Lactobacillus cerevisiae sp. nov., isolated from a spoiled brewery sample

Jennifer Koob, Fritz Jacob, Mareike Wenning and Mathias Hutzler

Abstract

A Gram-stain-positive, non-motile, rod-shaped bacterium, designated TUM BP 140423000-2250\(^{\top}\) (=DSM 100836\(^{\top}\)=LMG 29073\(^{\top}\)), was isolated from spoiled beer. This bacterium did not form spores, and was catalase-negative and facultatively anaerobic. Its taxonomic position was determined in a polyphasic study. The 16S rRNA gene sequence similarity data showed that the strain belonged to the Lactobacillus genus with the nearest neighbours being Lactobacillus koreensis DCY50\(^{\top}\) (sequence similarity 99.5 %), Lactobacillus yonginensis THK-V8\(^{\top}\) (99.2 %) and Lactobacillus parabrevis LMG 11984\(^{\top}\) (98.7 %). Sequence comparisons of additional phylogenetic markers, pheS and rpoA, confirmed the 16S rRNA gene sequence tree topology. The maximum rpoA sequence similarity was 92.3 % with L. yonginensis THK-V8. The DNA G+C content of the isolate was 50.0 mol%. The DNA–DNA relatedness showed that strain TUM BP 140423000-2250\(^{\top}\) could be clearly distinguished from L. koreensis DCY 50\(^{\top}\) (30.8±0.4 %) and L. yonginensis THK-V8\(^{\top}\) (23.6±5.9 %). The major fatty acids were C\(_{18:1}\)ω9c, summed feature 7 (comprised of C\(_{17:0}\) cyclo ω10c/C\(_{19:1}\) ω6c) and C\(_{16:0}\). Based on phenotypic and genotypic studies, the authors propose classifying the new isolate as a representative of a novel species of the genus Lactobacillus, Lactobacillus cerevisiae sp. nov. The type strain is deposited at the Research Centre Weihenstephan for Brewing and Food Quality as TUM BP 140423000-2250\(^{\top}\) (=DSM 100836\(^{\top}\)=LMG 29073\(^{\top}\)).

Every year, the damage caused by beer-spoilage microorganisms results in financial losses. The consequences of the growth of these micro-organisms in beer range from slight changes in smell and taste to the product becoming unpalatable including consumer complaints and refunds [1]. The primary task of microbiological quality control in breweries is to detect even traces of these spoilage microbes and identify them if necessary. A basic requirement for successful analytics is to be aware of/knowledgeable of all microbes with spoilage potential [2].

The group of beer-spoilage micro-organisms is a limited pool of bacteria and yeast species that are able to tolerate the adverse conditions of beer. These antimicrobial beer properties include especially high levels of alcohol and hop acids, low pH and an anaerobic atmosphere as well as low concentrations of utilizable carbon sources [2–7]. The mechanisms protecting beer-spoilage micro-organisms from the hostile beer conditions are still not fully understood. The dominant genus causing beer-spoilage incidents is the genus Lactobacillus. Species belonging to the beer-spoiling group newly described in the last decade are Lactobacillus backii, Lactobacillus rossiae and Lactobacillus paucivorans [8–11].

Recently, bacterial isolates were obtained from contaminated brewery samples that were first assigned to the species Lactobacillus parabrevis, which was not known to be a beer-spoilage bacterium [12]. One isolate (culture collection number TUM BP 140423000-2250\(^{\top}\), working number 2301\(^{\top}\)) was selected for further study. It was obtained from a bright beer tank sample exhibiting turbidity and slightly enhanced acidity, but no significant sensory changes. Phylogenetic analyses led to the conclusion that the isolate could not be assigned to Lactobacillus brevis, the most dominant beer-spoiling species [3, 13–16], or L. parabrevis. Physiological characteristics were also determined such as temperature, acid, alcohol and salt tolerance, the presence of known hop resistance genes as well as the degree of its beer-spoilage potential [12].

In this study, a polyphasic approach was used to demarcate the isolate TUM BP 140423000-2250\(^{\top}\) from related species based on the sequences of the 16S rDNA gene and two
housekeeping genes, pheS and rpoA. In addition, genomic relatedness, fatty acid profile and further phenotypic and physiological characteristics were determined, which resulted in the description of a novel species that belongs to the genus Lactobacillus. The closely related type strains Lactobacillus koreensis DCY50T and Lactobacillus yonginensis THK-V8T were obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ, Braunschweig, Germany), and Lactobacillus parabrevis DSM 11984T was obtained from the Belgian Co-ordinated Collections of Micro-organisms (BCCM, Gent, Belgium) [17–19].

All strains used in this study were separated on De Man, Rogosa and Sharpe agar (MRS; pH 6.2) and cultivated in an anaerobic atmosphere at 28±1°C [20]. Ten colonies of each were picked and combined to form the initial cultures followed by storage in cryostock at −80°C.

Cell morphology of 48-h-old broth cultures and spore-forming ability were examined by dark-field and phase-contrast microscopy [Nikon, Eclipse Ti microscope; Andor, (DIS) Zyla V - 3tap camera]. Motility was tested using the hanging-drop technique [21]. Gram staining was performed according to Buck [22]. Catalase activity was examined by bubble production in 3% (v/v) H2O2 solution, and oxidase activity was determined using Bactident strips (Merck). To determine the fermentation type of isolate TUM BP 140423000-2250T, tests for gas production from glucose and gluconate were carried out in triplicate in MRS broth with Durham tubes [3]. The production of D- and L-lactic acid was analysed using a D-lactic acid/L-lactic acid enzyme kit according to the manufacturer’s instructions (R-Biopharm). The test for NH3 production from arginine was carried out according to Back [23]. Carbohydrate fermentation pattern was determined using the API CHL 50 kit (BioMérieux), and enzyme activity using the API ZYM kit (BioMérieux). The growth behaviour in the presence of oxygen was determined by stab cultures in NBB agar (Döhler).

The fatty acid profile and the cell-wall composition, including the presence of meso-diaminopimelic acid, of strain TUM BP 140423000-2250T and the DNA–DNA relatedness values of the combinations of Lactobacillus cerevisiae sp. nov. TUM BP

![Fig. 1. Neighbour-joining tree based on the 16S rRNA gene sequences showing the phylogenetic relationship between L. cerevisiae sp. nov. TUM BP 140423000-2250T and related species of the genus Lactobacillus; Lactobacillus delbrueckii DSM 20074T was included as an outgroup species. Bootstrap values (expressed as percentages of 1000 replicates) ≥50% are shown. Bar, 0.01 substitutions per nucleotide position.](image-url)
The 16S rRNA gene sequence of strain TUM BP 140423000-2250\(^\top\) and \textit{L. koreensis} DCY50\(^\top\) as well as \textit{L. cerasiae} sp. nov. and \textit{L. yonginensis} THK-V8\(^\top\) were analysed at the DSMZ [24–36].

The 16S rRNA gene sequence was determined using the primer pairs 27\(\text{f}\) (5\' AGAGTTTGATCMTGGCTCAG 3\') and 1492\(\text{r}\) (5\' TACGGYTACCTTGTTACGACTT 3\') as well as 933\(\text{f}\) (5\' GCACAAGCCTGAGGATG 3\') and 1514\(\text{r}\) (5\' AAGGACGTGATCAGCCGA 3\') [37–40]. Since the sequences of the RNA polymerase alpha subunit (\textit{rpoA}) and the phenylalanine-tRNA synthase alpha subunit (\textit{pheS}) have a higher resolution potential with regard to the genera \textit{Enterococcus} and \textit{Lactobacillus} [41–43], both housekeeping gene sequences were determined from TUM BP 140423000-2250\(^\top\) and compared with the sequences of the nearest phylogenetic neighbours. The temperature protocol and the primer set (\textit{rpoA}-21-\text{F} and \textit{rpoA}-23-\text{R}) for \textit{rpoA} PCR were adopted from the study by Naser \textit{et al.} [44]. The temperature protocol and the primer set (\textit{pheS}-forward and \textit{pheS}-reverse) for \textit{pheS} gene PCR were adopted from the study by Ehrmann \textit{et al.} [10]. The sequencing was performed by GATC Biotech (Constance, Germany).

The 16S rRNA gene sequence of TUM BP 140423000-2250\(^\top\) was compared with those of related taxa of the genus \textit{Lactobacillus} obtained from the EzTaxon database [45]. \textit{rpoA} and \textit{pheS} housekeeping gene sequences of closely related species were obtained from the National Centre for Biotechnology Information (NCBI) database or determined in this study. Phylogenetic trees were reconstructed using the MEGA6 software [46–50].

The 16S rRNA gene sequence of strain TUM BP 140423000-2250\(^\top\) was a continuous stretch of 1477 bp. The neighbour-joining tree topology (Fig. 1) was evaluated and confirmed by the maximum-likelihood method (Fig. S1, available in the online Supplementary Material). The trees classified \textit{L. cerasiae} sp. nov. TUM BP 140423000-2250\(^\top\) as a member of the \textit{Lactobacillus buchnerii} group [51] with the two nearest neighbours, \textit{L. koreensis} and \textit{L. yonginensis}, showing sequence similarities of 99.5\% and 99.2\%, respectively. Due to the high 16S rRNA gene sequence similarities, alternative chronometers and DNA–DNA hybridization experiments were undertaken [25, 34–36, 52–55].

The analyses of the \textit{rpoA} (Fig. 2) and \textit{pheS} gene sequences (Fig. S2) had a higher resolution potential. Using continuous DNA hybridization and alternative chronometers experiment [25, 34–36, 52–55] and determined in this study. flap [46–50].

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
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<th>4</th>
<th>5</th>
<th>6</th>
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<td></td>
<td></td>
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<tr>
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<td>16.1</td>
<td>5.8</td>
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<td>7.8</td>
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<td>2.7</td>
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<tr>
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<td>24.5</td>
<td>6.4</td>
<td>10.3</td>
<td>13.8</td>
<td>10.2</td>
<td>11.4</td>
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<tr>
<td>C15:0(3\text{-OH})</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.7</td>
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<tr>
<td>Cyclo fatty acid</td>
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<tr>
<td>C17:0(\text{cyclo})</td>
<td>–</td>
<td>1.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.5</td>
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<tr>
<td>C19:0(\text{cyclo})</td>
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<td>–</td>
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<td>Summed features*</td>
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</tr>
<tr>
<td>3; C16:1(\text{ω7c/ω6c})</td>
<td>0.9</td>
<td>2.5</td>
<td>1.1</td>
<td>1.5</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>7; C19:0(\text{cyclo})</td>
<td>35.2</td>
<td>45.6</td>
<td>26.9</td>
<td>35.9</td>
<td>36.1</td>
<td>33.0</td>
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<tr>
<td>8; C18:1(\text{ω6c/ω5c})</td>
<td>8.9</td>
<td>3.5</td>
<td>4.1</td>
<td>5.5</td>
<td>4.4</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system.
stretches of 801 bp for \( rpoA \) and 371 bp for \( pheS \), both sequence comparisons indicated that the novel beer-spoilage isolate represents a novel species within the genus Lactobacillus. The nearest phylogenetic neighbours of \( L. \) cerevisiae sp. nov. TUM BP 140423000-2250\(^T \) regarding the \( rpoA \) sequence were \( L. \) yonginensis THK-V8\(^T \) and \( L. \) koreensis DCY 50\(^T \), showing 92.3 % and 91.2 % similarity, respectively.

DNA–DNA relatedness values between strain TUM BP 140423000-2250\(^T \) and \( L. \) koreensis DCY50\(^T \) as well as \( L. \) yonginensis THK-V8\(^T \) were 30.5 % (31.1) and 19.4 % (27.8), respectively (values in parentheses are results of measurements in duplicate). Both values were far below the threshold value of 70 % postulated by Wayne et al. for the description of a novel species [52]. The DNA G+C content of \( L. \) cerevisiae sp. nov. TUM BP 140423000-2250\(^T \) was determined as 50.0 mol% [12]. The major fatty acids were determined as \( C_{18:1} \) \( \omega 9c \), summed feature 7 (comprised of \( C_{19:0} \) cyclo \( \omega 10c/C_{19:1} \) \( \omega 6c \)) and \( C_{16:0} \). The comparison between the fatty acid content of \( L. \) cerevisiae sp. nov. TUM BP 140423000-2250\(^T \) and those of related type strains is shown in Table 1. The novel type strain can be clearly demarcated by the low level of the \( C_{14:0} \) fatty acid and the high amount of the \( C_{18:1} \) \( \omega 9c \) unsaturated fatty acid.

The analyses for the presence of meso-diaminopimelic acid and the cell-wall composition of the novel isolate proved difficult. Even after repeated attempts, only small quantities of protein-contaminated peptidoglycan cell-wall compound could be isolated by the DSMZ and this did not allow further structural analysis. The presence of meso-diaminopimelic acid was confirmed after highly sensitive gas chromatography/mass spectrometry (GC/MS) analysis [56], but it was not possible to determine the full structure of the cell-wall peptidoglycan.

Strain TUM BP 140423000-2250\(^T \) produced gas from glucose and gluconate. Cells were short or long, and slender rods that occurred singly, in pairs or in short chains (Fig. S3). Beige colonies appeared in two morphological forms: circular with either smooth or fringed edges (Fig. S4). Motility and spore formation could not be observed. The \( D/\)L-lactic acid ratio for strain TUM BP 140423000-2250\(^T \) was 4:6. Further physiological characteristics can be extracted from a previous study (e.g. alcohol, salt, acid and temperature tolerance, hop resistance genes \( horA, horC, hitA \)) [12].

With regard to the carbohydrate fermentation pattern, isolate TUM BP 140423000-2250\(^T \) was positive for acid production from \( \alpha\)-xylose, \( \alpha\)-galactose, \( \alpha\)-glucose, \( \alpha\)-fructose, \( N\)-acetylgalactosamine, maltose and potassium gluconate. It was weakly positive for acid production from \( \alpha\)-arabinose, \( \alpha\)-ribose, \( \alpha\)-mannitol, methyl \( \alpha\)-D-glucopyranoside and melibiose. The key difference between TUM BP 140423000-2250\(^T \) and its three closest phylogenetic neighbours was the weakly positive fermentation of \( \alpha\)-mannitol and the non-fermentation of \( \alpha\)-arabitol (Table 2). The enzymic profiles of TUM BP 140423000-2250\(^T \) and closely related species are summed up in Table S1. Therefore, based on morphology, and physiological and phylogenetic information it is proposed that the novel organism belongs to a novel species of the genus Lactobacillus for which the name Lactobacillus cerevisiae sp. nov. is proposed.

### Table 2. Differential characteristics between \( L. \) cerevisiae sp. nov. TUM BP140423000-2250\(^T \) and related type strains

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
</tr>
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<tbody>
<tr>
<td>Colony colour</td>
<td>Beige</td>
<td>Beige(^a)</td>
<td>Beige(^b)</td>
<td>Cream-coloured(^d)</td>
</tr>
<tr>
<td>Catalase activity</td>
<td>Negative</td>
<td>Negative(^a)</td>
<td>Negative(^b)</td>
<td>Negative(^e)</td>
</tr>
<tr>
<td>Motility</td>
<td>Negative</td>
<td>Positive(^a)</td>
<td>Negative(^b)</td>
<td>Negative(^e)</td>
</tr>
<tr>
<td>Ratio of ( D) and ( L)-lactic acid</td>
<td>4:6</td>
<td>3:7(^a)</td>
<td>4:6(^b)</td>
<td>1:1(^c)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>50.0</td>
<td>49.0(^a)</td>
<td>49.0(^b)</td>
<td>47.8(^c)</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl-( \beta)-xylopyranoside</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>( \alpha)-Mannitol</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Methyl ( \alpha)-D-glucopyranoside</td>
<td>w</td>
<td>–</td>
<td>v</td>
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<tr>
<td>( N)-Acetylglucosamine</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Aesculin ferric citrate</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Salicin</td>
<td>–</td>
<td>–</td>
<td>v</td>
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<tr>
<td>Maltose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Lactose</td>
<td>–</td>
<td>–</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>w</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>( \alpha)-D-Arabitol</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Potassium gluconate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

\(^a\)Data taken from: a, Bui et al. [17]; b, Vancanneyt et al. [18]; c, Yi et al. [19].
**DESCRIPTION OF LACTOBACILLUS CERESIACAE SP. NOV.**

*Lactobacillus ceresiacae* (ce.re.vi’siae. L. fem. gen. n. ceresiacae of beer).

Cells are Gram-stain-positive, rod-shaped, non-motile, non-spore-forming, catalase-negative, oxidase-negative, heterofermentative and facultatively anaerobic. Colonies on MRS agar after 48 h are beige and circular, with either smooth or fringed edges. Growth can be observed at temperatures between 4 and 37 °C, but not at 40 or 45 °C. Cells can grow at pH values between pH 4.0 and 7.8. Alcohol is tolerated up to at least 8.0 vol% in MRS broth. No growth is observed on MRS agar after 48 h at temperatures between 4 and 37 °C. The DNA G+C content of the type strain is 50.0 mol%.

**Conflicts of interest**
There does not exist any conflict of interest due to personal or financial relationship with other people or organisations.

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**References**


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