Lactobacillus rossii sp. nov., isolated from wheat sourdough

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Screening of sourdough lactic acid bacteria for bacteriocin production resulted in the isolation of a Gram-positive, catalase-negative, non-spore-forming, non-motile rod bacterium (strain CS1T) that could not be associated with any previously described species. Comparative 16S rRNA gene sequence analysis recognized strain CS1T as a distinct member of the genus Lactobacillus. By a species-specific PCR strategy, five additional strains previously isolated from sourdoughs were found to belong to the same species as strain CS1T, as confirmed by 16S rRNA gene sequence analysis. The closest related species were Lactobacillus durianis, Lactobacillus malefermentans and Lactobacillus suebicus, with which strain CS1T shared 93% sequence similarity. For a further characterization of strain CS1T, physiological (growth temperature, CO2 production, hydrolysis of arginine, isomeric type of lactate, sugar fermentation) and chemotaxonomic (G+C content and peptidoglycan structure) properties were determined. Phenotypic characterization showed that strain CS1T was a member of the obligately heterofermentative group of the genus Lactobacillus. The DNA G+C content was 44.6 mol%. The peptidoglycan was of the A3α (L-Lys–L-Ser–L-Ala2) type. Physiological, biochemical and genotypic data, as well as results of DNA–DNA hybridization with one of the closest phylogenetic relatives, L. durianis (34-3%), indicated that strain CS1T represents a novel species of the genus Lactobacillus for which the name Lactobacillus rossii sp. nov. is proposed. The type strain of this species is CS1T (= ATCC BAA-822T = DSM 15814T).

Sourdoughs are considered extremely complex ecosystems where lactic acid bacteria (LAB) represent the prevailing microflora. Typical sourdough LAB, responsible for the acidification of dough, are lactobacilli. They consist of obligately and facultatively heterofermentative and obligately homofermentative species (Hamnes & Vogel, 1995). The dominant Lactobacillus species in Italian wheat sourdoughs have been reported to be Lactobacillus sanfranciscensis, Lactobacillus brevis, Lactobacillus fermentum and Lactobacillus fructivorans, belonging to the obligately heterofermentative group of lactobacilli; Lactobacillus plantarum and Lactobacillus alimentarius, belonging to the facultatively heterofermentative group; and Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. delbrueckii and Lactobacillus farcininis, belonging to the obligately homofermentative lactobacilli (Gobbetti et al., 1994; Corsetti et al., 2001, 2003). Some recently described species such as Lactobacillus spicheri (Meroth et al., 2004), Lactobacillus mindensis (Ehrmann et al., 2003), Lactobacillus frumenti (Müller et al., 2000) and Lactobacillus paralimentarius (Cai et al., 1999) were also isolated from sourdough. In this complex system, the synthesis of bacteriocins and other antimicrobial compounds could regulate the interactions within the starter micro-organisms and between the starter and the contaminant microflora of the sourdough (Corsetti et al., 2004).

During a study on the production of antimicrobial...
substances by sourdough LAB, a bacteriocinogenic strain was isolated that could not be assigned to a hitherto-known species by the API 50 CH system and 16S rRNA gene sequence analysis. A species-specific primer pair was designed on the basis of its 16S rRNA gene and five additional isolates from different Italian wheat sourdoughs were found to show high similarity to this isolate. This study presents phenotypic and genotypic evidence to describe these strains as a novel Lactobacillus species, for which we propose the name Lactobacillus rossii sp. nov.

Strains CS1T, CR20, CF51, CD76, CI35 and CM17 were isolated from wheat sourdoughs of central Italy. Samples were subjected to serial dilution and plated onto modified de Man–Rogosa–Sharpe medium (mMRS) (maltose and fresh yeast extract were added at 1 and 10%, respectively, and the final pH was adjusted to 5–6) agar as described by Corsetti et al. (2001). The above strains were grown at 30°C for 24 h and maintained at −80°C in glycerol stocks. The purity of the cultures was checked microscopically and by preparing streak cultures. Reference strains were grown on regular MRS (Difco) or mMRS and incubated at the temperature recommended by the respective strain culture collection. Gram determination was performed by Gram staining (Merck) and Gregersen’s KOH method (Gregersen, 1978). Cell morphology was studied with a phase-contrast optical microscope (Leitz Laborlux S). Catalase activity was determined by transferring fresh colonies from mMRS agar to a glass slide and adding 5% H2O2. Growth at 15 and 45°C was tested in mMRS broth. CO2 production was detected in sourdough bacteria broth (Kline & Sugihara, 1971) containing glucose in place of maltose and supplemented with 10% gelatin powder in test tubes sealed with 2% sterile molten agar. Arginine hydrolysis was determined according to the method of Sharpe (1979). The isomeric type of lactate in fermented broth was determined enzymically using the DL-lactate test kit (Boehringer). A fermentation profile was determined using the API 50 CH System (bioMérieux). The peptidoglycan structure of the cell wall was determined at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) by one-dimensional and two-dimensional TLC (Schleifer & Kandler, 1972) followed by derivatization (MacKenzie, 1987). The approximate molar amino acid ratio was determined by GC as reported by Groth et al. (1996). The N terminus of the interpeptide bridge was determined as reported by Schleifer (1985). For the preparation of genomic DNA for PCR assays, cells from 2 ml of overnight cultures were harvested and DNA was extracted according to the method of De Los Reyes-Gavilán et al. (1992). The concentration and purity of DNA was assayed by determining A260 and A280 as described by Sambrook et al. (1989). 16S rRNA gene amplification was performed using the LacbF/LacbR primer pair following the method of Corsetti et al. (2004). PCR products were purified using the Concert Rapid PCR Purification System (Gibco). DNA sequencing reactions were performed by MWG Biotech AG. In order to determine the phylogenetic placement of strain CS1T, the nearly complete 16S rRNA gene sequences of 17 Lactobacillus species, most of them often reported as typical organisms in sourdough fermentation (Vogel et al., 1999; Müller et al., 2000; Corsetti et al., 2003), were aligned using the CLUSTAL W program (Thompson et al., 1994) and phylogenetic analysis was performed with MEGA version 2.1 (Kumar et al., 2001): the distance matrix was obtained with the Kimura two-parameter formula and tree reconstruction with neighbour-joining clustering. An analysis of the robustness of the tree was obtained by bootstrap analysis with 1000 replicates. A second distance-matrix analysis was performed using the program TREECON (van de Peer & de Wachter, 1994) with the Galtier–Gouy model as the distance formula and neighbour joining as the clustering option. A maximum-parsimony method was also applied with default options as implemented in PHYLIP DNAPARS (Felsenstein, 1993). A maximum-likelihood analysis was performed with the DNAML program in the PHYLIP software package (Felsenstein, 1993). The G+C content of the DNA (mol%) of strain CS1T was determined at the DSMZ by an HPLC analytical method. DNA was isolated and purified by chromatography on hydroxyapatite according to the procedure of Cashion et al. (1977) and was hydrolysed and dephosphorylated as reported by Mesbah et al. (1989). HPLC was carried out using the protocol described by Tamaoka & Komagata (1984). Wild-type phage lambda DNA was used as the standard (Mesbah et al., 1989). The G + C content was determined according to the method of Mesbah et al. (1989). Since a 93% identity value for 16S rRNA gene sequences is rather low, determination of DNA homology values was performed by the DSMZ between strain CS1T and the type strain of one of its closest phylogenetic relatives, Lactobacillus durianis. DNA was isolated and purified as for DNA base composition analysis. DNA–DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications described by Huss et al. (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992). Primers for the species-specific PCR assay were designed on regions of high sequence heterogeneity in the 16S RNA gene sequence alignment of lactobacilli included in the phylogenetic tree (see Fig. 1 for corresponding accession numbers) plus another 19 Lactobacillus species (GenBank/EMBL/DDBJ accession nos M58811, M58821, D86517, D16551, AY204893, AY204892, AY204894, AF089108, X94229, AF243177, X76328, M58805, Y17362, M58802, Y17361, M58814, AJ002515, AJ306297 and D79211). The primers used were LrosF (5’-GTATCTGAGAGTAACTGTTCAGA-3’) and LrosR (5’-AGGGAACTCGATCTCTCG-3’) and PCR was performed using the same reaction volume and reaction mixture composition as described for amplification with the LacbF/LacbR primer pair (Corsetti et al., 2004). The PCR programme comprised an initial template denaturation step for 4 min at 94°C followed by 30 cycles.
of denaturation for 45 s at 94 °C, annealing for 30 s at 58 °C and extension for 45 s at 72 °C. The final extension step was for 7 min at 72 °C. To exclude clonal relatedness, four oligonucleotides, P1 (5'-ACGCCGCCCT-3'), P4 (5'-CCGCAGCGTT-3'), P7 (5'-GAGGCTTACGCGTT-3') (Corsetti et al., 2003) and M13 (5'-GAGGGTGGCGGTTCT-3') (Stendid et al., 1994), with arbitrarily chosen sequences, were used to examine the six *L. rossii* strains by randomly amplified polymorphic DNA (RAPD)-PCR. DNA amplification was carried out in 30 µl PCR mixture containing 12 µl *Taq* PCR Master Mix, 1 pmol primer, 1 µl template DNA (~ 25 ng DNA) and sterile distilled H₂O. The PCR programme reported by Corsetti et al. (2003) was used for primers P1, P4 and P7, whereas that reported by Zapparoli et al. (1998) was used for primer M13.

Colonies of *L. rossii* CS1T grown on mMRS agar at 30 °C for 3 days were 1–1·5 mm in diameter, white, smooth and circular when they were grown on the surface of the agar medium. If colonies were within the agar medium the appearance changed to a convex shape. Cells were rods of approximately 0·5 × 1–1·5 µm, non-motile, non-spore-forming and occurring singly, in pairs and in short chains. All isolates stained Gram-positive and were negative for the catalase assay. Physiological and biochemical characteristics, as well as the sugar fermentation pattern of *L. rossii* strains and the reference LAB, are listed in Table 1. All *L. rossii* strains produced Dl-lactate (the D isomer comprised 50 ± 5 % of the total amount of lactic acid produced) and CO₂ from glucose, thus being obligately heterofermentative. Obligate heterofermentative species such as *L. sanfranciscensis*, *L. brevis*, *L. fermentum* and *L. fructivorans* are typically found in wheat-flour Italian sourdoughs (Gobbetti et al., 1994; Corsetti et al., 2001, 2003), as well as in traditional three-stage-processed doughs (Stolz, 1995), and for that reason they were chosen as reference organisms (Table 1).

The peptidoglycan structure was of the A3 type. Among *Lactobacillus* species this peptidoglycan type is only shown by *Weissella minor* (Hammes & Vogel, 1995), formerly ' *Lactobacillus minor* ' (Kandler et al., 1983; Collins et al., 1993). The highest similarity value of strain CS1T, i.e. 93 %, was found with a recently described species, *L. durianis* (Leisner et al., 2002), and with *Lactobacillus malefermentans* and *Lactobacillus suebicus*, while similarities of 92 % or lower were found with other species of the genus *Lactobacillus*. This clearly indicated that strain CS1T could represent a novel species since the similarity value was less than 97 % with the closest relative (Stackebrandt & Goebel, 1994). The resulting tree is shown in Fig. 1. Strain CS1T had a G+C content of 44·6 mol%. This value is within the range for the genus *Lactobacillus* (32–53 mol%) (Kandler & Weiss, 1986). The DNA–DNA homology value

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Fig. 1. Phylogenetic tree showing the relationships between *L. rossii* CS1T and several related *Lactobacillus* species on the basis of 16S rRNA gene sequences. The tree was created by aligning 1360 nt. Bar, 0·01 nucleotide substitution per site.

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Table 1. Phenotypic characteristics of the strains of L. rossii, the closest related species and sourdough-associated species of Lactobacillus

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<td>W</td>
<td>+/W</td>
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<td>–</td>
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<td>W</td>
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<td>L-Lys–L-Ser–L-Ala₂</td>
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<td>ND</td>
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<td>m-Dpm</td>
<td>L-Lys–Ala</td>
<td>L-Lys–D-Asp</td>
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<td>DNA G+C content (mol%)</td>
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<td>ND</td>
<td>43–2–43–3</td>
<td>41–2</td>
<td>40–40–7</td>
<td>36–38</td>
<td>38–41</td>
<td>44–47</td>
<td>52–54</td>
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*Gas is produced from gluonic acid but not from glucose.

between L. rossii CS₁ and L. durianis LMG 19193 was 34.3 %, thus demonstrating that strain CS₁ represents a novel species of the genus Lactobacillus. The specificity of the LrosF/LrosR primer pair was checked against the closest related species (L. durianis, L. malefermentans and L. suebicus), the species showing a high similarity of 16S rRNA gene sequences in the regions of primer annealing (Lactobacillus buchneri, L. delbrueckii subsp. delbrueckii, L. fermentum, Lactobacillus casei, Lactobacillus pentosus and L. plantarum) and some of the typical sourdough-associated species (L. alimentarius, L. brevis, L. farcininis, L. frumenti, Lactobacillus panis, Lactobacillus postis and L. sanfranciscensis). A group of strains isolated from several sourdoughs (Corsetti et al., 2004) and showing CS₁-like phenotypic characteristics was screened with this species-specific PCR assay and the 757 bp DNA amplification product was obtained from strain CS₁ and isolates CR20, CF51, CD76, CI35 and CM17 when specific primers were used, while no amplification product was obtained from the other species of Lactobacillus used as negative controls. Accessibility of DNA for amplification assays was ensured by a control PCR using primers LacBF and LacBR, designed to amplify a fragment of approximately 1370 bp from the 16S rRNA gene within the genus Lactobacillus. PCR amplification products, except for L. malefermentans and L. suebicus, are shown in Fig. A, available as supplementary material in IJSEM Online. Under the conditions used, primers LrosF and LrosR were selective for L. rossii strains. However, for further confirmation, 16S rRNA gene sequence analysis was performed and 100 % identity among the six sequences was found. RAPD-PCR is a PCR-based method used to evaluate microbial biodiversity and provides a reliable way of discriminating strains belonging to the same species (Vincent et al., 1998). RAPD-PCR analysis was successfully used to differentiate L. plantarum (Johansson et al., 1995) and L. sanfranciscensis (Zapparoli...
et al., 1998) at the intraspecies level. RAPD patterns obtained with the four primers for the six strains of L. rossii are shown in Fig. B, available as supplementary material in IJSEM Online. Even though RAPD-PCR using all four oligonucleotides as primers generated only a small number of DNA fragments, they were able to provide evidence of DNA polymorphisms among the strains tested. All four primers could distinguish strain CS1\textsuperscript{T} from strain CD76, and these two strains from the other L. rossii strains. Primer P4 could distinguish strain CI35 from strains CR20, CF51 and CM17. However, in addition to RAPD patterns, different biochemical features of strains CR20, CF51 and CM17, besides their different geographical origin, clearly allowed strain differentiation, thus excluding clonal relatedness.

On the basis of the results shown in this study, we propose that strain CS1\textsuperscript{T} is classified as a novel species and that strains CR20, CF51, CD76, CI35 and CM17 are placed in the following novel species of the genus Lactobacillus: Lactobacillus rossii sp. nov.

**Description of Lactobacillus rossii sp. nov.**

*Lactobacillus rossii* (ros.si’i. N.L. gen. n. rossii of Rossi, to honour Professor Jone Rossi, University of Perugia, Perugia, Italy, for her main contribution to dairy and sourdough microbiology).

Cells are Gram-positive rods of approximately 0.5-1.5 μm. Non-motile, non-spore-forming and occur singly, in pairs and in short chains. After growth for 3 days at 30°C on mMRS agar plates, colonies are 1-1.5 mm in diameter, white, smooth and circular or convex. Micro-aerophilic. Catalase-negative. Growth is observed at 15 but not at 45°C. Obligately heterofermentative. Ammonia is produced from arginine. Aesculin is not hydrolysed. Both D- and L-lactic acid are produced. Acid is produced from L-arabinose, ribose, D-glucose, D-fructose, N-acetylglucosamine and maltose. The majority of strains also ferment D-lyxose and weakly ferment galactose, D-mannose and L-arabinose, ribose, D-glucose, D-fructose, D-inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, cellobiose, lactose, sucrose, trehalose, inulin, melezitose, raffinose, amidon, glycogen, xylitol, β-gentiobiose, D-turanose, D-tagatose, D- and L-fucose, D- and L-arabinol, and 2- and 5-ketogluconate are not fermented. Peptidoglycan structure is A\textsubscript{3}β (L-Lys–L-Ser–L-Ala\textsubscript{2}) type. The DNA G+C content is 44.6 mol%. Isolated from wheat sourdough.

The type strain is CS1\textsuperscript{T} (= ATCC BAA-822\textsuperscript{T} = DSM 15814\textsuperscript{T}).

**References**


