aesculin	−	−	−	+
DNA G + C content (mol %)	46.6	46	49	52.6

from beer, its hop tolerance was evaluated by determining minimum inhibitory concentrations (MIC) of hop iso- α -acid as previously described (Behr & Vogel, 2009). Briefly, cells were precultured in modified MRS broth (pH 4.3) with reduced manganese (0.16 mg l^{-1}) and magnesium (98 mg l^{-1}) content at 30 °C for 4 days. Neither glucose nor cysteine were added.

The MIC test was performed in a 96-well microtitre plate. Five μl precultured cells (OD_{590} of 2) were used to inoculate the test volume of 200 μl . The concentration of iso- α -acid was increased gradually from 5 p.p.m. up to a concentration of 35 p.p.m. All experiments were performed in duplicate. After 48 h at 30 °C, visible growth was detectable only at 5 p.p.m. hop iso- α -acid (data not shown). This indicates a rather low beer-spoilage potential as compared to obligate beer-spoiling strains of *L. brevis* that are able to tolerate up to 11.6 p.p.m. (Behr & Vogel, 2009).

Metabolic activity in beer

Stationary phase cells were washed twice with 50 mM phosphate buffer (pH 6.8) and dissolved to an OD_{590} of 2 in beer (pilsner). Five microlitres of the cell suspension was mixed with 200 μl beer in a 96-well microtitre plate and overlaid with 100 μl paraffin oil. *L. brevis* TMW 1.313 (high spoilage potential) and TMW 1.6 (no spoilage potential) served as positive and negative controls, respectively. Active metabolism was monitored after incubation for 48 h at 30 °C by addition of 10 μl resazurin (0.01 mM in 500 mM Tris, pH 8.8) as indicator. After one hour of incubation, change of absorbance (decoloration of resazurin) was measured spectrophotometrically at 590 nm by using a Tecan plate reader. No activity was detected for strain TMW 1.1424^T.

Hop tolerance of beer-spoiling lactic acid bacteria is suggested to be a multi-gene trait (Fujii *et al.*, 2005). Thus, several genetic markers differentiating beer-spoilage ability were established. The *horA* and *horC* genes encoding multidrug transporters (Sakamoto *et al.*, 2001; Iijima *et al.*, 2006), as well as *hitA* encoding a divalent cation transporter (Hayashi *et al.*, 2001), have been reported to correlate strongly with hop tolerance.

A PCR screening with primers specific for *horA* (for: 5'-ATGCAAGCTCAGTCCAA-3'; rev: 5'-TCACCCGTTGCTCGTCGC-3') and *horC* (for: 5'-ATGTTTCGATGTAATTCGT-3'; rev: 5'-TTATTTAATTTTGCAGTG-3') verified the presence of both genes, whereas *hitA* (for: 5'-ATGAAAGAGGGTATTGAT-3'; rev: 5'-TTAACCAATCAGCCAAC-3') was not detected.

Phylogeny

The phylogenetic position of strain TMW 1.1424^T was determined by comparative sequencing of three phylogenetic marker genes (16S rRNA gene, *tuf* gene for elongation factor Tu and *pheS* gene for phenylalanyl-tRNA synthase alpha subunit). DNA was isolated with an E.N.Z.A. bacterial DNA kit (Omega Bio-Tek) according to the manufacturer's instructions. The complete 16S rRNA gene was amplified with primers 616V (5'-AGAGTTTGATYMTG-GCTCAG-3') and 630R (5'-CAKAAAGGAGGTGATCC-3'). PCR products were purified by using the QIAquick PCR purification kit (Qiagen) and were eluted with 60 µl elution buffer. DNA sequences were determined by the chain-termination method using the ABI Prism Dye Terminator Cycle Sequencing kit. The almost complete 16S rRNA gene sequence of 1513 bp (EMBL accession number FN185731) was compared with those of the most closely related species retrieved from GenBank. Levels of sequence similarity were found to be 97 % with *Lactobacillus parabrevis* LMG 11984^T, 95.9 % with *L. brevis* DSM 20054^T and 96.2 % with *Lactobacillus hammesii* DSM 16381^T. A 16S rRNA gene sequence similarity lower than 98.7 % has been determined sufficient evidence that

organisms belong to different species (Stackebrandt & Ebers, 2006).

A phylogenetic tree on the basis of a multiple alignment-based similarity matrix was reconstructed by the neighbour-joining method (Saitou & Nei, 1987) using the software package Bionumerics (Applied Maths). Unknown bases were discarded from the analyses. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree by using 100 bootstrap resamplings of the data (Fig. 1).

Additional neighbour-joining trees were reconstructed with *tuf* and *pheS* gene sequences (Fig. 2). The *tuf* gene is highly conserved in the bacterial genome. The amino acid sequence of this gene differs by no more than 27 % between even the most divergent eubacterial species (Lathe & Bork, 2001). For sequence analysis, *tuf* was partly (1041 bp) amplified with oligonucleotides complementary to conserved regions of other bacterial *tuf* genes. The forward primer was 5'-CCAYGWAAAYATYGGTAC-3' and the reverse primer was 5'-GTTTRTCRCCAGGCATAAC-3'. Annealing temperature was optimal at 56 °C. A 965 bp region was successfully sequenced (EMBL accession number FN185732). Sequence comparison revealed similarities of 94 % to *L. hammesii* DSM 16381^T and 92 % to *L. parabrevis* LMG 11984^T.

In addition to sequencing of the *tuf* gene, sequence analysis of the *pheS* gene, which has previously been proven to be a robust phylogenetic marker for lactic acid bacteria (Naser *et al.*, 2005, 2007), was performed. As published primers did not result in any PCR products, two new primers were designed on the basis of an alignment of available *pheS* genes from other lactobacilli: *phe_forward*, 5'-CASGAT-ACSTTCTACATYAC-3' and *phe_reverse*, 5'-ACCATAC-CRGCACCYARMACTTC-3'. Annealing temperature was optimal at 54 °C. A 361 bp amplicon of *pheS* of strain TMW 1.1424^T was sequenced (EMBL accession number FN185733). Phylogenetic neighbourhood trees of both genes (*tuf* and *pheS*) confirmed the position of strain TMW 1.1424^T determined using the 16S rRNA gene sequence.

Fig. 1. Neighbour-joining tree derived from 16S rRNA gene sequence analysis showing the relationship of the novel strain TMW 1.1424^T to related members of the genus *Lactobacillus*. The *L. buchneri*/*L. parabuchneri* clade was used as an outgroup. Approximately 1500 nt from each sequence was used for alignment. Bar, 1 % estimated sequence divergence. Numbers indicate bootstrap values greater than 50 % (percentage of 100 replications).

devouring few substrates, relating to the observation that the organism utilizes few carbohydrates).

Cells are Gram-stain-positive, catalase-negative, non-spore-forming and non-motile rods (1.5–5 µm in length and 0.9 µm wide), occurring singly, in pairs or in short chains. After 48 h, colonies are approximately 1–2 mm in diameter, whitish, circular, convex, smooth-edged or roughly textured with fringed edges. Grows at 15 °C, but not at 45 °C. Grows at 5 % NaCl but not at 10 % NaCl. Grows anaerobically and produces DL-lactic acid. Glucose is not fermented. Fructose is fermented without gas production. Gluconate is not fermented. Produces acid from D-fructose, D-ribose and D-xylose. Weak acid production is observable after 48 h from melezitose, β-gentiobiose and D-tagatose. No acid is produced from D- and L-arabinose, D- and L-arabitol, gluconate, N-acetylglucosamine, D-glucose, maltose, methyl β-xyloside, adonitol, amygdalin, arbutin, cellobiose, dulcitol, aesculin, erythritol, D- and L-fucose, 2- and 5-ketogluconate, glycerol, glycogen, inositol, inulin, D-lyxose, mannitol, D-mannose, methyl α-D-mannoside, melibiose, raffinose, rhamnose, sucrose, salicin, starch, sorbitol, L-sorbose, trehalose, turanose, xylitol, L-xylose, galactose, methyl α-D-glucoside or lactose. Arginine is not deaminated. The cell wall does not contain meso-diaminopimelic acid. The G + C content of DNA of the type strain is 46.4 mol%.

The type strain, TMW 1.1424^T (=DSM 22467^T =LMG 25291^T) was isolated from a storage tank of a brewery.

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