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 CS1^T) that could not be associated with any previously described species. Comparative 16S rRNA gene sequence analysis recognized strain CS1^T 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 CS1^T, as confirmed by 16S rRNA gene sequence analysis. The closest related species were *Lactobacillus durianis*, *Lactobacillus malefermentans* and *Lactobacillus suebicus*, with which strain CS1^T shared 93% sequence similarity. For a further characterization of strain CS1^T, 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 CS1^T 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 A3 α (L-Lys-L-Ser-L-Ala₂) type. Physiological, biochemical and genotypic data, as well as results of DNA–DNA hybridization of genomic DNA with one of the closest phylogenetic relatives, *L. durianis* (34.3%), indicated that strain CS1^T 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 CS1^T (= ATCC BAA-822^T = DSM 15814^T).

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 (Hammes & 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 farciminis*, belonging to the obligately homofermentative lactobacilli (Gobetti *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

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Abbreviations: LAB, lactic acid bacteria; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *L. rossii* CS1^T is AJ564009.

Figures showing analysis of *L. rossii* species-specific PCR and RAPD patterns are available as supplementary material in IJSEM Online.

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 CS1^T, 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 routinely 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 CS1^T, 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 CS1^T 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 CS1^T 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 rRNA 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'-GTATCTGAGAGTAACTGTTTCAGA-3') and LrosR (5'-AGGGAACGTCCGATCTCTCG-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

Fig. 1. Phylogenetic tree showing the relationships between *L. rossii* CS1^T 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.

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'-ACGCGCCCT-3'), P4 (5'-CCG-CAGCGTT-3'), P7 (5'-AGCAGCGTGG-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* CS1^T 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 A3α (L-Lys–L-Ser–L-Ala₂) 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 CS1^T, 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 CS1^T 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 CS1^T 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

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

–, Negative reaction; +, positive reaction; W, weak response; ND, not determined. 1, *L. rossii* CS1^T; 2, other *L. rossii* isolates ($n=5$); 3, *L. durianis* LMG 19193^T (data from Leisner *et al.*, 2002); 4, *L. malefermentans* NCDO 1410^T (Farrow *et al.*, 1986); 5, *L. suebicus* DSM 5007^T (Kleynmans *et al.*, 1989). The following data were from Hammes & Vogel (1995) and Kandler & Weiss (1986); these authors use the following symbols: +, >90% of strains are positive; –, >90% of strains are negative; d, 11–89% of strains are positive; 6, *L. sanfranciscensis*; 7, *L. fructivorans*; 8, *L. brevis*; 9, *L. fermentum*. Details of the variable fermentations of *L. rossii* strains: galactose and gluconate were fermented by strain CR20 and CM17 and only weakly by the other strains. D-Mannose was fermented by strain CD76, whereas weak positive reactions were detected for the other strains; CR20 produced acid from melibiose but was weakly fermented by strains CI35 and CF51. D-Xylose was fermented only by strains CS1^T and CD76, and D-lyxose was fermented by all strains except strain CS1^T.

Characteristic	1	2	3	4	5	6	7	8	9
Growth at:									
15 °C	+	+	+	+	+	+	+	+	–
45 °C	–	–	–	–	–	–	–	–	+
Production of:									
CO ₂ from glucose	+	+	–*	+	+	+	+	+	+
NH ₃ from arginine	+	+	–	+	ND	–	+	+	+
Aesculin hydrolysis	–	–	+	–	–	ND	–	d	–
Isomeric type of lactate	DL	DL	DL	ND	DL	ND	ND	ND	DL
Acid production from:									
L-Arabinose	+	+	+	–	+	–	–	+	d
Ribose	+	+	+	+	+	d	W	+	+
D-Xylose	+	+/-	+	–	+	–	–	d	d
Galactose	W	+/W	+	–	+	d	–	d	+
D-Glucose	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	–	–	–	–	+	+	+
D-Mannose	W	+/W	–	–	–	–	–	–	W
N-Acetylglucosamine	+	+	–	+	–	ND	ND	ND	ND
Maltose	+	+	+	+	+	+	d	+	+
Melibiose	–	+/W/–	–	–	+	–	–	+	+
D-Lyxose	–	+	+	–	–	ND	ND	ND	ND
Gluconate	W	+/W	W	+	–	ND	ND	ND	ND
Peptidoglycan type	L-Lys–L-Ser–L-Ala ₂	ND	ND	ND	m-Dpm	L-Lys–Ala	L-Lys–D-Asp	L-Lys–D-Asp	L-Orn–D-Asp
DNA G + C content (mol%)	44·6	ND	43·2–43·3	41–42	40–40·7	36–38	38–41	44–47	52–54

*Gas is produced from gluconic acid but not from glucose.

between *L. rossii* CS1^T and *L. durianis* LMG 19193^T was 34·3%, thus demonstrating that strain CS1^T 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. farciminis*, *L. frumenti*, *Lactobacillus panis*, *Lactobacillus pontis* and *L. sanfranciscensis*). A group of strains isolated from several sourdoughs (Corsetti *et al.*, 2004) and showing CS1^T-like phenotypic characteristics was screened with this species-specific PCR assay and the 575 bp DNA amplification product was obtained from strain CS1^T 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^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^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 × 1–1.5 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-xylose and weakly ferment galactose, D-mannose and gluconate. Some strains are able to ferment D-xylose and melibiose. Glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl β-xyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, cellobiose, lactose, sucrose, trehalose, inulin, melezitose, D-raffinose, amidon, glycogen, xylitol, β-gentibiose, D-turanose, D-tagatose, D- and L-fucose, D- and L-arabitol, and 2- and 5-ketogluconate are not fermented. Peptidoglycan structure is A3α (L-Lys–L-Ser–L-Ala₂) type. The DNA G+C content is 44.6 mol%. Isolated from wheat sourdough.

The type strain is CS1^T (= ATCC BAA-822^T = DSM 15814^T).

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