Leuconostoc palmae sp. nov., a novel lactic acid bacterium isolated from palm wine

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A novel lactic acid bacterium, strain TMW 2.694^T, was isolated among other lactic acid bacteria from palm wine, an alcoholic beverage produced from the sap of various palm tree species. Strain TMW 2.694^T is a Gram-positive, facultatively anaerobic, catalase-negative, non-spore-forming coccus, occurring in long chains. Phylogenetic analysis based on 16S rRNA gene sequencing positioned strain TMW 2.694^T in a distinct line of descent within the genus *Leuconostoc*, with the closest neighbours being *Leuconostoc lactis* JCM 6123^T (98.7 % sequence similarity) and *Leuconostoc citreum* DSM 5577^T (98.8 % sequence similarity). Comparative sequencing of the additional phylogenetic markers *dnaK* and *recA* confirmed the 16S rRNA gene tree topology. Genomic DNA–DNA similarities of strain TMW 2.694^T to *L. lactis* DSM 20202^T and *L. citreum* DSM 5577^T were 45.1 and 17.7 %, respectively. The DNA G+C content is 36.4 mol%. Thus, we propose a novel species within the genus *Leuconostoc*, with the name *Leuconostoc palmae* sp. nov. and the type strain TMW 2.694^T (=DSM 21144^T =LMG 24510^T).

Palm wine is an alcoholic beverage produced from the sap of various palm tree species. The drink is particularly common in parts of Africa, south India and the Philippines. In Africa, the sap is most often taken from wild date palms such as *Phoenix sylvestris* (the palmyra) and *Caryota urens*, from oil palms such as *Elaeis guineensis*, or from *Raphia*, kithul or nipa palms (Bassir, 1962). The initial white liquid is rich in sugars and begins to ferment spontaneously after collection, due to natural micro-organisms in the plant environment or residual micro-organisms left in the collecting container. The sugar composition of the unfermented sap from oil palm trees (e.g. *E. guineensis*) ranges from 9.59 to 10.59 % (w/v) sucrose, whereas the concentrations of glucose and fructose are 1.00 and 0.13–0.73 % (w/v), respectively (Eze & Ogan, 1988). Raffinose occurs in traces only (0.13–0.35 %, w/v). Within a few hours, fermentation yields an aromatic wine of up to 4 % alcohol content.

Besides fermenting yeasts belonging to various genera, e.g. *Saccharomyces*, *Candida*, *Endomycopsis*, *Hansenula*, *Pichia*, *Saccharomycodes* and *Schizosaccharomyces* (Faparusi, 1973; Owuama & Saunders, 1990; Ezeronye & Okerentugba, 2001), the dominant bacterial population of palm wine was previously described as lactic acid bacteria – strains of *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *L. mesenteroides* subsp. *dextranicum*. Acetic acid bacteria occurred only after day 3 of fermentation (Okafor, 1978; Amoa-Awua *et al.*, 2007).

The genus *Leuconostoc*, which at the time of writing consists of 11 species, encompasses a group of coccoid lactic acid bacteria. Recently, all of the rod-shaped species, i.e. *Leuconostoc fructosum*, *Leuconostoc durionis*, *Leuconostoc ficulneum* and *Leuconostoc pseudoficulneum*, were reclassified on the basis of phylogenetic and morphological differences into the genus *Fructobacillus* (Endo & Okada, 2008). The majority of members of both genera were isolated from sugar-rich substrates, e.g. fruits, vegetables and fermentations thereof and/or meat and dairy products.

In this study, we isolated strain TMW 2.694^T from a sample taken from palm wine prepared in Senegal. This strain was conspicuous during routine identification by its 16S rRNA gene sequence, which was different from previously published sequences.

Strain TMW 2.694^T was cultivated routinely at 30 °C on glucose–yeast extract–peptone (GYP) medium containing (l^{-1}): 10 g glucose, 3 g yeast extract, 10 g peptone, 10 g meat extract, 5 g NaCl, 2.0 g sodium acetate, 0.25 g Tween 80, 200 mg $MgSO_4.7H_2O$, 10 mg $MnSO_4.4H_2O$, 10 mg $FeSO_4.7H_2O$ (pH 6.8). Colonies are small (0.5–1.0 mm in diameter), circular, smooth, convex and whitish. Cells are Gram-positive, non-motile, non-spore-forming cocci that are 0.5–0.8 μm in diameter and occur in extremely long

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA* and *dnaK* gene sequences of *Leuconostoc palmae* TMW 2.694^T are AM940225–AM940227, respectively; those for the *recA* and *dnaK* gene sequences of *Leuconostoc holzapfelii* LMG 23990^T are AM940228 and AM940229, respectively; that for the *dnaK* gene sequence of *Leuconostoc lactis* JCM 6123^T is AM940230.

Supplementary figures showing maximum-parsimony and maximum-likelihood 16S rRNA gene trees are available with the online version of this paper.

chains of up to 40 cells. The coccoid cell shape that distinguishes strain TMW 2.694^T from the rod-shaped fructobacilli is shown in Fig. 1. In liquid cultures, cells show a strong tendency toward flocculation and sedimentation.

Physiological characterization

Strain TMW 2.694^T was able to grow at 15–45 °C, but not at temperatures above 45 °C. Optimal growth was observed at 30 °C. The strain tolerated up to 3 g NaCl l^{-1}. It is obligately heterofermentative and 94 % of the total lactic acid produced is of the D configuration, as determined enzymically by using a D/L-lactate test kit (Roche Diagnostics). As some *Leuconostoc* species are known to produce high amounts of tyramine (Moreno-Arribas *et al.*, 2003), decarboxylation of amino acids (arginine, aspartate, glutamate, histidine, lysine, ornithine, phenylalanine and tyrosine) was determined by following the procedure described by Bover-Cid & Holzapfel (1999). No decarboxylation of any amino acid by strain TMW 2.694^T was detected.

A sugar-fermentation profile was determined by using API 50 CHL galleries (bioMérieux) with minor modifications. The pH of the test medium was adjusted to 6.0. All tests were performed in duplicate. Acid is produced from D-glucose, D-fructose, sucrose and D-mannose. Acid is not produced from D-ribose, D-xylose, D-rhamnose, maltose, D-lactose, melibiose or trehalose. Summarized results of morphological, chemotaxonomic and physiological analyses are given in Table 1 and the species description.

Chemotaxonomic characterization

The DNA G+C content was determined by HPLC analyses at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany) following the protocol described by Tamaoka & Komagata (1984) and according to Mesbah *et al.* (1989). The DNA G+C content of strain TMW 2.694^T is 36.4 mol%, which is within the range reported for *Leuconostoc* species (Garvie, 1986). The peptidoglycan structure of the cell wall was determined at the DSMZ. Analysis of the cell-wall composition of strain TMW 2.694^T revealed the presence of alanine, glutamic acid and lysine (4.2 : 1.0 : 0.8) after total hydrolysis (4 M NaOH, 16 h, 100 °C). Peptides obtained by partial hydrolysis (4 M NaOH, 0.75 h, 100 °C) indicate peptidoglycan type A3α (L-Lys–L-Ala_2), corresponding to type A11.5 (http://www.dsmz.de/microorganisms/main.php?content_id=35).

Table 1. Physiological characteristics of *L. palmae* TMW 2.694^T useful in differentiating it from its nearest phylogenetic relatives

Taxa: 1, *L. palmae* TMW 2.694^T; 2, *L .citreum* DSM 5577^T (data from Takahashi *et al.*, 1992); 3, *L. lactis* JCM 6123^T (Garvie, 1986); 4, *L. holzapfelii* LMG 23990^T (De Bruyne *et al.*, 2007). All taxa produce acid from D-fructose.

Characteristic	1	2	3	4
Production of acid from:				
L-Arabinose	−	+	−	+
Maltose	−	+	+	+
D-Mannose	+	+	−	+
Cellobiose	−	+	−	−
D-Salicin	−	+	−	−
Trehalose	−	+	−	+
Melibiose	−	−	−	+
Raffinose	−	−	−	+
Sucrose	+	+	+	−
Gluconate	−	−	−	+
DNA G+C content (mol%)	36.4	43.9	43–45	43.5

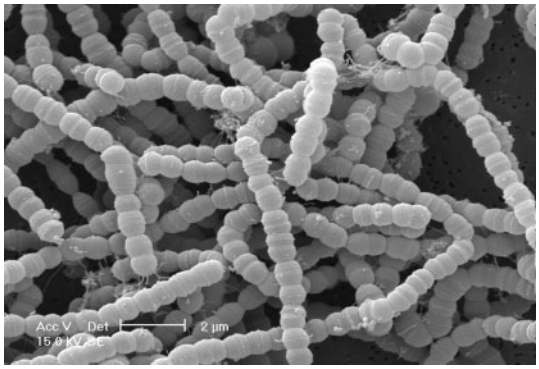

Fig. 1. Electron micrograph of cells of strain TMW 2.694^T grown overnight on GYP medium at 30 °C, showing its typical coccoid cell morphology forming long and tangled chains, which distinguishes it from rod-shaped species of the genus *Fructobacillus*. Bar, 2 μm.

Phylogenetic analyses

To determine the phylogenetic position of strain TMW 2.694^T, the 16S rRNA gene was sequenced over a continuous stretch of 1531 bp. DNA was isolated with an E.Z.N.A. Bacterial DNA kit (Omega Bio-Tek) according to the manufacturer's instructions.

PCR products amplified with primers 616V (5′-AGAGTTTGATYMTGGCTCAG-3′) and 6130R (5′-CAKAAAGGAGGTGATCC-3′) were purified by a QIAquick PCR Purification kit (Qiagen) and eluted with 60 μl elution buffer. DNA sequences were determined by the chain-termination method using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems). The 16S rRNA gene sequence of strain TMW 2.694^T was compared with those of the most closely related species retrieved from GenBank. A phylogenetic tree on the basis of a multiple alignment-based similarity matrix was constructed by the

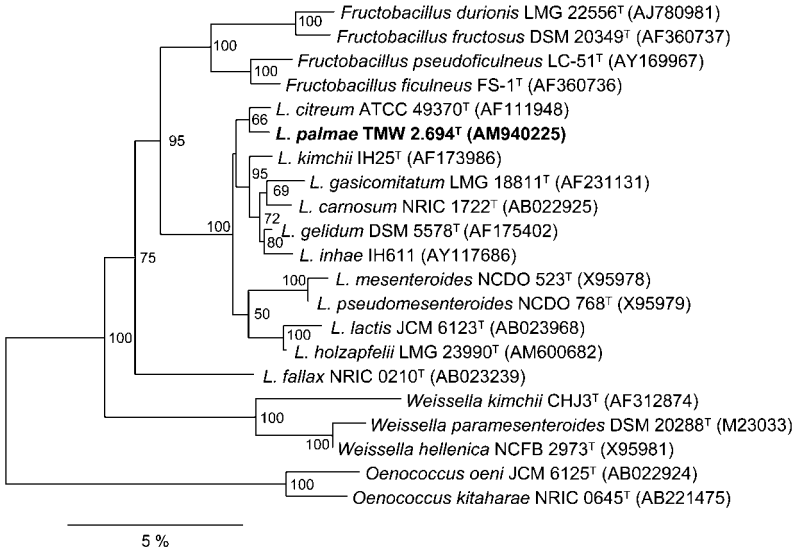

Fig. 2. Neighbour-joining tree derived from 16S rRNA gene sequence analysis, showing the relationship of *L. palmae* TMW 2.694^T to members of the genus *Leuconostoc*. The *Oenococcus* clade was used as an outgroup. Approximately 1500 nt from each sequence was used for the alignment. Bar, 5 % estimated sequence divergence. Numbers indicate bootstrap values >50 % (percentage of 100 replications).

neighbour-joining method (Saitou & Nei, 1987) by using the software package Bionumerics, 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 100 bootstrap resamplings of the data (Fig. 2). This analysis positioned strain TMW 2.694^T in a distinct line of descent within the genus *Leuconostoc*, with the closest

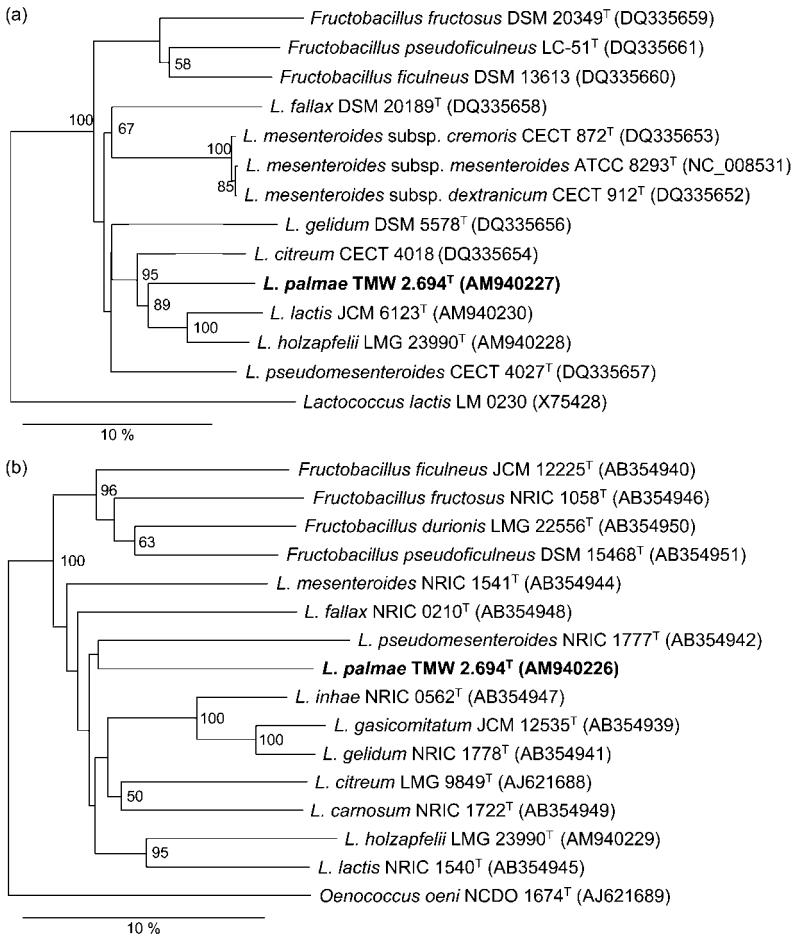

Fig. 3. Neighbour-joining trees obtained with partial protein-encoding sequences of the (a) *dnaK* (735 nt) and (b) *recA* (1810 nt) genes, showing the relationship of *L. palmae* TMW 2.694^T to members of the genera *Leuconostoc* and *Fructobacillus*. The sequences of *Lactococcus lactis* (*dnaK*) and *Oenococcus oeni* (*recA*) were used as outgroup representatives. Bars, 10 % estimated sequence divergence. Numbers indicate bootstrap values >50 % (percentage of 100 replications).

neighbours being *Leuconostoc lactis* JCM 6123^T (98.7 % similarity) and *Leuconostoc citreum* DSM 5577^T (98.8 % similarity). Alternative treeing methods (maximum parsimony and maximum likelihood) result in an essentially similar phylogenetic position (see Supplementary Figs S1 and S2, available in IJSEM Online).

A higher resolution than that provided by 16S rRNA gene sequence data at the species level is achievable by the simultaneous comparison of additional molecular markers throughout the bacterial chromosome. Multilocus sequencing of protein-encoding genes has become a common approach, especially in the resolution of close taxa (Konstantinidis & Tiedje, 2005). Therefore, comparative sequence analyses of the *recA* and *dnaK* genes were performed. Sequences for strain TMW 2.694^T and those of reference strains whose *recA* and *dnaK* sequences were not available in public databases (*Leuconostoc holzapfelii* and *L. lactis*) were amplified by using primers and PCR conditions as described by Dellaglio *et al.* (2005) and Chelo *et al.* (2007), respectively. Analysed sequence lengths were 1815 and 710 bp for *dnaK* and *recA*, respectively. Tree construction for phylogenetic analyses was performed as descibed above. The *recA* and *dnaK* trees revealed only moderate consistency with the 16S rRNA gene-derived topology, showing marked differences in the branching order (Fig. 3). Whilst in the *dnaK* tree, strain TMW 2.694^T clusters closely with *L. lactis*, *L. holzapfelii* and *L. citreum* in one coherent group, it clusters in the *recA* tree with *Leuconostoc pseudomesenteroides*, albeit rather distantly. Discrepancies may be explained by the different dataset (not all sequences of all species were available), different sequence lengths of genes, weak bootstrap support (especially within the *recA* tree) or differences in the apparent rate of evolution of protein-encoding genes with respect to the 16S rRNA gene in this and related genera (Chelo *et al.*, 2007). Nevertheless, each of these trees corroborates the separate species status of strain TMW 2.694^T.

To evaluate and establish a fast screening method, random amplification of polymorphic DNA (RAPD) analysis was carried out with DNA of strain TMW 2.694^T and 10 type strains of additional *Leuconostoc* species. All strains tested showed clearly different profiles (Fig. 4) with primer M13V (5′-GTTTTCCCAGTCACGAC-3′) under conditions described previously (Ehrmann *et al.*, 2003) with minor modifications. The cycling program was three cycles of 94 °C for 3 min, 40 °C for 5 min and 72 °C for 5 min; 32 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 3 min. *Taq* polymerase was obtained from MP Biomedical. The polymerase buffer contained 5 mM $MgCl_2$.

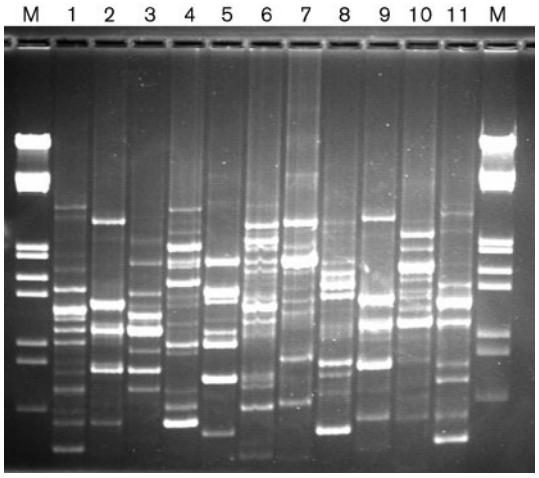

Fig. 4. RAPD profiles differentiating *L. palmae* TMW 2.694^T from other *Leuconostoc* species. Lanes: 1, *L. pseudomesenteroides* TMW 1.201 (isolated from palm wine); 2, *L. citreum* TMW 2.695 (palm wine); 3; *L. palmae* TMW 2.694^T (DSM 21144^T, palm wine); 4, *L. holzapfelii* LMG 23990^T; 5, *L. carnosum* DSM 5576^T; 6, *L. lactis* DSM 20202^T; 7, *L. fallax* DSM 20189^T; 8, *L. gelidum* DSM 5578^T; 9, *L. citreum* DSM 5577^T; 10, *L. mesenteroides* subsp. *dextranicum* DSM 20484^T; 11, *L. pseudomesenteroides* DSM 20193^T; M, DNA marker.

DNA–DNA hybridization

DNA–DNA similarity values were determined by using chromosomal DNA of strain TMW 2.694^T and its closest phylogenetic neighbours in the 16S rRNA gene tree, *L. citreum* and *L. lactis*. Renaturation kinetics were performed at the DSMZ after the protocol of De Ley *et al.* (1970) with modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). All experiments were performed in duplicate. The values of the DNA–DNA reassociation of TMW 2.694^T to *L. lactis* DSM 20202^T and *L. citreum* DSM 5577^T were 45.1 and 17.7 %, respectively. These values are far below the 70 % border recommended as the boundary value for isolates allocated to the same species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001).

Taken together, the presented phenotypic and genotypic data confirm that strain TMW 2.694^T represents a novel species in the genus *Leuconostoc*, for which we propose the name *Leuconostoc palmae* sp. nov.

Description of *Leuconostoc palmae* sp. nov.

Leuconostoc palmae (pal′mae. L. gen. n. *palmae* of a palm tree).

On GYP agar, colonies are small (0.5–1.0 mm in diameter), circular, smooth, convex and whitish. Cells are Gram-positive, non-motile, non-spore-forming cocci that are 0.5–0.8 μm in diameter and occur in very long chains of up to 40 cells. In liquid culture, cells show a strong tendency toward flocculation and sedimentation. Weak growth is observed at 15 °C and almost no growth occurs at 45 °C after 48 h. Optimal growth occurs at 30 °C. Facultatively anaerobic; produces almost exclusively D-lactic acid (94 %). Cells are catalase-negative and gas is produced from glucose. Growth occurs at up to 3 % NaCl. No growth is observed at 5 %

NaCl or higher. Acid is produced from D-glucose, D-fructose, sucrose and D-mannose. Acid is not produced from D-ribose, D-xylose, D-rhamnose, maltose, D-lactose, melibiose or trehalose. The DNA G+C content is 36.4 mol%. Arginine is not hydrolysed. Tyrosine is not decarboxylated. The peptidoglycan type is A3α (L-Lys–L-Ala$_2$).

The type strain, TMW 2.694^T (=DSM 21144^T =LMG 24510^T), was isolated from palm wine prepared in Senegal.

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