Lactobacillus rennini sp. nov., isolated from rennin and associated with cheese spoilage

Empar Chenoll,^{1,2} M. Carmen Macián² and Rosa Aznar^{1,2}

Correspondence

Rosa Aznar

rosa.aznar@uv.es

¹Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, PO Box 73, Burjassot, 46100 Valencia, Spain

²Departament de Microbiologia i Ecologia, Facultat de Biologia, Universitat de València, Campus de Burjassot, 46100 Valencia, Spain

Two bacterial strains, DSM 20253^T and DSM 20254, isolated from rennin and regarded as causing cheese spoilage, were deposited in the DSMZ as *Lactobacillus* sp. by J. Stadhouders. The strains show 99.9 % 16S rRNA gene sequence similarity and have less than 94.3 % similarity with any other species of the genus. *Lactobacillus coryniformis* is their closest phylogenetic neighbour. DNA–DNA hybridization experiments confirmed that the two strains are members of the same species with separate status within the genus *Lactobacillus*. The strains are homofermentative lactic acid bacteria and can be phenotypically and genotypically distinguished from their closest relatives. 16S rRNA gene-targeted specific primers were designed to enable PCR detection of the novel species. The name *Lactobacillus rennini* sp. nov. is proposed for the novel isolates, with strain CECT 5922^T (=DSM 20253^T) as the type strain.

The genus *Lactobacillus*, classified within the order 'Lactobacillales' in the last release of the Taxonomic Outline of the Prokaryotes (Garrity *et al.*, 2004), includes 97 recognized species and novel taxa are continuously arising within this group. Species of the genus *Lactobacillus* are widespread in nature and can be found on plants or material of plant origin, in manure and man-made habitats such as sewage, in fermenting or spoiling food and also in association with the intestinal tracts and mucous membranes of man and many animals. The identification of novel isolates as separate species is not always possible by classical methods of physiological testing as they may share many phenotypic traits. At present, it is well established that, for an unequivocal identification, DNA and RNA analysis (hybridization or gene sequencing) form the basis of systematic taxonomy (Hammes & Vogel, 1995).

Chenoll *et al.* (2003) obtained rDNA-RFLP profiles for 91 lactic acid bacteria (LAB) reference strains, including 45 species of *Lactobacillus* (of which 40 were type strains). The rDNA-RFLP profiles were derived from *DdeI* and *HaeIII* digests of the internal spacer region (ISR; ISR-*DdeI*, ISR-*HaeIII*) and amplified rDNA restriction analysis (ARDRA) of the 16S rRNA gene digested with the same restriction

enzymes (16S-ARDRA-*DdeI* and 16S-ARDRA-*HaeIII*). Analysis of the rDNA-RFLP profiles revealed that some reference *Lactobacillus* strains, deposited as *Lactobacillus* sp., had unique rDNA-RFLP profiles (Chenoll *et al.*, 2003). Based on 16S rRNA gene sequence analysis, it was suggested that these strains might represent novel species since the similarity values were clearly below 97 %. Two of these strains, CECT 5922 (=DSM 20253) and CECT 5923 (=DSM 20254) isolated from rennin and causing spoilage in cheese, were deposited at the DSMZ by J. Stadhouders. In 1967, Stadhouders and Veringa reported the presence of salt-tolerant lactobacilli multiplying in rennet that caused defects in Dutch cheese (Stadhouders & Veringa, 1967). The spoiled cheeses showed many eyes and holes and, at a later stage of ripening, also showed small and large cracks and often developed a phenolic flavour. Lactobacilli were isolated from inefficiently filtered rennet and appeared to be related to species *Lactobacillus casei* and *Lactobacillus plantarum*, but differed from them, amongst other things, in their higher resistance to salt. The lactobacillus strains were able to persist in cheese for a long time during ripening and remained active by their breakdown of amino acids, which led to the production of off-flavours, acetic acid, ammonia and gas (CO₂). By using this contaminated rennet, it was demonstrated that cheese could be produced which showed the defects in question (Stadhouders & Veringa, 1967).

The two strains, CECT 5922^T and CECT 5923, share 99.9 % 16S rRNA gene sequence similarity and have less than 94.3 % similarity with any other species of the genus; their nearest phylogenetic neighbour is *Lactobacillus coryniformis*

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Abbreviations: 16S-ARDRA, amplified rDNA restriction analysis; ISR, internal spacer region; LAB, lactic acid bacteria.

A table detailing the reference strains used to test *L. rennini*-specific PCR primers and a dendrogram derived from 16S-ARDRA analyses are available as supplementary material in IJSEM Online.

(Chenoll *et al.*, 2003). In this work, phenotypic and genetic characterization of the two strains has confirmed that they belong to a novel species in the genus *Lactobacillus*.

Strains CECT 5922^T and CECT 5923 were routinely grown in MRS (Oxoid) at 30 °C under microaerophilic conditions and stored in liquid growth medium containing 20 % (v/v) glycerol at –80 °C. Biochemical characterization of these strains, together with *L. coryniformis* subsp. *torquens*, was performed following standardized methods. All strains are Gram-positive and catalase-negative. Colonies of strains CECT 5922^T and CECT 5923 grown on MRS agar plates for 3 days are very small (up to 1 mm in diameter), grey, smooth, circular and with entire edges. Cells are non-motile and non-spore-forming, rod-shaped, approximately 2–4 × 1 µm in size, though occasionally longer cells are observed, and occur mainly singly or in pairs. Strains CECT 5922^T and CECT 5923 grow at 13, 25 and 37 °C, but not at 4 or 45 °C, and at pH 3·7, 4·5 and pH 8·0. Both strains grow in medium with 5 and 10 % (w/v) NaCl. Strains CECT 5922^T and CECT 5923 do not produce gas from glucose or gluconate as examined with Durham tubes, revealing their homofermentative metabolism. Production of ammonia from arginine, determined as described by Kato *et al.* (2000), is negative. The lactic acid isomer, determined enzymically using the DL-lactate test kit (Boehringer Mannheim), is L(D).

Carbohydrate fermentation patterns and aesculin hydrolysis were evaluated using API 50 CHL galleries (bioMérieux) according to the manufacturer's instructions. Strains were incubated at 30 °C for 48 h. Strains CECT 5922^T and CECT 5923 show similar fermentation profiles, but strain CECT 5922^T also ferments D-mannitol, maltose, lactose and sucrose and is able to hydrolyse aesculin. Differential phenotypic traits are shown in Table 1. *L. coryniformis* subsp. *torquens* CECT 4129^T differed from the novel strains in its ability to ferment 2-ketogluconate and in its inability to ferment L-arabinose, D-ribose or D-xylose and its inability to hydrolyse aesculin (Table 1).

As stated in the online catalogue of DSMZ (<http://www.dsmz.de>), the cell-wall peptidoglycan of strains CECT 5922^T and CECT 5923 is L-Lys–D-Asp.

The 16S rRNA gene sequences of about 1500 bp of strains CECT 5922^T (GenBank accession number AJ576007) and CECT 5923 (GenBank accession number AJ576008) were obtained in a previous study (Chenoll *et al.*, 2003). Comparative analysis was performed with the full dataset of more than 22 000 reference sequences from the ARB database (<http://www.arb-home.de>) using the ARB program package (Ludwig *et al.*, 2004). The ARB tools were used for automated sequence alignment and tree reconstruction. The alignment was checked visually and corrected manually using the sequence editor ARB_EDIT. Sequence similarity values were calculated by comparing nucleotides at corresponding positions and the high similarity between strains CECT 5922^T and CECT 5923 (99·9 %) was confirmed. A closely

Table 1. Phenotypic traits that differ between *Lactobacillus rennini* and its closest phylogenetic neighbour, *L. coryniformis*

For comparative purposes, some other homofermentative lactobacilli are included. Species: 1, *L. rennini* sp. nov. (this study); 2a, *L. coryniformis* subsp. *coryniformis* (Abo-Elnaga & Kandler, 1965); 2b, *L. coryniformis* subsp. *torquens* (this study); 3, *L. fornicialis* (Dicks *et al.*, 2000); 4, *L. fuchuensis* (Sakala *et al.*, 2002); 5, *L. kitasatonis* (Mukai *et al.*, 2003); 6, *L. kunkei* (Edwards *et al.*, 1998); 7, *L. suntoryeus* (Cachat & Priest, 2005). +, All strains positive; –, all strains negative; P, 11–89 % of the strains are positive; W, weak reaction; ND, no data.

Characteristic	1	2a	2b	3	4	5	6	7
Gas production from:								
Gluconate	–	+	+	–	–	–	–	–
Lactate isomer produced	L(D)	D(L)	D	DL	L(D)	DL	L	ND
Growth at:								
5 °C	–	ND	–	–	+	–	–	–
15 °C	W	+	+	–	ND	–	+	–
45 °C	–	–	–	–	–	+	–	+
Acid production from:								
N-Acetylglucosamine	+	+	+	ND	+	ND	–	+
Amygdalin	–	–	–	+	+	–	–	+
L-Arabinose	W	–	–	–	–	–	–	–
Arbutin	–	ND	–	ND	+	–	–	+
D-Cellobiose	–	–	–	+	+	P	–	–
D-Galactose	W	+	+	+	+	+	–	P
D-Mannose	+	+	+	+	+	+	–	+
D-Melezitose	–	–	–	+	–	–	–	–
L-Rhamnose	–	+	–	–	+	ND	–	–
D-Ribose	+	(W)*	–	–	+	–	–	–
D-Sorbitol	–	P	–	+	–	–	–	–
Starch	–	ND	–	–	+	ND	–	P
D-Trehalose	–	–	–	+	+	–	–	–
D-Xylose	W	–	–	–	+	–	–	–

*Result for type strain is in parentheses.

related sequence, submitted to EMBL by D. Ouwerkerk & A. V. Klieve in 2000 (GenBank accession number AF316586), has 99·8 % similarity with both novel strains. This sequence is documented as belonging to a feedlot manure isolate, YE07, and identified as *Lactobacillus* sp. However, strains CECT 5922^T and CECT 5923 had less than 94·3 % sequence similarity to any species with validly published names. The phylogenetic tree constructed using the neighbour-joining method shows that *L. coryniformis* subsp. *torquens* and *L. coryniformis* subsp. *coryniformis* are the closest phylogenetic neighbours to the novel strains (Fig. 1). Following the recommendations of Ludwig *et al.* (1998), alternative treeing methods (maximum-parsimony and maximum-likelihood) were used which confirmed the position of CECT 5922^T and CECT 5923 in the vicinity of *L. coryniformis* and *Lactobacillus bifermens* (data not shown).

Fig. 1. Phylogenetic tree constructed using the neighbour-joining method based on 16S rRNA gene sequences. *Lactobacillus rennini* sp. nov. strains CECT 5922^T and CECT 5923 and sequence AF316586 (corresponding to a feedlot manure isolate, YE07) are close to *L. coryniformis*. Bar, 5% nucleotide substitution.

Genotypic characterization by rDNA-RFLP profile analysis revealed that strains CECT 5922^T and CECT 5923 share 16S-ARDRA-*Dde*I, ISR-*Dde*I and ISR-*Hae*III profiles. The strains differ only in one band in the 16S-ARDRA-*Hae*III profile, due to one nucleotide difference in a restriction site. Compared with the phylogenetically closest species they showed unique profiles, apart from the ISR-*Hae*III-RFLP profile (Chenoll *et al.*, 2003) which was similar to those of *Lactobacillus parabuchneri* and *L. coryniformis* subsp. *torquens* (see Supplementary Fig. S1 in IJSEM Online).

DNA–DNA hybridization experiments were performed as described by Ziemke *et al.* (1998) with DNA of strains CECT 5922^T, CECT 5923 and *L. coryniformis* subsp. *torquens* CECT 4129^T. The reference DNA was that of strain CECT 5922^T and the results are expressed as mean percentage values based on three independent hybridization experiments. The relative binding ratio values of strain CECT 5922^T to strain CECT 5923 were always above 80% (mean 86.5, max. 93, min. 80, SD 9.2, *n*=3). The hybridization values between strain CECT 5922^T and *L. coryniformis* subsp. *torquens* CECT 4129^T were always below 60% (mean 55.5, max. 60, min. 46.3, SD 6.4, *n*=3). All these results confirm that strains CECT 5922^T and CECT 5923 are members of the same species, with separate status within the genus *Lactobacillus*.

Species-specific primers targeted to the 16S rRNA gene for the novel strains were designed using the appropriate ARB software package tool (Ludwig *et al.*, 2004). Following the nomenclature of Alm *et al.* (1996), they are S-S-Lren-0060-a-S (5'-GTCGCACGCACAACTGTT-3') as the sense primer and S-S-Lren-0175-a-A (5'-GTCGCGTATTTCTA-TGCG-3') as the antisense primer. The primers were proved

to be specific by testing *in silico* using the BLAST tool. In addition, specificity was tested *in vitro* by PCR amplification of 52 LAB reference strains (seven *Carnobacterium* species, 36 *Lactobacillus* species and nine *Leuconostoc* species). The strains used are listed in Supplementary Table S1 (see IJSEM Online). PCR was carried out at an annealing temperature of 56 °C, as described by Macián *et al.* (2004). The specific fragment of 133 bp was only amplified from strains CECT 5922^T and CECT 5923.

Based on the data reported here, strains CECT 5922^T and CECT 5923 are members of a novel species within the genus *Lactobacillus*. They show phenotypic characters that distinguish them from other homofermentative lactobacillus species and *L. coryniformis*, their closest phylogenetic relative (Table 1), as well as genotypic traits that enable them to be differentiated by any of the following rDNA-RFLP profiles: 16S-ARDRA-*Dde*I, 16S-ARDRA-*Hae*III, ISR-*Dde*I or ISR-*Hae*III (see Supplementary Fig. S1). We propose the name *Lactobacillus rennini* sp. nov. for the two novel strains isolated from rennin by J. Stadhouders. The type strain is CECT 5922^T (= DSM 20253^T) and CECT 5923 (= DSM 20254) is a reference strain.

Description of *Lactobacillus rennini* sp. nov.

Lactobacillus rennini (ren.ni'ni. N.L. n. *renninum* rennin, an enzyme that coagulates milk and is used in making cheese; N.L. gen. n. *rennini* of rennin).

Cells are rod-shaped, 1 µm wide by 2–4 µm long, Gram-positive, catalase-negative, non-spore-forming and non-motile. They are found singly and in pairs. Grows better in

MRS broth than on agar plates and under anaerobic conditions. After 3 days of incubation in MRS agar at 30 °C, colonies are small (up to 1 mm in diameter), circular, translucent and grey. Grows at 13 °C, but not at 4 or 45 °C. Homofermentative. No gas is produced from glucose or gluconate. Arginine is not hydrolysed. Both D- and L-lactic acid are produced. Strains ferment L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose and N-acetylglucosamine. Does not ferment glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl β -xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, salicin, D-cellobiose, melibiose, D-trehalose, inulin, melizitol, D-raffinose, starch, glycogen, xylitol, gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The type strain also ferments D-mannitol, maltose, lactose and sucrose and hydrolyses aesculin. Peptidoglycan type is L-Lys-D-Asp.

The type strain, CECT 5922^T (= DSM 20253^T), was isolated from rennin by J. Stadhouders. Strain CECT 5923 (= DSM 20254), also isolated from rennin by J. Stadhouders, is a reference strain.

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