Pediococcus stilesii sp. nov., isolated from maize grains

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A Gram-positive, coccus-shaped, lactic acid bacterium, strain LMG 23082^T, was isolated from steeped maize grains. The organism is homofermentative and produces D- and L-lactic acid from glucose. 16S rRNA gene sequence analysis revealed that the organism belongs to the genus *Pediococcus*, with *Pediococcus pentosaceus* and *Pediococcus acidilactici* as nearest neighbours. Genotypic fingerprinting, whole-cell protein electrophoresis, DNA–DNA hybridizations and physiological and biochemical tests allowed differentiation of strain LMG 23082^T from other established *Pediococcus* species. A remarkable feature was that, unlike other pediococci, this bacterium was capable of growth at pH 9.0. The strain studied represents a novel species for which the name *Pediococcus stilesii* sp. nov. is proposed with the type strain LMG 23082^T (= BFE 1652^T = FAIR-E 180^T = CCUG 51290^T), the only currently known isolate of the species.

The genus *Pediococcus* consists of seven species at the time of writing, *Pediococcus acidilactici*, *P. clausenii*, *P. damnosus*, *P. dextrinicus*, *P. inopinatus*, *P. parvulus* and *P. pentosaceus*. The former [*Pediococcus*] *urinaeequi* has recently been reclassified as *Aerococcus urinaeequi* on the basis of 16S rRNA gene sequencing and DNA–DNA hybridizations (Felis *et al.*, 2005). Based on the phylogeny, *P. dextrinicus* is distantly related to the other pediococci (Dobson *et al.*, 2002) and is also phenotypically distinguished from other *Pediococcus* species by the combination of the production of L(+) lactate as the end product of glucose metabolism and the production of CO₂ from gluconate (Back, 1978; Simpson & Taguchi, 1995). Although *P. dextrinicus* is still a member of the genus, it has been suggested that it should be reclassified in the genus *Lactobacillus* (Collins *et al.*, 1990; Dobson *et al.*, 2002). In the present study, we describe a novel *Pediococcus* species, isolated from steeped white maize grains.

Strain LMG 23082^T (= BFE 1652^T = FAIR-E 180^T) was isolated in 1997 in Lagos, Nigeria, from white maize grains,

steeped for 3 days. Isolation and purification conditions were MRS agar (de Man *et al.*, 1960) at 30 °C under aerobic conditions. Analogous cultivation conditions were used for further experiments, unless indicated otherwise.

Cell morphology was determined using phase-contrast microscopy. Cells of strain LMG 23082^T were cocci, with a cell diameter of 1.2 µm. Cells occurred singly, in pairs or tetrads, the latter being typical for pediococci as a result of dividing alternately in two perpendicular directions (Simpson & Taguchi, 1995).

The phylogenetic position of strain LMG 23082^T was determined by complete 16S rRNA gene sequence analysis as described by Vancanneyt *et al.* (2004) with the following modifications: PCR-amplified 16S rDNA was purified by using a NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). Sequencing reactions were purified using a Montage SEQ₉₆ Sequencing Reaction Clean-up kit (Millipore). Sample preparation was assisted using a Tecan Genesis Workstation 200 (Tecan). Electrophoresis of sequence reaction products was performed by using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequence (a continuous stretch of 1529 bp) and sequences of other pediococci, as well as *Lactobacillus* species belonging to the *Lactobacillus*/*Pediococcus* phylogenetic group which were retrieved from EMBL, were aligned and a phylogenetic tree was constructed by the neighbour-joining method using the BioNumerics software package,

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Abbreviation: FAFLP, fluorescent amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of LMG 23082^T is AJ973157.

Fig. 1. Distance matrix tree based on 16S rRNA gene sequence comparisons showing the phylogenetic relationships of *P. stilesii* sp. nov. LMG 23082^T within the *Pediococcus*/*Lactobacillus* phylogenetic group, including sequences of representative type strains of *Lactobacillus* species which occur in this group. The *Bifidobacterium bifidum* sequence was used as the outgroup. Bootstrap percentage values (500 tree replications) are indicated at branch points. EMBL accession numbers are shown. Bar, 10% sequence divergence.

version 3.50 (Applied Maths). Unknown bases were discarded for the analyses. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 500 bootstrap resamplings of the data (Fig. 1). Comparison of the sequence of strain LMG 23082^T with deposited sequences available in the EMBL database revealed highest similarities with *P. pentosaceus* and *P. acidilactici* (sequence similarities of 98.2 and 97.5%, respectively). Within the genus *Pediococcus*, the latter two taxa and *P. clausenii* occupy a distinct branch.

Strains of all recognized *Pediococcus* species were screened using SDS-PAGE of whole-cell proteins. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of protein profiles, and a numerical analysis were performed by using the GelCompar software package, versions 3.1 and 4.0, respectively (Applied Maths). A dendrogram confirmed that LMG 23082^T constitutes a separate branch, distinct from its nearest phylogenetic neighbours, *P. pentosaceus* and *P. acidilactici* (Fig. 2).

Because of its higher taxonomic resolution compared with SDS-PAGE of proteins (Gancheva *et al.*, 1999; Torriani *et al.*, 2001), pediococcal reference strains and LMG 23082^T were also investigated using fluorescent amplified-fragment length polymorphism (FAFLP) fingerprinting of whole genomes. FAFLP fingerprinting was performed as described by Thompson *et al.* (2001) with the following modifications: *EcoRI*/*TaqI* was used as the restriction enzyme combination and the primer combination E01/T01 (both having an adenosine extension at the 3'-end) was applied for selective PCR. The resulting electrophoretic patterns were tracked and normalized using the GeneScan 3.1 software (Applied Biosystems). Normalized tables of peaks, containing fragments of 50–536 bp, were transferred into the BioNumerics software package, version 3.5, and the computer-generated fingerprints were added to an existing database of FAFLP

fingerprints of lactic acid bacteria at the BCCM/LMG Bacteria Collection. For numerical analysis, data between the 75- and 500-bp bands of the internal standard were used. Similarity was calculated using the Dice coefficient and clustering was done using the UPGMA algorithm. The FAFLP fingerprints of all strains were compared with reference profiles of lactic acid bacteria taxa as currently available in the database. FAFLP analysis revealed a separate branch for strain LMG 23082^T (Fig. 3).

As a further genotypic approach, rep-PCR was conducted using the primer (GTG)₅ (5'-GTGGTGGTGGTGGT-3') following the method described by Gevers *et al.* (2001) for lactic acid bacteria. Total genomic DNA was isolated from the type strains of all recognized *Pediococcus* species according to the method of Pitcher *et al.* (1989), as modified by Björkroth & Korkeala (1996), relying on a combined lysozyme and mutanolysin treatment. DNA was amplified in 50 µl volumes containing 100 ng template DNA, 1× *Taq* DNA polymerase buffer (Amersham Pharmacia), 200 µM dNTPs, 50 pM primer, 4% DMSO (Sigma) and 1.5 U *Taq* DNA polymerase (Amersham Pharmacia). PCR products were separated by electrophoresis and images were visualized and analysed as described by Kostinek *et al.* (2005) using the BioNumerics (version 2.5) software (Applied Maths). Groupings of the rep-PCR fingerprints were performed by using the Pearson product-moment correlation coefficient (*r*) and the UPGMA clustering algorithm (Sneath & Sokal, 1973). Using rep-PCR, LMG 23082^T was clearly distinguished from the other type strains and had the closest, although not significant, match with *P. inopinatus* at a correlation level of *r*=0.61 (Fig. 4). It is not known whether the (GTG)₅ sequence, which is highly repetitive on the chromosome of *Escherichia coli* (Versalovic *et al.*, 1994) is indeed present and, if so, how often it would be present in the genomes of pediococci. Nevertheless, even if we assume that it is not, the resolution of the method would resemble a RAPD-PCR-type genotyping method. Therefore, in our study, as in the study of

Fig. 2. Protein profiles and corresponding dendrogram, derived from UPGMA linkage of correlation coefficients (r , expressed as a percentage value for convenience), of *P. stilesii* and other representative pediococci.

Gevers *et al.* (2001), the method was clearly suitable to discriminate among the pediococci at the species level.

For determination of the DNA base composition and DNA–DNA binding values, DNA was isolated from strains LMG 23082^T, *P. pentosaceus* LMG 11488^T and *P. acidilactici* LMG 11384^T and purified according to the method of Marmur (1961) as modified by Stackebrandt & Kandler (1979). The DNA base composition (G + C content) was determined from the thermal melting temperature (T_m) of DNA using a spectrophotometer (Varian Cary 100 Bio UV-Visible). DNA–DNA relatedness was determined spectrophotometrically from renaturation rates according to De Ley *et al.* (1970). DNA from LMG 23082^T was hybridized with DNA of *P. pentosaceus* LMG 11488^T and *P. acidilactici* LMG 11384^T. A low reassociation value of 14.5 % was obtained with the type strain of *P. acidilactici* and 21 % with the type strain of *P. pentosaceus*, values that were well below the recommended 70 % cut-off value that indicates separate species (Wayne *et al.*, 1987). The DNA G + C content of LMG 23082^T was 38.0 mol%.

Phenotypic characterization was performed according to Schillinger & Lücke (1987). D(–)- and L(+)-lactate were determined from culture supernatants after 48 h of growth

using an enzyme test kit (Roche Diagnostics). Maximum pH and NaCl tolerance were determined in MRS broth (Merck) after aerobic incubation for 5 days at 37 °C. The API 50 CHL identification system (bioMérieux) was used to determine the carbohydrate fermentation profile. Biochemical tests results are summarized in Table 1 and given in the species description below. LMG 23082^T is distinguished from *P. pentosaceus* by its inability to produce acid from arabinose and lactose, from *P. acidilactici* by its inability to ferment xylose, and from *P. clausenii* by its ability to produce acid from galactose and DL-lactate from glucose (Table 1). Acid production from ribose distinguishes LMG 23082^T from *P. damnosus*, *P. inopinatus* and *P. parvulus*. Unlike other pediococci, LMG 23082^T grew at pH 9.0 (Table 1) and even at pH 9.6 (result not shown), a phenotypic characteristic which is commonly used to distinguish pediococci, lactococci and streptococci from enterococci (Weiss, 1991; Hardie & Whiley, 1997). The physiological and ecological significance of this alkaliphilic trait is not known, especially when considering that only one strain was isolated, which makes interpretations of such properties difficult. The maximum NaCl concentration for growth after 5 days of incubation was 8 %, a value which is higher than those reported for *P. clausenii* (5 %), *P. damnosus* (5 %) and *P. dextrinicus* (6 %), but lower than

Fig. 3. FAFLP patterns and corresponding dendrogram, derived from the UPGMA linkage of Dice coefficients (expressed as a percentage value for convenience), of *P. stilesii* and other representative pediococci.

Fig. 4. (GTG)₅-PCR fingerprints and corresponding dendrogram, derived from UPGMA linkage of correlation coefficients (r , expressed as a percentage value for convenience), of *P. stilesii* and other representative pediococci.

those for *P. pentosaceus* (10%) and *P. acidilactici* (10%) (Simpson & Taguchi, 1995; Holzapfel *et al.*, 2005).

All results obtained in the present study allowed us to assign strain LMG 23082^T to a novel species, for which we propose the name *Pediococcus stilesii* sp. nov.

Description of *Pediococcus stilesii* sp. nov.

Pediococcus stilesii (stile'si.i. N.L. gen. n. *stilesii* named in honour of Prof. emerit. Michael E. Stiles, a food microbiologist who specialized in food preservation with bacteriocinogenic lactic acid bacteria).

Cells are cocci, 0.6–1.2 µm in diameter. They are Gram-positive, non-motile, do not form spores and occur singly, in pairs or in tetrads. Colonies are white, smooth and circular with a convex elevation and an entire margin. Acid is produced from glucose, ribose, galactose, fructose, mannose, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, gentiobiose and tagatose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β-D-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol,

Table 1. Phenotypic differentiating features of *Pediococcus* species

Species: 1, *P. acidilactici*; 2, *P. pentosaceus*; 3, *P. claussenii*; 4, *P. damnosus*; 5, *P. dextrinicus*; 6, *P. inopinatus*; 7, *P. parvulus*; 8, *P. stilesii* LMG 23082^T. Data partially adapted from Simpson & Taguchi (1995) and Holzapfel *et al.* (2005). +, 90% or more strains positive; –, 90% or more strains negative; d, 11–89% of strains positive.

Characteristic	1	2	3	4	5	6	7	8
Growth at:								
pH 4.5	+	+	+	+	–	+	+	+
pH 7.0	+	+	+	–	+	d	d	+
pH 8.0	+	+	+	–	+	–	–	+
pH 9.0	–	–	–	–	–	–	–	+
35 °C	+	+	+	–	+	+	+	+
40 °C	+	d	+	–	+	d	–	+
45 °C	+	d	–	–	–	–	–	+
48 °C	+	–	–	–	–	–	–	–
Maximum NaCl concentration for growth	10	10	5	5	6	8	8	8
Lactic acid configuration	DL	DL	L(+)*	DL	L(+)	DL	DL	DL
Acid from:								
Arabinose	d	+	–	–	–	–	–	–
Galactose	+	+	–	+	d	+	d	+
Lactose	d	+	–	–	d	+	–	–
Melezitose	–	–	–	d	–	–	–	–
Maltose	–	+	d	d	+	+	d	+
Ribose	+	+	+	–	–	–	–	+
Xylose	+	d	–	–	–	–	–	–
G+C content of DNA (mol%)	42	38	40.5	38.5	40–41	39.5	41	38.0

*Data obtained for the type strain only.

turanose, lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The G+C content of the DNA is 38.0 mol%.

The type strain is LMG 23082^T (=BFE 1652^T=FAIR-E 180^T=CCUG 51290^T), isolated from white maize grains.

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