Weissella ghanensis sp. nov., isolated from a Ghanaian cocoa fermentation

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During a study on lactic acid bacteria (and their species diversity) in spontaneous heap fermentations of Ghanaian cocoa beans, two strains, designated 215^T and 194B, were isolated. A phylogenetic analysis based on 16S rRNA gene sequences demonstrated that these strains represented a distinct lineage close to the genus *Weissella* and showing only 92.1 % 16S rRNA gene sequence similarity with respect to their closest neighbour, *Weissella soli* LMG 20113^T. Whole-cell protein electrophoresis, fluorescent amplified fragment length polymorphism fingerprinting of whole genomes and physiological and biochemical tests confirmed the unique taxonomic position of the two novel isolates. On the basis of the results of the morphological and biochemical tests and 16S rRNA gene sequence analysis, strains 215^T and 194B represent the most peripheral lineage of the genus *Weissella*, for which we propose the name *Weissella ghanensis* sp. nov. The type strain is 215^T (=LMG 24286^T=DSM 19935^T).

The genus *Weissella* was created by Collins *et al.* (1993), in the course of a phylogenetic analysis of some unknown *Leuconostoc*-like lactic acid bacteria, to accommodate five heterofermentative *Lactobacillus* species and a species formerly classified as *Leuconostoc paramesenteroides*. Novel species of the genus *Weissella* have mainly been isolated from fermented foods: *Weissella hellenica* strains were isolated from fermented sausages (Collins *et al.*, 1993), *Weissella kimchii* (a later heterotypic synonym of *Weissella cibaria*) (Ennahar & Cai, 2004) and *Weissella koreensis* strains were isolated from kimchi (Choi *et al.*, 2002; Lee *et al.*, 2002), and *Weissella thailandensis* strains were isolated from fermented fish (Tanasupawat *et al.*, 2000). The exceptions are *Weissella soli* strains, which were isolated from soil (Magnusson *et al.*, 2002), and some *W. cibaria* strains that were detected in both fermented food and clinical samples (Björkroth *et al.*, 2002).

Abbreviations: FAFLP, fluorescent amplified fragment length polymorphism; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain 215^T and strain 194B are AM882997 and AM882998, respectively.

An extended neighbour-joining phylogenetic tree (based on 16S rRNA gene sequences) and whole-cell protein profiles for the novel strains, type strains of recognized *Weissella* species and other closely related taxa are available as supplementary figures with the online version of this paper.

The population dynamics of spontaneous heap fermentations of cocoa beans in Ghana during the main cropping season of 2005 (October–December) were studied by Camu *et al.* (2007). The levels of lactic acid bacteria species diversity of different fermentation heaps and different fermentation times were signalled by the relative intensities of bands obtained using a culture-independent technique, namely 16S rRNA gene sequence-based PCR-denaturing gradient gel electrophoresis. This analysis detected an unidentified bacterium that appeared in five of the seven heap fermentations studied, from the beginning of the fermentation up to 48 h after the start of the fermentation process. The 16S rRNA gene sequence of this bacterium was determined. Only three isolates (215^T, 194B and 225) that had 16S rRNA gene sequences identical to that of the unidentified bacterium were obtained (and subsequently cultivated) from the same fermenting cocoa bean heap in New Tafo, Ghana (Camu *et al.*, 2007). Isolate 225 lost viability during subsequent cultivation. To check clonality using randomly amplified polymorphic DNA (RAPD; Chen *et al.*, 2001), DNA from each isolate was prepared by alkaline lysis (Coenye *et al.*, 2002) and the DNA solutions were stored at –20 °C. Fingerprinting using RAPD was performed with primers RAPD-270 (5'-TGCGCGCGGG-3') and RAPD-272 (5'-AGCGGGCCAA-3'), as described previously (Mahenthiralingam *et al.*, 1996). Isolates 215^T and 194B were found to be non-clonal strains (data not shown).

The taxonomic positions of strain 215^T and strain 194B were first investigated by means of 16S rRNA gene sequence analysis, as described by Vancanneyt *et al.* (2004) but with the following modification: the PCR-amplified 16S rRNA gene was purified using a NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). Sequencing reactions were purified using a Montage SEQ96 Sequencing Reaction Clean-up kit (Millipore). Electrophoresis of sequence reaction products was performed by using an ABI Prism 3100 genetic analyser (Applied Biosystems). A BLAST search and phylogenetic analysis based on 16S rRNA gene sequence similarity showed that strain 215^T was related to members of the genus *Weissella*, the highest sequence similarity (92.1 %) being found with respect to *W. soli* LMG 20113^T. The 16S rRNA gene sequences obtained (continuous stretches of 1541 bp and 1538 bp) and sequences of the type strains from all species of the genus *Weissella* were aligned using CLUSTAL_X (version 1.81; Thompson *et al.*, 1997) and clustered using the BioNumerics software package, version 5.10 (Applied Maths). The maximum-parsimony and maximum-likelihood tree-building methods revealed similar topologies to those obtained in a phylogenetic tree constructed using the neighbour-joining approach (Fig. 1), which was calculated using bootstrapping based on 200 replications. The neighbour-joining analysis revealed strain 215^T as a distinct phylogenetic lineage close to the genus *Weissella*. A broader phylogenetic analysis including all of the phylogenetically closest neighbours of the genus *Weissella*, namely the genera *Pediococcus*, *Oenococcus* and *Leuconostoc*, is shown in Supplementary Fig. S1 (available in IJSEM Online). This analysis demonstrated that strains 215^T and 194B can be considered as representing the most peripheral branch of the genus *Weissella*. On the basis of the data from additional analyses described below, these strains were not assigned to a novel genus but were recognized as representing a novel species of the genus *Weissella*. This peripheral position could be the result of

differences in the rate of evolution of strains 215^T and 194B with respect to *Weissella* species. The large divergence in 16S rRNA gene sequence (>7.4 %) demonstrates unambiguously that the two strains represent a novel species that is closely related to the recognized species of the genus *Weissella*.

SDS-PAGE of whole-cell proteins and fluorescent amplified fragment length polymorphism (FAFLP) techniques were used to characterize these novel bacteria in more detail and were performed as described by Pot *et al.* (1994) and Franz *et al.* (2006), respectively. Protein profiles for *Weissella* reference strains were taken from Björkroth *et al.* (2002). To complete the protein profile reference data for all *Weissella* species, protein profiles for *W. koreensis* LMG 21853^T and *W. soli* LMG 20113^T were generated. The whole-cell protein profiles of strains 215^T and 194B formed a distinct branch within the dendrogram obtained (see Supplementary Fig. S2, available in IJSEM Online). Similarly, reference FAFLP data for all type strains were supplemented with data for *W. koreensis* LMG 21853^T, *W. soli* LMG 20113^T, *W. thailandensis* LMG 19821^T and *Weissella kandleri* LMG 18979^T. The unique taxonomic position of strains 215^T and 194B was again confirmed by numerical analysis of the FAFLP fingerprints (Fig. 2).

The DNA G + C content of strain 215^T was determined, as described by Mesbah *et al.* (1989), using a Waters Breeze HPLC system and an XBridge Shield RP18 column. The solvent used was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % (v/v) acetonitrile. *Escherichia coli* LMG 2093 DNA was used as the control and non-methylated lambda phage DNA (Sigma) was used as the calibration reference. The DNA G + C content of strain 215^T was found to be 40.0 mol%, which falls within the range for the genus *Weissella* (37–47 mol%; Collins *et al.*, 1993).

Cell and colony morphology were investigated after growth on MRS agar (pH 5.4, Oxoid) and 48 h aerobic incubation

Fig. 1. Phylogenetic neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strains 215^T and 194B with respect to members of the genus *Weissella*. Bootstrap percentages (based on 200 replications) are shown at branch points. *Pediococcus damnosus* DSM 20331^T was used as the outgroup. Bar, 2 % sequence divergence.

Fig. 2. FAFLP patterns and dendrogram based on UPGMA linkage of Dice coefficients of strains 215^T and 194B and reference strains from all of the recognized species of the genus *Weissella*.

at 28 °C, unless stated otherwise. Standard biochemical tests and growth experiments were determined in MRS broth (pH 5.4, Oxoid). All tests were performed in triplicate, unless stated otherwise. Growth was tested at 4, 10, 15, 37 and 45 °C, in the presence of 5, 6, 7 and 8 % NaCl and at pH 3.9 at 37 °C. The production of gas from 2 % glucose and 2 % gluconate in modified MRS broth (pH 5.4, without the addition of triammonium citrate) was investigated using Durham tubes. Arginine hydrolysis was evaluated in a medium containing 0.5 % tryptone, 0.5 % yeast extract, 0.3 % L-arginine, 0.05 % glucose, 0.2 % K₂HPO₄ (pH 7.0), with methyl orange as an indicator. Metabolites from glucose were determined in duplicate by means of HPLC (Waters apparatus). The proportions of D- and L-lactate were determined enzymically (R-Biopharm). Carbohydrate-fermentation tests were performed using the API 50 CHL system (bioMérieux). Other physiological and biochemical characteristics were determined using the API 20E system (bioMérieux); both strips were analysed in duplicate, according to the manufacturer's instructions. For the detection of glucose metabolites and D/L lactate isomers, strains were grown for 96 h at 30 °C in MRS broth (pH 5.4, Oxoid). The results of this characterization are given in the species description.

In spite of the peripheral position of strains 215^T and 194B in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1), the biochemical characteristics observed for the two strains (i.e. their cell and colony morphology, motility, catalase activity, heterofermentative metabolism, arginine hydrolysis and growth temperatures) and their DNA G + C content conformed to the expected profile of members of the genus *Weissella* (Collins *et al.*, 1993). The two novel strains could be clearly distinguished from homofermentative strains (e.g. pediococci) by the formation of gas from carbohydrates. The ability of strains 215^T and 194 to hydrolyse arginine and to form DL-lactate served to differentiate them from strains of the genus *Leuconostoc*. The novel isolates could be differentiated from recognized

Weissella species on the basis of phylogeny, whole-cell protein profiles, AFLP results and biochemical data (Table 1). Thus, strains 215^T and 194 represent a novel species of the genus *Weissella*, for which the name *Weissella ghanensis* sp. nov. is proposed.

Description of *Weissella ghanensis* sp. nov.

Weissella ghanensis (gha.nen'sis. N.L. fem. adj. *ghanensis* pertaining to Ghana).

Cells are Gram-positive, catalase-negative and facultatively anaerobic. They do not form spores. Gliding motility is not observed. Cells are small rods (1.0 µm wide and 2.0–4.0 µm long) and appear singly, in pairs or in short chains. Colonies are beige, opaque, smooth, circular or irregular with low convex elevation and entire margins and are 1.5–2.0 mm in diameter. Growth is obtained at 15–37 °C. No growth is observed in MRS broth supplemented with 5 % NaCl. For strain 215^T, lactic acid (D/L, 90 : 10), acetic acid, ethanol and gas formation are detected after growth on glucose, indicating the heterofermentative nature of this strain. Strain 194B produces different end metabolites. Both lactic acid and acetic acid are detected as end products of glucose metabolism. Mainly D-lactate (D/L, 95 : 5) is produced and no gas production is observed. Strain 215^T and strain 194B both produce slime from sucrose and are able to hydrolyse arginine. Acid is produced from glucose, fructose, mannose, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, trehalose and gentiobiose, but not from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-D-xylopyranoside, galactose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate or 5-ketogluconate. Strain 215^T produces acid from sucrose; strain 194B does not produce acid from sucrose.

Table 1. Differential characteristics of *Weissella ghanensis* sp. nov. and other species of the genus *Weissella*

Taxa: 1, strains 215^T and 194B; 2, *W. halotolerans*; 3, *W. minor*; 4, *W. viridescens*; 5, *W. soli*; 6, *W. kandleri*; 7, *W. koreensis*; 8, *W. cibaria*; 9, *W. confusa*; 10, *W. thailandensis*; 11, *W. hellenica*; 12, *W. paramesenteroides*. Data partially adapted from Collins *et al.* (1993), Tanasupawat *et al.* (2000), Björkroth *et al.* (2002), Choi *et al.* (2002), Lee *et al.* (2002), Magnusson *et al.* (2002) and Ennahar & Cai (2004). +, Positive for at least 90 % of the strains; –, negative for at least 90 % of the strains; d, positive for 11–89 % of strains; NT, not tested.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Acid from:												
Arabinose	–	–	–	–	+	–	+	+	–	+	+	d
Cellobiose	+	–	+	–	–	–	–	+	+	–	–	d
Fructose	+	+	+	+	–	+	NT	+	+	+	+	+
Galactose	–	–	–	–	–	+	–	–	+	+	–	+
Maltose	+	+	+	+	+	–	–	+	+	+	+	+
Melibiose	–	–	–	–	+	–	–	–	–	+	–	+
Raffinose	–	–	–	–	+	–	–	–	–	+	–	d
Ribose	–	+	+	–	+	+	+	–	+	+	–	d
Salicin	+	–	–	–	+	–	NT	+	+	–	–	–
Sucrose	d	–	+	d	+	–	–	+	+	d	+	+
Trehalose	+	–	+	d	+	–	–	–	–	d	+	+
Xylose	–	–	–	–	+	–	+	+	+	–	–	d
Hydrolysis of aesculin	+	–	+	–	+	–	–	+	+	NT	NT	d
NH ₃ from arginine	+	+	+	–	+	+	+	+	+	–	–	–
Dextran formation	+	NT	–	NT	–	+	+	+	+	–	–	–
Lactic acid configuration	DL	DL	DL	DL	D	DL	D	DL	DL	D	D	D
DNA G + C content (mol%)	40	45	44	41–44	43	39	37	44–48	45–47	38–41	39–40	37–38

The type strain, 215^T (=LMG 24286^T=DSM 19935^T), was isolated from Ghanaian cocoa bean heaps undergoing fermentation. The DNA G + C content of the type strain is 40.0 mol%. The two available strains (215^T and 194B) both originated from the same cocoa bean heap undergoing fermentation in New Tafo, Ghana. However, Camu *et al.* (2007) detected this organism in five out of seven spontaneous heap fermentations of cocoa beans studied, indicating that this species is common in Ghanaian cocoa bean heaps that are undergoing fermentation.

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