Pediococcus cellicola sp. nov., a novel lactic acid coccus isolated from a distilled-spirit-fermenting cellar

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Two novel *Pediococcus* strains, Z-1 and Z-8^T, were isolated from a distilled-spirit-fermenting cellar in Hebei Province, China. The cells were Gram-positive, non-spore-forming, non-motile cocci, occurring in pairs or tetrads. The strains were facultatively anaerobic and produced 50 % (–)-D-/50 % (+)-L-lactic acid exclusively from glucose fermentation, but did not produce catalase. Both strains fermented rhamnose, ribose, xylose, lactose, maltose, trehalose and sucrose, and tolerated 10 % ethanol. The mean G + C content of the two strains was 38 ± 1 mol% (37 mol% for the type strain, Z-8^T). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain Z-8^T clustered within the *Pediococcus damnosus*–*Pediococcus inopinatus*–*Pediococcus parvulus* group, with 98.3–98.5 % 16S rRNA gene similarity. DNA–DNA relatedness between strain Z-8^T and *P. damnosus* LMG 11484^T, *P. inopinatus* LMG 11409^T and *P. parvulus* LMG 11486^T was 19 ± 4, 35 ± 5 and 31 ± 5 %, respectively. Therefore, based on the distinctive phenotypic characteristics, and genotypic and phylogenetic analyses, a novel *Pediococcus* species, *Pediococcus cellicola* sp. nov., is proposed. The type strain is Z-8^T (= AS 1.3787^T = LMG 22956^T).

Pediococci are spherical lactic acid bacteria, with cells arranged mainly in pairs or tetrads. They produce lactic acid exclusively from glucose fermentation, and inhabit fermentable-sugar-rich niches, such as plant materials and fermented foods (Garvie, 1986), especially pickles and preserved meat. In the food industry, they have been used as starter cultures in some kinds of sausage fermentation. However, *pediococci* can also cause quality problems for brewers when present in beer and wines (Gardner *et al.*, 2001; Lonvaud-funel, 1999).

To date seven *Pediococcus* species have been described: *Pediococcus acidilactici*, *Pediococcus claussenii* (Dobson *et al.*, 2002), *Pediococcus damnosus*, *Pediococcus dextrinicus*, *Pediococcus inopinatus*, *Pediococcus parvulus* and *Pediococcus pentosaceus*. A species that was previously designated as belonging to the genus *Pediococcus*, *Pediococcus urinaeequi*, was recently reclassified as *Aerococcus urinaeequi* (Felis *et al.*, 2005). On the basis of 16S rRNA gene similarity, the genus *Pediococcus* was grouped in the *Lactobacillus casei* branch of the lactic acid bacteria (Collins *et al.*, 1991). Within the

genus *Pediococcus*, *P. damnosus*, *P. inopinatus* and *P. parvulus* formed a closely related cluster, whereas *P. acidilactici*, *P. claussenii* and *P. pentosaceus* formed a second cluster, and *P. dextrinicus*, a distinct *Pediococcus* species, was clustered with some *Lactobacillus* species.

Chinese liquor is a kind of wine produced by solidified fermentation of grain. During an investigation of lactic acid bacteria in Chinese wine breweries, we isolated several dozen *Pediococcus* strains. Two strains from a brewery in Hebei Province, China, exhibited phenotypic characteristics that differed from those of all existing *Pediococcus* species. Phylogenetic analysis based on 16S rRNA gene sequence similarity and DNA–DNA hybridization also indicated that the two strains represented a novel species of the genus *Pediococcus*.

P. damnosus LMG 11484^T, *P. inopinatus* LMG 11409^T and *P. parvulus* LMG 11486^T were kindly provided by the Laboratory of Microbiology, Ghent University (BCCM/LMG, Ghent, Belgium). Strains Z-1 and Z-8^T were originally isolated from the walls of a distilled-spirit-fermenting cellar in Hebei Province, China. They were isolated and purified by streaking the samples on de Man–Rogosa–Sharpe (MRS) agar (de Man *et al.*, 1960). The strains were routinely grown aerobically on the same medium at 37 °C unless stated otherwise. The end-products of glucose fermentation in

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tryptone-peptone-yeast extract-glucose (TPYG) liquid were detected by using a gas chromatograph (GC-14B; Shimadzu). Isomers of lactate formed from glucose were determined by using a D-/L-lactic acid test kit (Roche Diagnostics). Catalase activity was determined by adding 15% (v/v) hydrogen peroxide to the fresh cultures. Temperature profiles were determined by using a water bath with a temperature gradient. Growth at various pH values was determined in TPYG liquid adjusted with HCl or NaOH. Tolerance of ethanol was determined by monitoring growth in TPYG liquid with different concentrations of ethanol (v/v). The generation time was determined by monitoring the OD₆₀₀ of an MRS liquid culture at 37 °C for 24 h. Sugar fermentation patterns were determined by using both conventional methods (Garvie, 1986) and the API 50 CH system (bioMérieux) after 24 h incubation at 37 °C. All of the tests were performed in duplicate.

Genomic DNA was extracted and purified as described by Marmur (1961). The 16S rRNA gene was amplified by PCR using the genomic DNA as template, and sequenced using an ABI PRISM 377XL DNA sequencer. The 16S rRNA gene sequences of the most-related species were retrieved from GenBank and aligned, and similarity analysis was performed using the CLUSTAL_X program (Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), implemented in the MEGA2 program (Kumar *et al.*, 2001). The stability of the clustering of the tree was evaluated by bootstrap analysis of 1000 datasets. DNA G + C content was determined by using the thermal denaturation method (Marmur & Doty, 1962). DNA–DNA relatedness was determined on the basis of DNA–DNA liquid reassociation rate (De Ley *et al.*, 1970), using a DU-800 spectrophotometer (Beckman) with a thermal controller.

Cells of the two isolates were Gram-positive, non-spore-forming, non-motile cocci, occurring in pairs or tetrads. Catalase activity was negative and the strains were facultatively anaerobic. These characteristics indicated that the two isolates were members of the genus *Pediococcus*. Product determination showed that the two strains produced lactic acid [about 50% (–)-D-/50% (+)-L-] exclusively from glucose fermentation without gas. By using both conventional methods and the API 50 CH system, the two strains were shown to use rhamnose, ribose, D-xylose and L-xylose as sole carbon and energy sources, which differentiated them from phylogenetically closely related species (Table 1). Fermentation of D-arabinose, L-arabinose and methyl α -D-glucoside was variable in the two strains. Both strains hydrolysed aesculin but not arginine. In addition, the two strains tolerated 10% ethanol (v/v), in accordance with their original habitat. The mean generation time of the two strains was 1.09 ± 0.06 h when cultured aerobically, and 1.01 ± 0.06 h when cultured anaerobically.

The complete 16S rRNA gene sequence of the novel strain Z-8^T was compared with those of the most closely related species retrieved from GenBank. A phylogenetic tree rooted

with *Bacillus subtilis* NCDO 1769^T was constructed (Fig. 1). It was shown that strain Z-8^T clustered within the *P. damnosus*–*P. inopinatus*–*P. parvulus* group, with 16S rRNA gene sequence similarities ranging from 98.3 to 98.5%. The two novel isolates had a partial 16S rRNA gene sequence similarity of 99.5%. The mean DNA G + C content of the two novel strains was 38 ± 1 mol% (37 mol% for strain Z-8^T and 39 mol% for strain Z-1), which was within the range for the genus *Pediococcus* (34–42 mol%). DNA–DNA relatedness between the novel strains and their phylogenetically closest related species was determined at a hybridization temperature of 67 °C. The DNA–DNA relatedness between the two novel isolates, Z-1 and Z-8^T, was 91%, indicating a homogeneous genetic group. However, the DNA–DNA relatedness between strain Z-8^T and *P. damnosus* LMG 11484^T, *P. inopinatus* LMG 11409^T and *P. parvulus* LMG 11486^T was 19 ± 4 , 35 ± 5 and 31 ± 5 %, respectively, lower than the DNA–DNA relatedness threshold (70%) for a species.

Although strain Z-8^T had higher 16S rRNA gene sequence similarities with *P. damnosus* LMG 11484^T (98.3%), *P. inopinatus* LMG 11409^T (98.5%) and *P. parvulus* LMG 11486^T (98.3%), the following characteristics verified their separate species status: low DNA–DNA relatedness, different phenotypic characteristics such as fermentation of rhamnose, ribose, D-xylose and L-xylose, and the ability to grow in 10% ethanol (v/v) (Table 1).

Based on the phenotypic characteristics, phylogenetic relationship and DNA–DNA relatedness, a novel species

Fig. 1. Dendrogram based on 16S rRNA gene sequence similarity in a consensus length of 1400 bp, showing the phylogenetic relationships between *P. cellicola* sp. nov. Z-8^T and related lactic acid bacterial species. The tree was rooted with *B. subtilis* NCDO 1769^T and constructed using the neighbour-joining method with bootstrap values calculated from 1000 trees. Numbers at branch points are percentage levels of bootstrap support. GenBank accession numbers of 16S rRNA gene sequences are given in parentheses. Bar, 3% sequence divergence.

Table 1. Differential phenotypic characteristics of *P. cellicola* sp. nov. and phylogenetically closely related species

Taxa: 1, *P. cellicola* Z-8^T; 2, *P. cellicola* Z-1; 3, *P. damnosus* (data from Back, 1978); 4, *P. inopinatus* (Back, 1978); 5, *P. parvulus* (Gunther & White, 1961). The data were also verified in this study by using conventional methods. +, >90 % of strains positive; −, <10 % of strains positive; D+, 50–89 % of strains positive. In acid production tests: C, by using conventional methods; API, by using the API 50 CH system; no indication, by using both methods.

Characteristic	1	2	3	4	5
Acid production from:					
D-Arabinose	+	−	− (C)	− (C)	− (C)
L-Arabinose	+	−	− (C)	− (C)	− (C)
Dextrin	− (C)	− (C)	− (C)	D+ (C)	− (C)
Lactose	+	+	− (C)	+	− (C)
Maltose	+	+	D+ (C)	+	+
Melezitose	− (C)	− (C)	D+ (C)	− (C)	− (C)
Rhamnose	+	+	− (C)	− (C)	− (C)
Ribose	+	+ (C), − (API)	− (C)	− (C)	− (C)
Sucrose	+	+	D+ (C)	D+ (C)	− (C)
D-Xylose	+	+	− (C)	− (C)	− (C)
L-Xylose	+	+	− (C)	− (C)	− (C)
Methyl α-D-glucoside	− (C)	+ (C)	D+ (C)	D+ (C)	D+ (C)
Growth at/in:					
pH 7·0	+	+	−	+	+
10 % Ethanol (v/v)	+	+	+*	−*	−*
30 °C	+	+	−	+	+

*Data from this study.

of the genus *Pediococcus*, *Pediococcus cellicola* sp. nov., is proposed.

Description of *Pediococcus cellicola* sp. nov.

Pediococcus cellicola (cel.li.co'la. L. n. *cella* a storeroom for wine and food; L. suff. *-cola* from L. n. *incola* an inhabitant, dweller; N.L. n. *cellicola* an inhabitant of a storeroom, indicating that the strains were originally isolated from a distilled-spirit-fermenting cellar).

Gram-positive, non-spore-forming, non-motile, spherical cells, 0·6–1 µm in diameter after 24 h incubation in aerobic MRS liquid at 37 °C. Cells occur in pairs or tetrads. Colonies are greyish white, low-convex, circular, entire, opaque and about 1 mm in diameter after 24 h cultivation on MRS plates at 37 °C. Catalase-negative. Facultatively anaerobic. Lactic acid [about 50 % (−)-D-/50 % (+)-L-] is produced from glucose fermentation, but not gas. Growth occurs at 13–44 °C, with an optimum at 30–37 °C. Optimum pH for growth is 6·0–6·8; no growth occurs at pH 3·8 and 8·2. Grows in 10 % ethanol (v/v) but not in 10 % NaCl (w/v). Aesculin but not arginine is hydrolysed. Acid is produced from galactose, glucose, fructose, mannose, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, arbutin, salicin, cellobiose, gentiobiose, D-tagatose, 2-ketogluconate, rhamnose, ribose, D-xylose, L-xylose, lactose,

maltose, trehalose and sucrose. Acid is not produced from glycerol, erythritol, adonitol, methyl β-D-xyloside, sorbose, dulcitol, inositol, mannitol, sorbitol, amygdalin, aesculin, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 5-ketogluconate or dextrin. Fermentation of D-arabinose, L-arabinose and methyl α-D-glucoside is variable. Strain Z-8^T ferments D-arabinose and L-arabinose but not methyl α-D-glucoside, whereas the reverse applies for strain Z-1. Mean DNA G+C content is 38 ± 1 mol% (37 mol% for strain Z-8^T).

The type strain is Z-8^T (=AS 1.3787^T=LMG 22956^T), which was isolated from a distilled-spirit-fermenting cellar in Hebei Province, China.

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