Pediococcus ethanolidurans sp. nov., isolated from the walls of a distilled-spirit-fermenting cellar

Limeng Liu,^{1,2} Bin Zhang,¹ Huichun Tong¹ and Xiuzhu Dong¹

Correspondence

Xiuzhu Dong

dongxz@sun.im.ac.cn

¹State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, P. R. China

²College of Life Science, Hebei University, Baoding, Hebei 071002, P. R. China

Two novel *Pediococcus* strains, Z-9^T and Z-17, were isolated from a distilled-spirit-fermenting cellar in Hebei Province, China. The cells were Gram-positive, non-spore-forming, non-motile cocci and occurred in pairs or tetrads. The strains were facultatively anaerobic and produced only lactic acid [D(–) and L(+) isomers in the ratio 50 : 50] from glucose fermentation. Catalase activity was not present. Both strains were able to grow in 6.5 % ethanol and at pH 3.5, but not in 4 % NaCl. The mean genomic G + C content of the two strains was 39.5 ± 0.5 mol% (39 mol% for the type strain, Z-9^T). The levels of 16S rRNA gene sequence similarity between the two novel strains and related species of the genus *Pediococcus* ranged from 98.3 to 98.7 %. The levels of DNA–DNA relatedness between strain Z-9^T and the phylogenetically closely related *Pediococcus* *Pediococcus damnosus* LMG 11484^T, *Pediococcus inopinatus* LMG 11409^T, *Pediococcus parvulus* LMG 11486^T and *Pediococcus cellicola* LMG 22956^T were 14.6, 33, 28.7 and 16.8 %, respectively. On the basis of phenotypic, genotypic and phylogenetic analyses, a novel species, *Pediococcus ethanolidurans* sp. nov., is proposed, with strain Z-9^T (= AS 1.3889^T = LMG 23354^T) as the type strain.

The *Pediococcus* are a group of lactic acid bacteria with spherical cells that are typically arranged in tetrads (Garvie, 1986). Traditionally, they have been used in the fermentation of vegetables and meats and as additives for animal feeds (Knorr, 1998; Tannock, 1997). To date, the genus *Pediococcus* contains nine recognized species: *Pediococcus damnosus*, *P. acidilactici*, *P. pentosaceus*, *P. parvulus*, *P. inopinatus*, *P. dextrinicus*, *P. clausenii* (Dobson *et al.*, 2002), *P. cellicola* (Zhang *et al.*, 2005) and *P. stilesii* (Franz *et al.*, 2006) (two other species have been reclassified as *Tetragenococcus halophilus* and *Aerococcus urinaequi*). Most of the species were isolated from fermenting vegetables and beer; *P. cellicola* was the only one isolated from a distilled-spirit-fermenting cellar in China.

Chinese liquors are produced by a solidified grain fermentation and have special fragrances as a result of fermentation by a diverse microbial flora. As lactic acid bacteria were believed to produce aromatic substances during lactic acid fermentation, in this work, these microorganisms were surveyed on behalf of Chinese wine breweries. Among the lactic acid bacteria isolated, several ethanol-tolerant *Pediococcus* strains were obtained, including the novel species *P. cellicola* (Zhang *et al.*, 2005). In the

present study, we isolated and characterized another two *Pediococcus* strains from a brewery in Hebei Province, China. They exhibited distinct phenotypic characteristics, a divergent 16S rRNA gene sequence, and lower rates of DNA–DNA hybridization. All of these characteristics distinguish them from existing *Pediococcus* species, including *P. cellicola*.

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, Belgium; *P. cellicola* LMG 22956^T was from our laboratory. Strains Z-9^T and Z-17 were originally isolated from the walls of a distilled-spirit-fermenting cellar in Hebei Province, China. They were isolated and then purified by streaking the samples on MRS agar (De Man *et al.*, 1960). The strains were routinely grown aerobically on the same medium at 37 °C unless indicated otherwise. Determinations of the end products of glucose fermentation, the lactate isomers, catalase activity and other physiological and biochemical characteristics were carried out as described previously (Zhang *et al.*, 2005).

Genomic DNA was extracted and purified as described by Marmur (1961). The 16S rRNA gene was amplified by means of a PCR using the genomic DNA as the template, and was sequenced using an ABI PRISM 377XL DNA sequencer. The 16S rRNA gene sequences of the most closely related species were retrieved from GenBank and then aligned; the similarity analysis was performed using the

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Z-9^T and Z-17 are AY956789 and DQ400914, respectively.

CLUSTAL_X program (Thompson *et al.*, 1997). The distance matrix was calculated by using the Kimura distance formula implemented in the MEGA3 program (Kumar *et al.*, 2004) and the PHYLIP package (Felsenstein, 1993); the global gap removal item was selected. A reduced phylogenetic tree was produced from trees constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood and maximum-parsimony methods. The stability of the tree clustering was evaluated by means of a bootstrap analysis of 1000 datasets. The DNA G + C content was determined by using the thermal denaturation method (Marmur & Doty, 1962). DNA–DNA relatedness was determined on the basis of the DNA–DNA liquid reassociation rate (De Ley *et al.*, 1970), using a DU-800 spectrophotometer (Beckman) with a thermal controller.

The cells of the two isolates were Gram-positive, non-spore-forming, non-motile cocci occurring in pairs or tetrads. Lactic acid [D(–) and L(+) isomers in the ratio 50 : 50] was the sole product of glucose fermentation; gas was not produced. The strains grew as facultative anaerobes. Catalase activity was not present. These characteristics identified the two strains as members of the genus *Pediococcus*. Conventional methods and the API 50 CH system showed that the two strains could use several monosaccharides and disaccharides as sole carbon and energy sources (see Table 1 and the species description). The

fermentation of rhamnose, lactose and D-turanose was variable between the two strains. The mean generation time of the two strains was 3.15 ± 0.15 h when they were cultured aerobically. In addition, strain Z-9^T and strain Z-17 were able to tolerate 7 and 6.5 % ethanol (v/v), respectively, this being consistent with their natural niche habitat.

The complete 16S rRNA gene sequences of strain Z-9^T and Z-17 were compared with those of *Pediococcus* species retrieved from GenBank. A reduced phylogenetic tree rooted with *Bacillus subtilis* NCDO 1769^T was constructed using the neighbour-joining, maximum-likelihood and maximum-parsimony methods (Fig. 1). The novel strains clustered in the *P. damnosus*–*P. inopinatus*–*P. parvulus*–*P. cellicola* group, showing 16S rRNA gene sequence similarities ranging from 98.3 to 98.7 %.

The meangenomic DNA G + C content of the two novel strains was 39.5 ± 0.5 mol% (39 mol% for type strain Z-9^T), which was within the range for the genus *Pediococcus* (34–42 mol%). The level of DNA–DNA hybridization between strains Z-9^T and Z-17 was 87.5 %, while those between strain Z-9^T and *P. damnosus* LMG 11484^T, *P. inopinatus* LMG 11409^T, *P. parvulus* LMG 11486^T and *P. cellicola* LMG 22956^T were 14.6, 33, 28.7 and 16.8 %, respectively, i.e. below the DNA–DNA relatedness threshold (70 %) recommended for species delineation (Wayne *et al.*, 1987).

Table 1. Differential phenotypic characteristics of strains Z-9^T and Z-17 and phylogenetically related species

Taxa: 1, strain Z-9^T; 2, strain Z-17; 3, *P. cellicola* (Zhang *et al.*, 2005); 4, *P. damnosus* (Back, 1978); 5, *P. inopinatus* (Back, 1978); 6, *P. parvulus* (Gunther & White, 1961); 7, *P. dextrinicus* (Garvie, 1986). The data were also verified in this study by using conventional methods. +, Positive; –, negative; d, 50–89 % of strains positive; ND, no data available. Results of acid-production tests are indicated as: C, data obtained using conventional methods; API, data obtained using the API 50 CH system; no symbol shown, both conventional methods and the API 50 CH system were used.

Characteristic	1	2	3	4	5	6	7
Acid production from:							
Arabinose	+ (C), – (API)	–	+	– (C)	– (C)	– (C)	– (C)
Ribose	–	+ (C), – (API)	+	– (C)	– (C)	– (C)	– (C)
Xylose	–	+ (C), – (API)	+	– (C)	– (C)	– (C)	– (C)
Rhamnose	+	–	+	– (C)	– (C)	– (C)	– (C)
Lactose	+	–	+	– (C)	+	– (C)	d (C)
Methyl α-D-glucoside	+	+	–	d (C)	d (C)	d (C)	+
2-Ketogluconate	–	–	+	ND	ND	ND	ND
Amygdalin	+	+	–	ND	ND	ND	ND
Growth at/in:							
pH 3.8	+	+	+	–	–	–	–
pH 7.5	+	+	+	–	+	+	+
30 °C	+	+	+	–	+	+	+
45 °C	+	+	+	–	–	–	–
4 % NaCl	–	–	+	–	+	+	+
6.5 % NaCl	–	–	–	–	d	+	–
6.5 % Ethanol (v/v)	+	+	+	+	–	–	–
7 % Ethanol (v/v)	+	–	+	+	–	–	–
7.5 % Ethanol (v/v)	–	–	+	+	–	–	–

Fig. 1. Neighbour-joining phylogenetic dendrogram of strain Z-9^T and related lactic acid bacterial species based on 16S rRNA gene sequence similarity. The tree was rooted with *B. subtilis* NCDO 1769^T. Solid circles indicate coincident branches supported by trees constructed using the maximum-likelihood and maximum-parsimony methods. Numbers at branch points are levels of bootstrap support (%) based on analysis of 1000 resampled datasets with the three methods. GenBank accession numbers are given in parentheses. Bar, 1% sequence divergence.

Strain Z-9^T showed higher levels of 16S rRNA gene sequence similarity with *P. damnosus* LMG 11484^T (98.6%), *P. inopinatus* LMG 11409^T (98.3%), *P. parvulus* LMG 11486^T (98.3%) and *P. cellicola* LMG 22956^T (98.5%). However, low levels of DNA–DNA relatedness together with some phenotypic features distinguished strains Z-9^T and Z-17 from these *Pediococcus* species, thus confirming the two novel strains as representing a separate species. Strain Z-9^T, strain Z-17 and *P. cellicola* were all isolated from a Chinese wine brewery, and they differed from *P. damnosus*, *P. inopinatus* and *P. parvulus* in having a higher growth temperature (45 °C), a tolerance of 6.5–7.5% ethanol and a lower final pH (3.8). However, strains Z-9^T and Z-17 differed from *P. cellicola* in fermenting methyl α -D-mannoside, 2-ketoglucuronate, ribose, D-xylose, aesculin and amygdalin and having different tolerances for ethanol and NaCl. The characteristics that differentiated the novel strains from phylogenetically close *Pediococcus* species are shown in Table 1.

Phylogenetic relationships were also analysed on the basis of partial *ftsZ* gene sequence similarity (data not shown). Strain Z-9^T was again shown to cluster in the *P. damnosus*–*P. inopinatus*–*P. parvulus*–*P. cellicola* group, with *ftsZ* gene similarities in the range 77.7–89.6%; the similarity between strain Z-9^T and *P. cellicola* LMG 22956^T (which were isolated from the same environment) was 85.3%, implying that the two species could be closely related.

On the basis of their phenotypic characteristics, phylogenetic relationships and levels of DNA–DNA relatedness, strains Z-9^T and Z-17 represent a novel species of the genus *Pediococcus*, for which the name *Pediococcus ethanolidurans* sp. nov. is proposed.

Description of *Pediococcus ethanolidurans* sp. nov.

Pediococcus ethanolidurans (e.tha.nol.i.dur'ans. N.L. n. ethanol, ethanolis ethanol; L. part. adj. durans insensible, resisting; N.L. part. adj. ethanolidurans ethanol-resisting).

Cells are Gram-positive, non-spore-forming, non-motile, spherical and 0.7–1.0 μ m in diameter after 24 h in MRS

liquid under aerobic conditions 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 [D(–) and L(+) isomers in the approx. ratio 50:50], but not gas, is produced from glucose fermentation. Growth occurs at 15–45 °C, with optimum growth occurring at 32–34 °C. The optimum pH for growth is 6.0–6.8 (range, pH 3.5–8.3). Strains Z-9^T and Z-17 can grow with NaCl at 7 and 6.5%, respectively, but not with 4% NaCl. Aesculin is hydrolysed but arginine is not. Acid is produced from galactose, glucose, fructose, mannose, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, sucrose, trehalose, gentiobiose and D-tagatose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -D-xyloside, sorbose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Strain Z-9^T ferments rhamnose and lactose but not D-turanose, whereas strain Z-17 is the reverse of this. The mean genomic DNA G + C content is 39.5 $\pm$ 0.5 mol% (39 mol% for strain Z-9^T).

The type strain, Z-9^T (= AS 1.3889^T = LMG 23354^T), was isolated from a distilled-spirit-fermenting cellar in Hebei Province, China.

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