Lactobacillus secaliphilus sp. nov., isolated from type II sourdough fermentation

Matthias A. Ehrmann,¹ Markus Brandt,² Peter Stolz,² Rudi F. Vogel¹ and Maher Korakli¹

Correspondence

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

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

²Ernst BÖCKER GmbH & Co. KG Postfach 21, D-32378 Minden, Germany

Two strains of Gram-positive, catalase-negative, lactic acid bacteria, strains TMW 1.1309^T and TMW 1.1313, were isolated at an interval of several years from an industrial type II sourdough. They occurred at cell numbers of 8×10^8 c.f.u. g⁻¹ and therefore were considered to be one of the dominant members of the microbiota in this type of fermentation. Cells of both strains grow exclusively on modified MRS containing trypsin-digested rye-bran extract. Both strains possessed identical 16S rRNA gene sequences, but could be discriminated by RAPD fingerprints. Comparative 16S rRNA and *tuf* gene sequence analyses positioned strain TMW 1.1309^T as part of the *Lactobacillus reuteri* phylogenetic group within the genus *Lactobacillus*. The 16S rRNA gene sequence similarities to the closest related species, *Lactobacillus coleohominis* and *Lactobacillus ingluviei* were 97.1 and 95.4 %, respectively. The DNA G + C content of strain TMW 1.1309^T was 48 mol%. Growth characteristics, biochemical features and DNA–DNA hybridization values below 70 % with all the nearest neighbours demonstrated that the isolates represent a novel *Lactobacillus* species. The name *Lactobacillus secaliphilus* sp. nov. is proposed for the novel isolates, with the type strain TMW 1.1309^T (= DSM 17896^T = CCUG 53218^T).

Sourdough is a mixture of flour and water that is fermented by lactic acid bacteria and used to improve the bakeability of rye and to ameliorate the dough properties, bread texture and flavour in doughs from other cereals (Corsetti *et al.*, 1998; Hammes & Gänzle, 1998; Rosenquist & Hansen, 1998; Vogel *et al.*, 1999). The typical bacterial microbiota of sourdough consists of various species of lactobacilli (Ehrmann & Vogel, 2005). Its composition is highly dependent on physical fermentation parameters as well as the type of raw materials. Based on common principles used in artisanal and industrial processes, sourdoughs are classified as type I, II and III sourdoughs (Meroth *et al.*, 2003).

Abbreviation: RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TMW 1.1309^T and TMW 1.1313 are AM279150 and AM411002, respectively. The GenBank/EMBL/DDBJ accession numbers for the *tuf* gene sequences of *L. mucosae* DSM 13345^T, *L. coleohominis* CCUG 44007^T, *L. secaliphilus* DSM 17896^T, *L. ingluviei* DSM15946^T, *L. cellobiosus* DSM 20055^T, *L. pontis* DSM 8475^T, *L. frumenti* DSM 13145^T, *L. panis* DSM 6035^T and *L. oris* DSM 4864^T are AM295057–AM295065, respectively.

Additional phylogenetic trees based on maximum-likelihood and maximum-parsimony analyses of the 16S rRNA gene sequence of strain TMW 1.1309^T are available as supplementary figures in IJSEM Online.

Type I sourdoughs are characterized by continuous daily propagation at ambient temperatures of 20–30 °C resulting in a distinct tendency to a relatively high biodiversity. The predominant lactic acid bacterium in type I sourdoughs is *Lactobacillus sanfranciscensis*, but other species of the genera *Weissella*, *Lactococcus*, *Leuconostoc* and *Enterococcus* are also found (Rocha & Malcata, 1999; Corsetti *et al.*, 2001, 2003; De Vuyst *et al.*, 2002; Ehrmann *et al.*, 2003; Meroth *et al.*, 2003; De Vuyst & Neysens, 2005).

The industrialization of the baking process for rye bread has led to the development of type II sourdoughs, which serve mainly as dough acidifiers. Type II sourdoughs are fermented for long periods (up to 5 days) at temperatures of up to 50 °C and have a high water content. The species established in this environment are less numerous, differ from type I organisms and are highly adapted to the selective fermentation conditions. Key organisms of type II doughs include *Lactobacillus pontis*, *Lactobacillus amylovorus*, *Lactobacillus frumenti* and *Lactobacillus panis* (Vogel *et al.*, 1994; Müller *et al.*, 2000a, b). These cereal-associated lactobacilli are characterized by their strong thermotolerance and remarkable acid tolerance.

In the course of a study to comprehensively describe these microbiota, cultivation of micro-organisms was achieved using adaptations to standard media and growth conditions.

To allow as many micro-organisms to grow as possible, we routinely used MRS that was enriched with cereal components, e.g. rye bran and malted wheat flour. Modified MRS (mMRS) medium was MRS supplemented with an aqueous extract of trypsin-digested rye-bran as described by Vogel *et al.* (1994). This rye-bran extract replaced 90 % of the water of standard MRS. The rye-bran extract was prepared by incubation (24 h at 50 °C) of 40 g rye bran l⁻¹, 2 g malted wheat meal l⁻¹ and 0.8 g trypsin l⁻¹ (Sigma-Aldrich) in water. The resulting turbid liquid was clarified by centrifugation (5000 g, 15 min) and fluted paper filtration. Plated samples of a type II sourdough were incubated at 40 °C under a modified atmosphere (90 % N₂/10 % CO₂, v/v). This strategy enabled us to isolate two novel strains, TMW 1.1309^T and TMW 1.1313, which occurred at high cell numbers of 8 × 10⁸ c.f.u. g⁻¹ and were apparently some of the dominant members of the microbiota in this type of fermentation. Strain TMW 1.1309^T was isolated several years before strain TMW 1.1313, but the fermentation conditions had remained unchanged.

Colonies of strains TMW 1.1309^T and TMW 1.1313 appeared after 2–3 days of incubation and were typically small (0.5–1 mm in diameter), flat and non-pigmented. This type of colony constituted about 10–20 % of the total bacterial cell count in this fermentation. Other colonies were identified as *L. amylovorus*, *L. pontis* and *L. frumenti*.

Strains TMW 1.1309^T and TMW 1.1313 were routinely cultivated on mMRS at 40 °C. The physiology, chemotaxonomy and phylogenetic positions of the novel isolates were analysed as described below.

Cell morphology was observed by phase-contrast microscopy with a light microscope (Zeiss) at × 1000 with cells grown for 3 days at 40 °C on mMRS agar. Strains TMW 1.1309^T and TMW 1.1313 subcultured on mMRS were able to grow at 35–45 °C, but not at 20 °C or above 45 °C. No growth was observed on MRS or on Columbia blood agar. Both novel strains tolerate up to 30 g NaCl l⁻¹. Both strains are obligately heterofermentative and 94 ± 1.5 % of total lactic acid produced is of the L configuration. Additional results of morphological, chemotaxonomical and physiological analyses are given in Table 1 and in the species description.

A sugar fermentation profile was determined using API 50 CHL galleries (bioMérieux) with some minor modifications. API inoculation medium was enriched by up to 40 % with trypsin-digested rye-bran to allow growth of the strains. The pH was adjusted to 6.0. All tests were performed in duplicate. The formation of lactate isomers was determined enzymically using the DL-lactate test kit (Roche Diagnostics). Both strains produced acid from ribose. Strain TMW 1.1313 produced acid from sucrose.

The DNA G + C content was determined for strain TMW 1.1309^T by HPLC analysis by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) following the protocol previously described by Tamaoka & Komagata (1984). Wild-type λ phage DNA was used as a standard (Mesbah *et al.*, 1989). The peptidoglycan structure of the cell wall was determined by the DSMZ. The DNA G + C content of strain TMW 1.1309^T was 48 mol%, which is within the range of 32–55 mol% previously reported for

Table 1. Physiological characteristics that differentiate strain TMW 1.1309^T and phylogenetic relatives of the genus *Lactobacillus*

Strains: 1, strain TMW 1.1309^T; 2, *L. coleohominis* DSM 14060^T (data from Nikolaitchouk *et al.*, 2001); 3, *L. ingluviei* DSM 15946^T (Baele *et al.*, 2003); 4, *L. thermotolerans* DSM 14792 (Niamsup *et al.*, 2003; Felis *et al.* 2006). +, Positive; –, negative; ND, not determined; v, variable, dependent on strain; w, weakly positive; mDpm, *meso*-diaminopimelic acid.

Characteristic	1	2	3	4
Acid produced from:				
L-Arabinose	–	–	+	+
D-Fructose	–	ND	+ / –	+
Gluconate	–	ND	w	+
D-Glucose	–	+	– / +	+
D-Maltose	–	+	+ / –	ND
Melibiose	–	–	+	+
D-Raffinose	–	–	+ / –	+
D-Ribose	+	+	+	+
Sucrose	v	–	+	–
Lactic acid isomer	DL*	DL	ND	DL
Cell wall peptidoglycan	A4α L-Lys-D-Asp	A1γ, mDpm	ND	no mDpm
DNA G + C content (mol%)	48	ND	49	50.5

*95 % of total lactic acid is of the L configuration.

Lactobacillus (Kandler & Weiss, 1986). Analysis of the cell-wall composition of strain TMW 1.1309^T revealed the presence of lysine and aspartic acid, indicating the presence of the A4 α L-Lys-D-Asp peptidoglycan type.

To determine the phylogenetic position of strains TMW 1.1309^T and TMW 1.1313, the 16S rRNA genes were sequenced over a continuous stretch of 1551 bp. Both sequences were identical. DNA was isolated according to a protocol described by Marmur (1961), except that lysis was performed overnight at 4 °C. Amplification and sequencing of the 16S rRNA gene was carried as described by Müller *et al.* (2000a). The primer-binding sites of primers 616V and 630R were at positions 8–27 and 1593–1608 of the 16S rRNA gene sequence according to the *Escherichia coli* numbering system (Brosius *et al.*, 1981). PCR products were purified by the QIAquick PCR purification kit (Qiagen) and were eluted with 60 μ l elution buffer. DNA sequences were determined by the chain-termination method using the ABI Prism Dye Terminator Cycle sequencing kit (Perkin Elmer) on an ABI 373 stretch-sequencing system. The 16S rRNA gene sequences for the novel isolates (1518 bp) and sequences of reference strains retrieved from EMBL were aligned and a phylogenetic tree was constructed by the neighbour-joining method using the BioNumerics version 3.50 software package (Applied Maths). Unknown bases were discarded for the analyses. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 500 bootstrap resamplings of the data (Fig. 1). Alternative treeing methods (maximum-parsimony and maximum-likelihood) were also applied (see Supplementary Figs S1 and S2 available in IJSEM Online).

The highest gene sequence similarities, of 97.1 and 95.4 %, were obtained to the sequences of *Lactobacillus coleohominis* (GenBank accession no. AJ292530) and *Lactobacillus ingluviei* (AF333975), respectively (Fig. 1). These values clearly indicate that strain TMW 1.1309^T belongs to the *reuteri* group of the genus *Lactobacillus*. A value of 95.0 %

gene sequence similarity was found between the novel strains and *Lactobacillus thermotolerans*. It was recently proposed that this species is a later synonym of *L. ingluviei* on the basis of its high genetic similarity (Felis *et al.*, 2006). Lower sequence similarities (<95 %) were found with all other recognized species of the genus *Lactobacillus*. The use of additional treeing methods (maximum-parsimony and maximum-likelihood) resulted in essentially similar phylogenetic positions (see Supplementary Figs S1 and S2 in IJSEM Online).

Simultaneous comparisons of additional molecular markers from throughout the bacterial chromosome can achieve a higher resolution at the species level than 16S rRNA gene sequence data alone (Gevers *et al.*, 2005). Even though the case for isolate TMW 1.1309^T representing a separate species was clear on the basis of its 16S rRNA gene sequence, we followed the trend towards multilocus sequence analyses and also included partial sequences for the elongation factor Tu in the phylogenetic analyses for a more robust classification at the species level. The *tuf* universal primers used to amplify a target region of 803 bp were those previously described by Ke *et al.* (1999). A 735-bp portion of the *tuf* gene of strain TMW 1.1309^T was sequenced (GenBank accession no. AM295059) and phylogenetic analysis was performed as described for the 16S rRNA gene sequence data.

The topology obtained with the *tuf* gene sequences was not identical in every case with that based on the 16S rRNA gene sequence data (Fig. 2), but the tree obtained confirmed the separate species status of strain TMW 1.1309^T. As found in the 16S rRNA gene sequence analysis, the closest relative based on *tuf* sequences is *L. coleohominis* (90.78 % sequence similarity). In contrast to the topology based on the 16S rRNA gene sequence, *L. panis* and *L. frumenti* were the second closest relatives, each showing 88.3 % gene sequence similarity. As expected, the *tuf* gene sequences were more discriminatory than the 16S rRNA gene sequences.

Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequence analysis showing the relationship of *L. secaliphilus* sp. nov. to members of the *Lactobacillus reuteri* subgroup of lactobacilli. The sequence of *L. sanfranciscensis* was used as an outgroup. Approximately 1500 nt from each sequence were used for the alignment. Numbers indicate bootstrap values >50 % (percentage of 500 replications). Bar, 1 % estimated sequence divergence.

Fig. 2. Neighbour-joining phylogenetic tree obtained with partial sequences of the elongation factor Tu-encoding *tuf* gene showing the relationship of *L. secaliphilus* sp. nov. to members of the *Lactobacillus reuteri* subgroup of lactobacilli. The sequence of *L. brevis* was used as an outgroup. Approximately 735 nt from each sequence were used for the alignment. Numbers indicate bootstrap values >50 % (percentage of 500 replications). Bar, 2 % estimated sequence divergence.

In order to differentiate the two novel isolates, TMW 1.1309^T and TMW 1.1313, a randomly amplified polymorphic DNA analysis (RAPD) was performed with the M13V primer (5'-GTTTTCACGTCACGAC-3') as described previously (Ehrmann *et al.*, 2003). Strains TMW 1.1309^T and TMW 1.1313 showed distinguishable fingerprints that demonstrated their genotypic differences (Fig. 3). DNAs of other species of lactobacilli showed quite different patterns.

DNA–DNA relatedness values were determined by using chromosomal DNA of strain TMW 1.1309^T and its closest neighbours based on 16S rRNA gene sequence analysis, *L. coleohominis* and *L. ingluviei*. Renaturation kinetics were performed by the DSMZ following the protocol of De Ley *et al.* (1970), with modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). Experiments were performed in duplicate.

DNA–DNA relatedness values of strain TMW 1.1309^T with *L. coleohominis* DSM 14060^T and *L. ingluviei* DSM 15946^T were 50.4 and 23.7 %, respectively. These values are far below the 70 % threshold repeatedly recommended as the lowest value for isolates representing the same species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001).

Taken together, the phenotypic and genotypic data presented here confirm that strains TMW 1.1309^T and TMW 1.1313 represent a novel species, for which we propose the name *Lactobacillus secaliphilus*. The name reflects the need for rye extract to be added to the culture media to achieve growth of the novel species.

As a concluding remark, it may be mentioned that lactobacilli belonging to the phylogenetically defined *Lactobacillus reuteri* group are either found in association with the intestinal tracts and mucous membranes of humans (e.g. *Lactobacillus vaginalis*, *Lactobacillus anstrumi*, *Lactobacillus gastricus*, *Lactobacillus oris*, *L. coleohominis* and *Lactobacillus mucosae*) and many animals (e.g. *L. ingluviei* and *L. thermotolerans*) or in fermented cereals (*L. pontis*, *L. panis*, *L. frumenti* and *L. secaliphilus* sp. nov.). Only one species of the *L. reuteri* group has been found so far that is

native to both of these environments. The prevailing conditions in these environments resemble each other in terms of similar temperatures, low pH and anaerobiosis and these conditions may lead to the selection of organisms of a closely defined phylogenetic group with similar physiological properties.

Description of *Lactobacillus secaliphilus* sp. nov.

Lactobacillus secaliphilus (se.ca.li.phi'lus. L. n. *secale* rye; Gr. adj. *philos* loving; N.L. masc. adj. *secaliphilus* rye-loving).

Cells are Gram-positive, non-motile, non-spore-forming rods that are 0.5–1 µm in width and 2.0–4.0 µm in length and occur singly, in pairs or in filaments. When the organism is grown on mMRS agar at 37 °C for 2 days,

Fig. 3. M13 RAPD-fingerprint of *L. secaliphilus* strains and other related lactobacillus species. 1, *L. secaliphilus* TMW 1.1309^T; 2, *L. secaliphilus* TMW 1.1313; 3, *L. coleohominis* DSM 14060^T; 4, *L. ingluviei* DSM 15946^T; 5, *L. panis* DSM 6035^T; 6, *L. pontis* DSM 8475^T; 7, *L. reuteri* DSM 20016^T; 8, *L. frumenti* DSM 13145^T; 9, *L. mucosae* TMW 1.81; 10, *L. vaginalis* DSM 5837^T; 11, *L. oris* DSM 4864^T; M, size marker (marker III, MBI Fermentas).

colonies are small (0.5–1 mm in diameter), colourless, circular to slightly irregular to rough and flat in form. No growth is observed at 15 °C or at 45 °C. The organism is facultatively anaerobic and produces almost only L-lactic acid (94 %) heterofermentatively. Catalase is not produced. Growth occurs in up to 3 % NaCl. Acid is produced from D-ribose. Acid production from sucrose is variable. Acid is not produced from D-glucose, L-arabinose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol, methyl β -D-glucoside, salicin, D-cellobiose, D-fructose, melibiose, melezitose, lactose, D-raffinose, maltose, galactose, D-xylose, β -gentiobiose, gluconate or D-turanose. The DNA G+C content of the type strain is 48 mol%. The peptidoglycan type is A4 α (L-Lys–D-Asp).

The type strain, TMW 1.1309^T (=DSM 17896^T=CCUG 53218^T), was isolated from a type II sourdough in Germany.

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