Lactobacillus ghanensis sp. nov., a motile lactic acid bacterium isolated from Ghanaian cocoa fermentations

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Three Gram-positive, catalase-negative, motile, rod-shaped strains, designated L486, L489^T and L499, were isolated from fermenting cocoa. These organisms produced DL-lactic acid from glucose without gas formation. Ammonia was not produced from arginine. Acid was produced from amygdalin, D-cellobiose, aesculin, D-fructose, D-glucose, D-galactose, D-mannitol, D-mannose, N-acetylglucosamine, L-rhamnose, sucrose, salicin and D-trehalose. The cell walls contained peptidoglycan of the D-meso-diaminopimelic acid type. A 16S rRNA gene sequence analysis revealed that the isolates belong phylogenetically to the genus *Lactobacillus* and are closely related to *Lactobacillus nagelii*, *Lactobacillus vini* and *Lactobacillus satsumensis*. Low DNA–DNA reassociation values were obtained between the isolates and the phylogenetically closest neighbours. On the basis of the genetic and phenotypic results, the isolates are considered to represent a novel species, for which the name *Lactobacillus ghanensis* is proposed. The type strain is L489^T (= DSM 18630^T = CCUG 53453^T).

Cocoa beans, the principal raw material of chocolate, have to be fermented, dried and roasted to produce the characteristic cocoa flavour and taste. The fermentation of cocoa is a microbiologically complex process involving the activities of yeasts, lactic acid bacteria and acetic acid bacteria (Schwan & Wheals, 2004). During an investigation of the micro-organisms involved in the fermentation of cocoa beans, a number of isolates with unusual properties were isolated from MRS agar and tentatively identified as *Lactobacillus* species (Nielsen *et al.*, 2007). This study presents the morphological, biochemical and molecular characterization of three of these isolates, designated L486, L489^T and L499.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains L486, L489^T and L499 are DQ867003, DQ523489 and DQ867004, respectively.

Maximum-likelihood and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences showing the phylogenetic position of strains L486, L489^T and L499 within the *L. casei* cluster are available as supplementary figures with the online version of this article.

Strains L486, L489^T, L499 and the reference strains *Lactobacillus nagelii* DSM 13675^T, *Lactobacillus satsumensis* DSM 16230^T and *Lactobacillus vini* DSM 20605^T were grown in MRS broth (Merck) at 30 °C for 2–3 days; for long-term storage, 20 % glycerol was added and the cultures were stored at –80 °C.

The cell morphologies of cultures grown overnight in MRS broth (30 °C) were determined using phase-contrast microscopy and scanning electron microscopy. For the latter, 30 µl culture diluted 10-fold with sterile MilliQ water was filtered through a polycarbonate filter (pore size, 0.2 µm), exposed to osmium vapour for 1 h and coated with gold–palladium. Cells were observed in a FEI Quanta 200 scanning electron microscope at 15 kV. The cells were rod-shaped (0.7–0.8 × 1.4–2.5 µm), occurring either singly, in pairs or in short chains of three to four cells (Fig. 1). The cells were observed to be highly motile under the phase-contrast microscope. Scanning electron microscopy showed the presence of peritrichous flagella (Fig. 1).

Fig. 1. Scanning electron micrograph of cells of strain L489^T grown overnight in MRS broth at 30 °C, showing the presence of flagella. Bar, 2 µm.

Colonies grown on MRS agar (Merck) incubated anaerobically for 4 days at 30 °C were 2–3 mm in diameter, white to creamy white, smooth, circular, convex and with entire or slightly uneven margins. If cultures were incubated aerobically (4 days, 30 °C), the colonies were pinpoint-sized.

The Gram reaction and catalase activity were tested using standard methods. Growth at 15 and 45 °C (in MRS broth), in the presence of 6.5 % NaCl and at pH 3.9 and 8.0, gas production from glucose (in MRS broth, with inverted Durham tubes, and determined at 30 °C), the production of ammonia from arginine and the presence of D-meso-diaminopimelic acid in the cell wall were tested by following the protocol of Schillinger & Lücke (1987). The configuration of the lactic acid enantiomer produced was determined enzymically (Boehringer Mannheim) (Schillinger & Lücke, 1987).

The carbohydrate-fermentation patterns of strains L486, L489^T and L499 were determined in microtitre plates by following the protocol of Jayne-Williams (1976) and, in addition, using the API 50 CHL identification system (bioMérieux).

For 16S rRNA gene sequencing, DNA was extracted using the method of Björkroth & Korkeala (1996) and the almost-complete 16S rRNA gene was amplified using primers 7f and 1510r (Lane, 1991; Björkroth & Korkeala, 1996). All reactions were carried out in a 50 µl volume containing 1.25 U *Taq* DNA polymerase (Amersham Biosciences), 5 µl 10 × PCR buffer (Amersham Biosciences), 200 µM each dNTP (Amersham Biosciences), 3.0 mM MgCl₂ (Amersham Biosciences), 0.1 µM each primer (DNA Technologies), 1 % (v/v) formamide (Merck), 0.1 % (w/v) BSA (New England Biolabs), 20 ng DNA template and sufficient sterile MilliQ water to adjust the volume to 50 µl. The PCR was performed using the following thermocycling program: 5 min initial denaturation at 94 °C, 30 cycles of 94 °C for 90 s, 52 °C for 30 s and 72 °C for 90 s and then a final elongation step at 72 °C for 7 min. Following purification (using the Qiagen PCR purification kit), the PCR products were sequenced in both directions using a CEQ 2000 automated sequencer (Beckman Coulter) and a CEQ 2000 Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter) or sent to a commercial sequencing facility (DNA Technology A/S, Aarhus, Denmark). Sequences were corrected manually and aligned using Vector NTI Suite 7 (Informax). 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 L486 and L489^T had 100 % identical 16S rRNA gene sequences, whereas L499 differed by one nucleotide from the former two. The 16S rRNA gene sequences of strains L486, L489^T, L499 and the sequences of the closest phylogenetic relatives retrieved from the GenBank database were aligned and phylogenetic trees constructed with the neighbour-joining, maximum-likelihood and maximum-parsimony methods using BIONUMERICS, version 3.5 (Applied Maths). Unknown bases were disregarded for the analysis; 1450 nt were included. The statistical reliability of the topology of the phylogenetic trees was evaluated using bootstrap resampling of the data (1000 resamplings) (Fig. 2; also see Supplementary Figs S1 and S2 available in IJSEM Online). In comparisons between the 16S rRNA gene sequence of L489^T and those of type strains in the GenBank

Fig. 2. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing the phylogenetic position of strains L486, L489^T and L499 within the *Lactobacillus casei* cluster of micro-organisms (accession numbers are shown in parentheses). Bootstrap percentages (based on 1000 replications) are shown at nodes.

database, the highest levels of similarity were with *L. nagelii* DSM 13675^T (98.0 %), *L. satsumensis* DSM 16230^T (95.5 %) and *L. vini* DSM 20605^T (93.7 %) (Table 1).

For determinations of the G + C content and DNA–DNA hybridization levels, DNA was extracted from L489^T, L499, *L. nagelii* DSM 13675^T and *L. satsumensis* DSM 16230^T and purified according to the protocol of Marmur (1961), as modified by Stackebrandt & Kandler (1978). Strain L486 was not included in the DNA–DNA hybridization studies, as the 16S rRNA gene sequence is 100 % identical to that of strain L489^T. The DNA G + C content was determined from the thermal melting temperature (T_m) of the DNA by using a Cary 100 Bio UV-visible spectrophotometer (Varian). DNA–DNA relatedness was determined spectrophotometrically from renaturation rates (De Ley *et al.*, 1970). DNAs from strains L489^T and L499 were hybridized with each other and with DNA from *L. nagelii* DSM 13675^T and with DNA from *L. satsumensis* DSM 16230^T. The reassociation value between L489^T and L499 was 92.5 %, indicating that the strains are conspecific. Reassociation values of 18–44 % were obtained with respect to *L. nagelii* DSM 13675^T and values of 0–12 % were obtained with respect to *L. satsumensis* DSM 16230^T. All of the DNA–DNA hybridization

values obtained for the closest relatives, namely *L. nagelii* DSM 13675^T and *L. satsumensis* DSM 16230^T, were therefore well below the 70 % cut-off value that indicates separate species (Wayne *et al.*, 1987). DNA reassociation values were not determined for L489^T and L499 with respect to the phylogenetically closely related species *L. vini*, as *L. vini* does not contain D-meso-diaminopimelic acid in the cell wall (Table 1). The DNA G + C content for strain L489^T and for strain L499 was 37.8 mol%, while those for *L. nagelii* DSM 13675^T and *L. satsumensis* DSM 16230^T were 37.7 and 40.2 mol%, respectively (Table 1).

The three strains were also investigated genotypically by using repetitive element palindromic-PCR with the primer GTG₅ according to the method of Gevers *et al.* (2001) for lactic acid bacteria, as described previously (Franz *et al.*, 2006). Strains L486, L489^T and L499 clustered closely ($r=85$ %) and were only distantly related to the type strains of *L. nagelii*, *L. satsumensis* and *L. vini* (Fig. 3).

On the basis of the above-mentioned data, it can be concluded that strains L486, L489^T and L499 form a homogeneous, distinct genetic group and are most closely related to *L. nagelii* and *L. satsumensis*. Phenotypically, strains L486,

Table 1. Differential phenotypic features of strains L486, L489^T and L499 and motile *Lactobacillus* species and *L. mali*

Taxa: 1, strains L486, L489^T and L499; 2, *L. nagelii*; 3, *L. satsumensis*; 4, *L. vini*; 5, *L. agilis*; 6, *L. rumi-nis*; 7, *L. curvatus* subsp. *curvatus*; 8, *L. mali*. *L. mali* is non-motile but is phylogenetically closely related to strains L486, L489^T and L499 and is thus included for comparison. Data are taken in part from Edwards *et al.* (2000), Endo & Okada (2005), Hammes & Hertel (2006), Kato *et al.* (2000), Rodas *et al.* (2006) and Torriani *et al.* (1996). +, Positive; –, negative; w, weak; d, strain-dependent; ND, no data.

Characteristic	1	2	3	4	5	6	7*	8†
Growth in MRS broth in/at:								
15 °C	w	+	+	–	–	–	+	+
45 °C	+	+	+	+	+	d	–	–
6.5 % NaCl	–	+	+	+	ND	ND	ND	–
pH 3.9	w	+	+	+	ND	ND	ND	ND
pH 8.0	–	+	ND	+	ND	ND	ND	–
Lactate isomer(s)	DL	DL	L	DL	L	L	DL	L
Acid from:								
L-Arabinose	–	–	–	+	–	–	–	+
D-Cellobiose	+	+	–	+	+	+	d	+
Mannitol	+	+	+	–	+	–	–	–
Raffinose	–	–	–	–	+	+	–	–
L-Rhamnose	+	+	+	–	–	–	–	d
Sorbitol	d	+	–	–	d	–	–	–
Dextran from sucrose	–	+	+	–	ND	ND	ND	+
D-meso-Diaminopimelic acid in cell wall	+	+	+	–	+	+	–	+
DNA G + C content (mol%)	37.8	37.7	40.2	39.4	43–44	44–47	42–44	32–34
16S rRNA gene sequence similarity (%) with respect to strain L489 ^T	99.9–100	98.0	95.5	93.7	90.3	92.3	89.9	91.9

*Some strains are motile but lose their motility when subcultured (Torriani *et al.*, 1996).

†Some strains have been reported to be motile (Kaneuchi *et al.*, 1998).

Fig. 3. (GTG)₅-PCR fingerprints and corresponding dendrogram, derived from UPGMA linkage of correlation coefficients (*r*, expressed as a percentage for convenience), for strains L486, L489^T and L499 and related lactobacilli.

L489^T and L499 are closely related to *L. nagelii*, but the absence of growth at pH 8.0 and with 6.5 % NaCl, absent or weak growth at pH 3.9, the inability to produce acid from sorbitol and tagatose (although delayed positive reactions were observed in strain L499) and the absence of dextran production from glucose are features that serve to differentiate strains L486, L489^T and L499 from *L. nagelii* (Table 1).

The results obtained in the present study clearly indicate that the strains studied represent a novel species within the genus *Lactobacillus*, for which the name *Lactobacillus ghanensis* sp. nov. is proposed. The type strain is L489^T (=DSM 18630^T=CCUG 53453^T).

Description of *Lactobacillus ghanensis* sp. nov.

Lactobacillus ghanensis (ghan.en'sis. N.L. masc. adj. *ghanensis* pertaining to Ghana, where the micro-organism was first isolated).

Cells are rod-shaped, 0.7–0.8 × 1.4–2.5 µm in size and occur singly, in pairs or in short chains comprising three to four cells. Gram-positive, catalase-negative, motile with peritrichous flagella and non-spore-forming. Colonies are 2–3 mm in diameter, white to creamy white, smooth, circular, convex and with entire or slightly uneven edges after 3–4 days of anaerobic growth. Weak growth at 15 °C; good growth at 45 °C. No growth occurs at 6.5 % NaCl. Weak growth at pH 3.9; no growth at pH 8.0. Ammonia is not produced from arginine. No gas is produced from glucose. D(–)- and L(+)-lactic acid are produced as the end products from glucose metabolism. Acid is produced from amygdalin, D-cellobiose, aesculin, D-fructose, D-glucose, D-galactose, D-mannitol, D-mannose, *N*-acetylglucosamine, L-rhamnose, sucrose, salicin and D-trehalose. Acid is not produced from D-adonitol, starch, D-arabinose, L-arabinose, arbutin, dulcitol, erythritol, D-fucose, L-fucose, gentiobiose, gluconate, glycogen, inositol, inulin, D-lyxose, D-melezitose, D-melibiose, methyl β-xyloside, D-raffinose, D-ribose, D-sorbitol, D-turanose, xylitol, D-xylose, L-xylose, 2-ketogluconate or 5-ketogluconate. Acid production from glycerol, D-lactose, D-maltose, methyl α-D-glucoside (delayed reaction), D-sorbitol (delayed reaction), L-sorbose and D-tagatose (delayed reaction) is strain-dependent. Does not produce dextran from sucrose. The cell wall contains peptidoglycan of the D-meso-diaminopimelic acid type. The DNA G + C content is 37.8 mol%.

The type strain, L489^T (=DSM 18630^T=CCUG 53453^T), and all other known strains of the species were isolated from cocoa fermentations in Tafo, Ghana. The description of the type strain corresponds to the description of the species except that no acid is produced from glycerol, maltose, methyl α-D-glucoside, D-lactose, sorbitol or tagatose. Strain L499 (=DSM 18631=CCUG 53454) is a reference strain.

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