Weissella beninensis sp. nov., a motile lactic acid bacterium from submerged cassava fermentations, and emended description of the genus Weissella

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Four Gram-positive, catalase-negative, short rod-shaped or coccolid, heterofermentative lactic acid bacterial strains (2L24P13T, 1L48P15, 1L24P31 and 1L24P34) with unusual phenotypic and genotypic properties were isolated from submerged fermenting cassava on MRS agar. All strains were motile, grew at 15 °C, produced DL-lactic acid from glucose with gas formation and produced ammonia from arginine. Acid was produced from D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melibiose, D-raffinose, sucrose, N-acetylglucosamine and D-mannitol, but not from D-arabinose or xylose. 16S rRNA gene sequence analysis revealed that the strains belonged to the genus Weissella and were most closely related to Weissella ghanensis LMG 24286T. Low DNA–DNA reassociation values were obtained between the isolates and W. ghanensis DSM 19935T. Based on the genetic and phenotypic results, the strains are considered to represent a novel species, for which the name Weissella beninensis sp. nov. is proposed. The type strain is 2L24P13T (=DSM 22752T =LMG 25373T).

Cassava, the most widespread root crop in developing countries, is processed in several ways before consumption. A well-known method that is widely practiced in Africa is lactic acid fermentation (Cooke et al., 1987; Nout & Motarjemi, 1997). Cassava fermentation is a complex process that involves the activities of various micro-organisms and leads to the production of various food products such as gari, agbelima, attiekhé, fufu and lafun. Several types of micro-organisms have been reported to be associated with such fermentations but lactic acid bacteria (LAB) predominate (Amoa-Awua et al., 1996; Caplice & Fitzgerald, 1999; Nout & Sarkar, 1999; Kostinek et al., 2005; Coulin et al., 2006). In a previous study, we identified the micro-organisms associated with submerged cassava root fermentation for lafun production to species level, among which four LAB strains, isolated from MRS agar (Merck), had unusual phenotypic and genotypic properties and were tentatively identified as species of the genus Weissella (Padonou et al. 2009). This study reports the morphological, biochemical and molecular characterization of these strains, designated 2L24P13T, 1L48P15, 1L24P31 and 1L24P34. The four isolates and the reference strains Weissella koreensis DSM 15830T, W. kandleri DSM 20593T, W. soli DSM 14420T and W. ghanensis DSM 19935T were maintained in MRS broth at 30 °C for 2–3 days and stored in MRS medium with 20 % (v/v) glycerol at −80 °C.

Abbreviations: LAB, lactic acid bacteria; SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2L24P13T is EU439435.
After growth for 4 days at 30 °C on MRS agar under anaerobic conditions, all four isolates exhibited colonies that were 1–2 mm in diameter, white to creamish white, smooth, circular and convex. Cell morphology was determined after overnight growth in MRS broth at 30 °C using phase-contrast microscopy and scanning electron microscopy (SEM). A 30 μl sample of the culture was diluted 10-fold with sterile MilliQ-water and filtered through a polycarbonate filter (pore size 0.2 μm). While still attached to the filter, the bacteria were dehydrated with a graded ethanol series, dried with hexamethyldisilazane, fixed with glutaraldehyde and osmium tetroxide, coated with gold/palladium and observed using a FEI Quanta 200 microscope at an acceleration voltage of 30 kV. Cells were short rod-shaped or coccoid, 0.7–0.9 μm wide and 0.9–1.5 μm long, occurring singly, in pairs or in short chains (Fig. 1a, b). Motility was observed for all four isolates under the phase-contrast microscope, although the motility was variable and sometimes only a small fraction of a population was actively moving. Peritrichous flagella were observed using SEM (Fig. 1b, c).

The Gram reaction was determined using the KOH method (Gregersen, 1978). Catalase activity was tested by the standard method using 3 % H2O2. Growth was determined (Gregersen, 1978). Catalase activity was tested by the following the protocol of Jayne-Williams (1976) and using the API 50CH system (bioMe`rieux). The results of these phenotypic tests are given in Table 1 and the species description.

For all four isolates, the nearly complete 16S rRNA gene sequence was determined as described below. DNA was extracted according to the method of Pitcher et al. (1989), as modified for Gram-positive bacteria (Björkroth & Korkeala, 1996). The 16S rRNA gene was amplified by PCR and amplification products were purified and commercially sequenced at GATC Biotech as described previously (Kostinek et al., 2005). Sequences were manually corrected and aligned using Vector NTI Suite 10. The closest phylogenetic relatives were determined by aligning the corrected sequences with 16S rRNA gene sequences in the GenBank database by using the BLAST algorithm (Altschul et al., 1997). Strains 2L24P13T, 1L48P15, 1L24P31 and 1L24P34 had identical 16S rRNA gene sequences. The 16S rRNA gene sequences of the four isolates and their closest phylogenetic relatives were aligned and phylogenetic trees were constructed with the neighbour-joining, maximum-likelihood and maximum-parsimony methods using Bionumerics version 4.5 (Applied Maths). Unknown bases were discarded from the analysis and 1450 nucleotides were included. The statistical reliability of the topology of the phylogenetic trees was evaluated using bootstrap calculations with 1000 resamplings. As seen from the maximum-parsimony tree (Fig. 2), the four strains, represented here by strain 2L24P13T, and W. ghanensis LMG 24286T formed a distinct subgroup within the genus Weissella. Similar results were obtained with the neighbour-joining and maximum-likelihood methods (results not shown). Comparison of the 16S rRNA gene sequence of strain 2L24P13T revealed the highest 16S rRNA gene sequence similarity with W. ghanensis LMG 24286T (97.3 %) and lower similarities with W. confusa JCM 1093T, W. cibaria NRIC 0136 and W. koreensis S-5623T (92–94 %).

PCR with genus-specific primers demonstrated that the four isolates belonged to the genus Weissella: all of them produced a 1.2 kb fragment with the Weissella-specific primer Weissgrp (5‘-GATGGTTCTGCTACCACTAAG-3‘), using the procedure described by Schillinger et al. (2008). The four isolates were further investigated by two genotyping methods: repetitive element palindromic (rep)-PCR, using the primer GTG5 (5‘-GATGGTTCTGCTACCACTAAG-3‘) and following the method of Gevers et al. (2001) for LAB as described by Franz et al. (2006), and randomly amplified

**Fig. 1.** Scanning electron micrographs of cells (a, b and c) and flagella (b and c) of strain 2L24P13T grown overnight in MRS broth at 30 °C. Bars, 1 μm (a, b), 0.5 μm (c).
polymorphic DNA (RAPD)-PCR, using primer M13 (5'-GAGGGTGCGGTTCT-3') or primer AP4 (5'-TCAC-GCTGCA-3'; Andrighetto et al., 2001). A dendrogram was derived using the unweighted pair group method with arithmetic averages linkage of correlation coefficients (Fig. 3). Strains 2L24P13T, 1L48P15, 1L24P31 and 1L24P34 clustered closely together (r=90–95%) and clustered with strains of W. koreensis, W. kandleri, W. soli and W. ghanensis with a low correlation (r=12–20%).

For determination of G+C content and DNA–DNA relatedness, DNA was extracted from the four isolates and W. ghanensis DSM 19935T and purified following the protocol of Marmur (1961) as modified by Stackebrandt &

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Table 1. Differential characteristics of Weissella beninensis sp. nov. strains and other Weissella species

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*Delayed reaction.

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polymorphic DNA (RAPD)-PCR, using primer M13 (5'-GAGGGTGCGGTTCT-3') or primer AP4 (5'-TCAC-GCTGCA-3'; Andrighetto et al., 2001). A dendrogram was derived using the unweighted pair group method with arithmetic averages linkage of correlation coefficients (Fig. 3). Strains 2L24P13T, 1L48P15, 1L24P31 and 1L24P34 clustered closely together (r=90–95%) and clustered with

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![Fig. 2. Maximum-parsimony phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the phylogenetic positions of strain 2L24P13T and strains of the species of the genus Weissella. Percentages at nodes are bootstrap values based on 1000 resamplings. Bar, 2% sequence divergence.](http://ijs.sgmjournals.org)
Kandler (1979). The G+C content of DNA was determined by a modification of the fluorometric method described by Gonzalez & Saiz-Jimenez (2002) using a iQ5 real-time thermocycler (Bio-Rad). Thermal denaturation was performed in 1 × standard saline citrate with 25 μg genomic DNA and 20 × EvaGreen (Biotrend Chemicals) as the fluorescent dye. DNA of six LAB strains with completely sequenced genomes was used to create a calibration curve of G+C values versus the melting temperatures (Tm values) measured with the thermocycler. Regression analysis was performed to derive a formula for the calculation of the G+C content in mol%. DNA–DNA relatedness was determined using the fluorometric method of Jakava-Viljanen et al. (2008) with EvaGreen and a real-time thermocycler. Genomic DNA from strains 2L24P13T, 1L48P15, 1L24P31 and 1L24P34 were hybridized to each other and to W. ghanensis DSM 19935T. The G+C contents of strains 2L24P13T, 1L48P15, 1L24P31 and 1L24P34 were 37.0–37.2 mol%, which can be compared with the G+C content of 40 mol% for W. ghanensis DSM 24286T (De Bruyne et al., 2008; Table 1). The DNA–DNA relatedness values between strains 2L24P13T, 1L48P15, 1L24P31 and 1L24P34 were 84–122 %, which indicated that the strains are con-specific, and between the four isolates and W. ghanensis DSM 19935T they were 19–42 %. Thus, DNA–DNA relatedness values between each isolate and its closest relative were well below the 70 % cut-off value recommended to indicate separate species (Wayne et al., 1987).

Based on the morphological, physiological and genetic characteristics described above, strains 2L24P13T, 1L48P15, 1L24P31 and 1L24P34 clearly formed a homogeneous genetically distinct group that was most closely related to W. ghanensis. In addition to their genotypic characteristics, the four isolates could be clearly differentiated from their phenotypic and genotypic closest relatives by their motility and ability to produce acid from raffinose, melibiose and lactose. In conclusion, the isolates represent a novel species in the genus Weissella, for which the name Weissella beninensis sp. nov. is proposed, with strain 2L24P13T as the type strain. Weissella beninensis sp. nov. represents the first description of a motile species within the genus Weissella. Therefore, the genus Weissella described by Collins et al. (1993) should be emended.

**Emended description of the genus Weissella Collins et al. 1993**

This description is based on that given by Collins et al. (1993). Cells are generally short rods with rounded to tapered ends or coccoid in shape occurring singly, in pairs or in short chains. Cells are Gram-positive and non-motile, with the exception of W. beninensis where all known strains are motile. Endospores are not formed. Catalase and cytochromes are not produced. Chemo-organotrophs with complex nutritional requirements. Heterofermentative. The configuration of lactic acid produced from glucose is either Dl- or (−)-D, depending on the species. Acidoduric. Growth occurs at 15 °C, but not at 45 °C (with the exception of some strains of W. confusa). Strains of some species hydrolyse arginine. The cell-wall peptidoglycan is based upon lysine; the interpeptide bridge contains alanine or serine and alanine as typical constituents. The G+C content of DNA is 37–47 mol%. The type species is Weissella viridescens.

**Description of Weissella beninensis sp. nov.**

Weissella beninensis (ben.in.en’sis. N.L. fem. adj. beninensis pertaining to Benin, where the type strain was isolated). Cells are Gram-positive, catalase-negative and short rod-shaped or coccoid (0.7–0.9 × 0.1–1.5 μm). Motile with peritrichous flagella. Non-spor-forming. Occur singly, in pairs or in short chains. Colonies are 1–2 mm in diameter, white to creamish white, smooth, circular and convex after 3–4 days of anaerobic growth. Grows at 15 °C, at pH 3.9–8.0 and with 4 % NaCl. No growth at 45 °C. Ammonia is produced from arginine and gas is produced from glucose. (−)-Dl- and (+)-L-lactic acid are produced as end products of glucose metabolism. Dextran is formed...
from sucrose by most strains. Acid is produced from L-arabinose, D-fructose, D-glucose, D-galactose, β-gentiobiose, lactose, maltose, D-mannose, melibiose, D-raffinose, sucrose, D-mannitol, sorbitol, N-acetylglucosamine, amygdalin, arbutin, aesculin. Acid production from ribose, salicin (delayed reaction), cellobiose and trehalose is strain-dependent. Does not ferment D-arabinose, D- or L-fucose, D-lyxose, rhamnose, L-sorbose, D-tagatose, trehalose, D-turanose, D- or L-xylose, adonitol, D- or L-arabitol, dulcitol, erythritol, glycerol, inositol, xylitol, methyl D-D-gluco-side, methyl D-D-mannoside, methyl β-xyloside, inulin, starch, gluconate, glycogen or 2- or 5-ketogluconate. The G+C content is 37.0–37.2 mol%.

The type strain is 2L24P13T (=DSM 22752T=LMG 25373T), isolated from cassava fermentations in Ketou, Benin.

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References


