Lactobacillus manihotivorans sp. nov., a new starch-hydrolysing lactic acid bacterium isolated during cassava sour starch fermentation

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Two *Lactobacillus* strains were isolated from sour cassava starch fermentation. The cells were Gram-positive, catalase-negative, non-spore-forming, non-motile rods. They produced only L( )lactate and were homofermentative. Growth occurred at pH values of 5·0–7·0 and optimum growth occurred at pH 6·0. Growth was positive at 15 and 45 °C. The DNA G C content was 48·4 0·2 mol%. Sequence analysis of the 16S rRNA gene revealed that strains OND 32^T and YAM 1 clustered with, but were separate from *Lactobacillus casei*-related taxa. Protein pattern and sequence analyses of the 16S rRNA gene confirmed that the two new isolates represent a new *Lactobacillus* species, for which the name *Lactobacillus manihotivorans* is proposed; strain OND 32^T is the type strain of this species.

Keywords: lactic acid bacteria, *Lactobacillus manihotivorans* sp. nov., amylase, cassava, sour starch

INTRODUCTION

To date, only a few amylolytic lactic acid bacteria (ALAB) have been isolated, such as *Lactobacillus amylophilus* and *Lactobacillus amylovorus* from swine and cattle waste–corn fermentations, respectively, in the USA (Nakamura, 1981; Nakamura & Crowell, 1979), *Lactobacillus plantarum* A6 from retted cassava roots in Congo (Giraud *et al*., 1991), *Lactobacillus* strains LEM 220, 207 and 202 from chicken crop in France (Champ *et al*., 1983), *Leuconostoc* strains from fish silage in Sweden (Lindgren & Refai, 1984), and *L. plantarum* from fermented fish and rice food in Japan (Olympia *et al*., 1995). Among the most representative species, *L. amylovorus* had higher amylase activity than *L. amylophilus* (Pompeyo *et al*., 1993) and both species were studied for their ability to produce lactic acid from starch (Mercier *et al*., 1992; Yumoto & Ikeda, 1995; Zhang & Cheyran, 1991). Furthermore, it was shown that *L. plantarum* A6 was able to ferment raw starch (Giraud *et al*., 1991). The fact that amylolytic *Lactobacillus* strains were most often isolated from food-related ecosystems is congruent with a recent survey of Damelin *et al*. (1995), who reported that these bacteria constituted up to 65% of the isolates from different types of foods.

Cassava sour starch is a sun-dried fermented food product in Latin America with an unusual bread-making capacity. It is produced mainly in Colombia (Dufour *et al*., 1995) and in Brazil (Chuzel *et al*., 1995) where it is named 'almidon agrio' and 'polvilho azedo', respectively. Production of cassava sour starch involves lactic acid fermentation and a decrease in pH to 3·5 (Dufour *et al*., 1995). Nevertheless, the quality of sour starch is quite variable and this affects the bread-making capacity. Direct use of starch with selected starters could be utilized to better control the process.

Tropical fermented foods made from starch-containing substrates like cassava or corn are potential sources of new strains of ALAB which produce L(+)lactic acid. These strains would be more acceptable for human food applications than those isolated from waste products or animal guts. Furthermore, the systematic isolation and characterization of ALAB from different food-related eco-

Abbreviations: ALAB, amylolytic lactic acid bacteria; RDP, Ribosomal Database Project.

The GenBank accession numbers for the 16S rRNA sequences of strains OND 32^T and YAM 1 reported in this paper are AF000162 and AF000163, respectively.

systems would help in the future to establish the ecological significance of this particular group of bacteria. This work reports the isolation of a new ALAB species from sour starch production in Colombia for which the name *Lactobacillus manihotivorans* is proposed.

METHODS

Origin of the isolates. Strains OND 32^T and YAM 1 were isolated during sour starch production in Colombia on agar plates containing MRS medium with soluble starch (Prolabo) as substrate using the same procedure described for *L. plantarum* A6 (Giraud *et al.*, 1991). Strains OND 32^T and YAM 1 were selected for their ability to degrade soluble starch. All strains were stored in 20 % glycerol at −80 °C. The two isolates obtained (strains OND 32^T and YAM 1) were deposited in the Belgian Coordinated Collections of Microorganisms/Laboratorium voor Microbiologie Universiteit Gent (BCCM/LMG) culture collection as strains LMG 18010^T and LMG 18011, respectively.

Culture conditions. MRS medium (with glucose or soluble starch as substrate) was routinely used for general cultivation of bacteria (De Man *et al.*, 1960). The ability to grow under anaerobic or aerobic conditions was determined in Hungate tubes containing MRS broth (Difco), without shaking. For anaerobic growth, the medium was prepared under strict anaerobic conditions (Balch *et al.*, 1979) with O_2-free N_2 in the headspace. The temperature range for growth was determined with thermostatically controlled water baths, and the pH range for growth was determined at 30 °C in 2 l fermenters (Biolafitte) with pH control.

The API 50 CHL system (bioMérieux) was used to determine acid production from carbohydrates. Reproducibility was verified by repeated analysis using cultures grown on MRS agar and subcultured in liquid MRS medium prior to inoculation.

Analytical techniques. Cell density of culture was measured at 600 nm with a Spectronic 401 (Milton Roy) following the procedure of Koch (1981). Catalase activity was tested as described previously (Combet-Blanc *et al.*, 1995). Lactic acid configuration and concentration were determined using commercial enzymic test combinations (Boehringer Mannheim). The metabolic products in the supernatant were determined by HPLC using an Aminex HPX 87H column (Bio-Rad) with 6 mM H_2SO_4 at a flow rate of 0·8 ml min^{-1} and 65 °C with refractive index detection. Extracellular α-amylase activity was assayed in the supernatant of centrifuged cultures by measurement of the iodine-complexing ability of starch at pH 5·5 and 55 °C as previously described (Giraud *et al.*, 1994). All tests were done in duplicate.

Morphological characteristics. Cell morphology was observed by phase-contrast microscopy. Gram staining was studied using a Gram-colour coloration set (Merck) and by the KOH method of Gregersen (1978).

DNA base composition. The G+C content of the DNA was determined at the Deutsche Sammlung von Mikroorganism und Zellkulturen, Braunschweig, Germany. After disruption with a French pressure cell, the DNA was isolated and purified by chromatography on hydroxyapatite. The G+C content was determined by HPLC as described by Meshbah *et al.* (1989); non-methylated lambda DNA (Sigma) was used as the internal standard.

SDS-PAGE. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described previously (Pot *et al.*, 1994a). Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of strains with the Pearson product–moment correlation coefficient (Pearson & Lipman, 1988), and an unweighted pair group cluster analysis using average linkage were performed using the techniques described by Pot *et al.* (1994a) with the GELCOMPAR software package (version 4.0) (Vauterin & Vauterin, 1992). The protein profile of strain OND 32^T and YAM 1 was compared with a database consisting of normalized protein fingerprints derived from reference strains belonging to almost all previously described species of lactic acid bacteria (Pot & Janssens, 1993).

Purification of genomic DNA and standard electrophoresis. Genomic DNA was prepared in agarose blocks, similarly to preparation of DNA samples for pulsed field gel electrophoresis. An overnight culture of strains OND 32^T and YAM 1 was diluted (10^{-1}) in fresh MRS broth and grown at 30 °C for 4 h. The cells were harvested by centrifugation (10 min, 5000 r.p.m.), washed twice in TS buffer (50 mM Tris/HCl, pH 7·5, 25 % saccharose, w/w). The cells were then suspended in TS buffer to an A_{600} value of about 3. The cell suspension (1 ml) was treated with lysozyme (100 mg ml^{-1}) for 1 h at 37 °C. The protoplasts thus obtained were harvested by centrifugation (10 min, 5000 r.p.m.), resuspended in 500 µl SE buffer (75 mM NaCl, 25 mM EDTA) and incubated for 1 h at 37 °C. The agarose blocks were prepared and digested with *Eco*RI endonuclease according to the method of McClelland (1987). Standard electrophoresis of the DNA samples was performed in a large electrophoresis cell (30 × 20 cm) with 1 % agarose gel (w/v), in TAE buffer (40 mM Tris, 40 mM acetate, 2·0 mM EDTA, pH 8) at 4 V cm^{-1} and 10 °C for 15 h.

16S rRNA gene sequencing. The almost complete 16S rRNA gene was amplified by PCR by using domain *Bacteria*-specific primers 27f and 1425r (Lane, 1991). PCR reactions were done in a final volume of 100 µl containing: 0·1–0·5 µg DNA, deoxyribonucleoside triphosphate (200 µM each), primers (0·8 µM each), $MgCl_2$ (1·5 mM), 1 U *Taq* DNA polymerase (Promega), and the buffer supplied with the enzyme. The amplification conditions were as follows: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation for 30 s at 94 °C; annealing for 30 s at 55 °C; and elongation at 72 °C for 7 min. The amplified fragments were purified by a Wizard procedure and ligated into vector pGEM-T (Promega).

Both strands of the inserts of the clones were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) with the ABI PRISM Dye Terminator kit (Perkin Elmer). Sequence products were loaded and analysed on a 373 DNA sequencer (Applied Biosystems).

Phylogenetic analysis of the sequence data. Phylogenetic analyses were performed using data from the Ribosomal Database Project (RDP; Maidak *et al.*, 1996), the CLUSTAL W multi-alignment program (Thomson *et al.*, 1994) and PHYLIP (Phylogeny Inference Package version 3.5c; Felsenstein, 1993).

To determine the closest known relative of the two new 16S rDNA sequences, preliminary searches were performed in public data libraries (GenBank and RDP) with the FASTA (Devereux *et al.*, 1984) and RDP programs (Maidak *et al.*, 1996). Then, the new sequences were aligned with 24 related sequences using the CLUSTAL W program (Thomson *et al.*,

1994). Positions of sequence alignment uncertainty were omitted from the sequence analyses. A distance matrix was obtained by using the DNADIST program (Felsenstein, 1993) and the method of Jukes & Cantor (1969). The stability of the groups was determined by performing bootstrap analysis with 100 bootstrapped data sets and with the programs DNABOOT, DNAPARS, DNAML, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1993).

RESULTS

Colony and cell morphology

Colonies of strains OND 32^T and YAM 1 on MRS agar were white, convex and opaque with smooth edges. Clear zones of starch hydrolysis were observed on exposure of colonies grown on MRS agar containing starch (15 g l^{-1}) to iodine vapour.

Cells of strains OND 32^T and YAM 1 were non-spore-forming, non-motile rods that occurred in short, straight to curled, small crisped chains. The Gram stain reaction was positive.

DNA base composition

The mean G+C content of strain OND 32^T DNA, based on three determinations, was 48·4 ± 0·2 mol%.

Growth conditions and metabolic properties

Strains OND 32^T and YAM 1 were catalase-negative. Both strains grew well in liquid or solid MRS media in the presence of air and also under strict anaerobic conditions. Strains OND 32^T and YAM 1 produced only L(+)lactic acid. In MRS medium containing starch as the energy source at pH 6·0 and 30 °C, the specific growth rate of strain OND 32^T was 0·31 ± 0·02 h^{-1}. Strain OND 32^T required complex nitrogen sources for growth, such as meat extract and pancreatic peptone. For strain OND 32^T, growth occurred at 15 and 45 °C and was optimal at approximately 30 °C. The strain grew at pH values of 5·0–7·0, the optimum being 6·0. Strain OND 32^T was able to grow at NaCl concentrations of 20–65 g l^{-1} and did not grow at 100 g NaCl l^{-1}.

Amylolytic activity in strain OND 32^T was extracellular, whereas strain YAM 1 had a cell-linked amylase activity and did not produce extracellular amylolytic enzyme.

Fermentation of sugars by strain OND 32^T and YAM 1

The following sugars were fermented within 24 h: galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, D-raffinose, starch, glycogen, *β*-gentobiose and D-turanose.

Glycerol, erythritol, D-arabinose, D-ribose, D-xylose, L-xylose, adonitol, methyl *β*-xyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, inulin, melezitose, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate were not fermented.

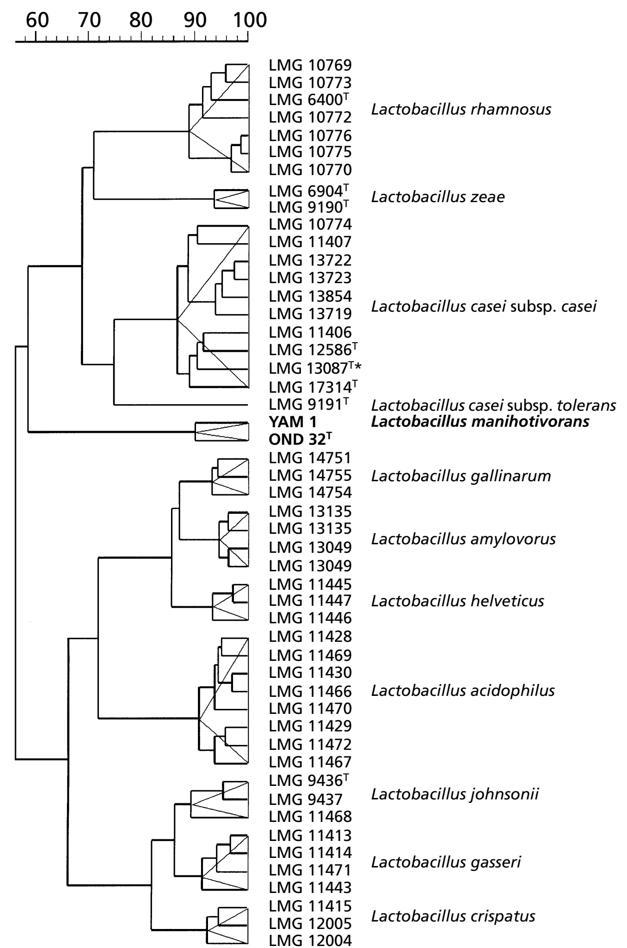

Fig. 1. Dendrogram derived from SDS-PAGE protein pattern analysis. The mean correlation coefficient is expressed as a percentage ($r \times 100$) and represented as a dendrogram calculated by the unweighted mean pair grouping method, for a number of relevant *Lactobacillus* species. Species are delineated by triangles. Points 10–325 of the 400 point traces were used to calculate similarities between the individual pairs of traces. LMG, BCCM/LMG culture collection, University of Gent, Gent, Belgium; *, type strain of *L. paracasei* as described by Collins *et al.* (1989).

SDS-PAGE

Both isolates were included in an identification procedure using SDS-PAGE of whole-cell proteins. The protein patterns of both strains were compared to a database containing the SDS-PAGE fingerprints of almost all known species of lactic acid bacteria (Pot & Janssens, 1993; Pot *et al.*, 1994a). Numerical analysis of the SDS-PAGE protein patterns, represented as a dendrogram and including representative strains of some phenotypically and phylogenetically relevant species, is presented in Fig. 1. The dendrogram obtained clearly shows the separate species status of

Table 1. Similarity values (%) for the 16S rRNA genes of strains OND 32^T and YAM 1, selected lactic acid bacteria and *E. coli*

Similarity values were determined using Olsen's modification of the method of Jukes & Cantor (1969) (see Methods). 1, OND 32^T; 2, YAM 1; 3, *Lactobacillus agilis*; 4, *L. amylophilus*; 5, *L. amylovorus*; 6, *Lactobacillus bifermentans*; 7, *Lactobacillus brevis*; 8, *Lactobacillus buchneri*; 9, *L. casei*; 10, *Lactobacillus coryniformis*; 11, *L. delbrueckii*; 12, *Lactobacillus fermentum*; 13, *Lactobacillus fructivorans*; 14, *Lactobacillus hilgardii*; 15, *L. mali*; 16, *Lactobacillus murinus*; 17, *Lactobacillus pentosus*; 18, *L. plantarum*; 19, *L. ruminis*; 20, *Lactobacillus sakei*; 21, *L. sharpeae*; 22, *Lactobacillus vermiforme*; 23, *Leuconostoc cremoris*; 24, *Pediococcus acidilactici*; 25, *Pediococcus pentosaceus*; 26, *E. coli*.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	100																									
2	99·4	100																								
3	91·6	90·6	100																							
4	90·5	90·0	88·7	100																						
5	89·3	89·0	87·0	90·9	100																					
6	93·4	93·1	90·3	90·7	87·9	100																				
7	93·3	93·1	90·9	90·6	87·0	92·0	100																			
8	93·5	93·3	91·4	90·2	87·7	92·6	95·2	100																		
9	94·9	94·6	90·7	90·2	87·5	91·6	92·1	91·9	100																	
10	93·8	93·5	91·9	90·5	87·7	95·3	92·4	92·3	93·2	100																
11	89·5	89·3	87·4	89·6	92·4	88·8	89·1	87·6	88·6	87·9	100															
12	90·7	90·5	89·2	90·3	87·8	89·8	91·6	89·4	90·1	88·7	88·7	100														
13	92·6	92·6	90·0	89·0	86·9	90·9	94·5	93·1	92·5	90·3	88·4	91·1	100													
14	92·8	92·5	91·1	90·0	87·3	91·9	94·8	96·3	93·6	91·7	88·2	90·9	94·8	100												
15	92·4	92·3	94·4	89·8	87·3	91·7	93·3	92·9	92·7	92·7	88·0	90·3	93·5	92·9	100											
16	91·4	91·5	94·3	88·7	86·2	90·4	92·1	91·1	91·8	91·1	88·2	89·8	92·1	90·9	94·5	100										
17	93·3	93·1	91·5	89·7	85·7	92·5	94·8	91·5	92·0	91·9	87·6	90·7	93·7	92·2	92·4	88·8	100									
18	93·3	93·1	91·5	89·7	85·7	92·5	94·8	91·5	92·0	91·9	87·6	90·7	93·7	92·2	92·4	88·8	100	100								
19	90·8	90·8	93·7	88·5	86·2	90·0	92·1	91·0	90·5	90·1	87·5	89·8	91·8	90·9	93·2	91·3	92·3	92·3	100							
20	93·4	93·1	90·3	90·0	87·2	92·2	92·4	91·5	93·6	93·5	88·2	90·7	92·7	91·7	92·3	89·3	93·1	93·1	90·4	100						
21	93·9	93·4	89·0	89·4	88·4	91·8	92·9	91·7	92·3	91·8	89·1	90·6	91·7	90·6	91·1	88·4	91·7	91·7	89·8	92·5	100					
22	92·7	92·5	91·4	90·2	87·4	92·1	95·0	96·7	93·8	91·9	88·2	90·9	94·8	98·5	93·7	89·6	93·7	93·7	92·1	92·7	91·3	100				
23	86·4	86·3	85·4	84·7	83·6	86·0	85·5	84·0	85·9	85·9	84·3	85·9	84·7	84·0	85·3	83·2	85·9	85·9	84·7	86·7	86·5	83·8	100			
24	93·3	92·9	90·5	89·8	87·2	92·0	94·3	93·1	93·3	91·7	87·8	91·9	94·4	92·5	93·4	89·6	94·2	94·2	90·9	93·5	93·3	92·7	85·3	100		
25	93·2	92·8	90·1	88·9	86·4	91·3	94·0	92·0	93·1	91·0	87·4	90·9	94·0	92·1	92·8	89·1	93·9	93·9	90·9	92·5	92·2	92·2	85·2	97·3	100	
26	77·0	76·9	75·8	76·0	74·8	76·6	76·0	75·5	77·4	75·6	76·5	75·9	76·9	75·3	76·0	74·3	76·9	76·9	77·2	76·2	76·2	75·1	76·8	75·7	76·3	100

the two strains LMG 18011 (YAM 1) and LMG 18010^T (OND 32^T). Both strains show a correlation value ($r \times 100$) of 90·2 % with each other and less than 58 % with the other lactobacilli included in the dendrogram.

Reproducibility of the SDS-PAGE method was estimated by studying duplicate lanes of a single protein extract on one gel (97 % reproducibility), on separate gels ($\geqslant$ 94 %; see duplicate samples of the two reference strains of *L. amylovorus* LMG 13135 and 13049, Fig. 1) or by comparing duplicate extract preparations applied on different gels ($\geqslant$ 93 %; see duplicate samples of the independently received subcultures of the type strains of *Lactobacillus zeae* LMG 6904^T and LMG 9190^T, and *Lactobacillus casei* subsp. *casei* LMG 12586^T and LMG 17314^T, Fig. 1). These values are in agreement with those reported previously for other lactic acid bacteria (Pot *et al.*, 1993, 1994b).

Whole-genome restriction fragment analysis

Whole genomic DNA fingerprinting was used only to distinguish both strains OND 32^T and YAM 1. Digestion of total genomic DNA with *Eco*RI gave complex DNA patterns which were very reproducible. DNA fingerprints obtained for each strain confirmed the separate species status of strains OND 32^T and YAM 1 (data not shown) as suggested by SDS-PAGE analysis.

16S rRNA sequence and phylogenetic analysis

The 16S rDNA fragments from strains OND 32^T and YAM 1 were amplified by PCR using the primer pair 27f/1525r defined by Lane (1991). The fragments obtained, corresponding to the almost complete sequence of 16S rDNA, were sequenced (1559 bases for OND 32^T and 1565 bases for YAM 1). Data searches indicated that OND 32^T and YAM 1 were members of the subphylum containing Gram-positive bacteria with DNA G+C contents less than 55 mol%, which included members of the genera *Lactobacillus*, *Pediococcus*, *Streptococcus* and *Carnobacterium*. Similarity values for a 1390 nucleotide region [positions 10–1400 of the *Escherichia coli* numbering system (Neef *et al.*, 1990)] of these two new sequences and homologous regions of 24 selected reference strains from major phylogenetic lines within the lactic acid bacteria were determined. A matrix of representative sequence similarities is shown in Table 1. Strains OND 32^T and YAM 1 showed a high level of similarity (99·4 %) and this suggests that both strains belong to the same species. For both strains, the highest level of sequence relatedness was observed with *L. casei* (levels of similarity were approximately 94·7 and 94·3 % with OND 32^T and YAM 1, respectively) and with other members of the *L. casei*/*Pediococcus* cluster (Collins *et al.*, 1991) (similarities were approximately 90·8–94·7 %). These data show that OND 32^T and YAM 1

Table 2. 16S rRNA sequence similarity values (%) for strains OND 32ᵀ, YAM 1 and *L. casei*-related species

Similarity values were determined using Olsen's modification of the method of Jukes & Cantor (1969).

Species	OND 32ᵀ	YAM 1	*L. casei*	*L. paracasei*	*L. rhamnosus*	*L. zeae*
L. manihotivorans OND 32ᵀ	100					
L. manihotivorans YAM 1	99·4	100				
L. casei	94·7	94·3	100			
L. paracasei	94·9	94·5	99·3	100		
L. rhamnosus	94·8	94·5	98·6	98·7	100	
L. zeae	95·2	94·9	99·8	99·3	99·2	100

Table 3. Sequence signatures in the 16S rDNAs of strains OND 32ᵀ and YAM 1 and of *L. casei*-related taxa

Strain	Nucleotide at position:*												
	73	75	76	84	85	96	97	98	104	107	108	110	111
L. casei NCDO 161ᵀ	T	G	G	A	C	C	T	G	C	C	T	A	A
L. zeae ATCC 15820ᵀ	T	G	G	A	C	T	C	G	C	C	T	A	A
L. paracasei JCM 8130ᵀ	C	C	G	T	C	C	C	G	C	C	A	G	G
L. rhamnosus DSM 20021ᵀ	C	G	A	A	A	T	C	T	T	T	T	T	G
L. manihotivorans OND 32ᵀ	T	T	A	A	-†	G	T	A	T	C	T	A	A
L. manihotivorans YAM 1	T	T	A	A	-†	G	T	A	T	C	T	A	A

* Nucleotide position from the 5′ end of the 16S rDNA sequence of *L. casei*.

† To achieve optimal alignment, no base was found in this position.

clearly belong to the genus *Lactobacillus* and are closely related to *L. casei* group, which contains the subspecies *L. casei* subsp. *casei*, *L. casei* subsp. *tolerans*, *Lactobacillus rhamnosus* and *L. zeae* (Dicks *et al*., 1996). The 16S rRNA sequences of strains OND 32ᵀ, YAM 1 and four representative strains of the *L. casei* group, were aligned and a distance matrix was assembled (Table 2). The levels of sequence similarity between the *L. casei* strains are much higher (98·6–99·8 %) than the level of similarity between the *L. casei* strains and the OND 32ᵀ and YAM 1 strains (94·3–95·2 %). The most drastic structural difference occurred at positions 66–103 (*E. coli* numbering system).

To differentiate the four *L. casei*-related taxa, Mori *et al*. (1997) have defined sequence signatures in the 16S rRNAs sequences at positions 66–103 (*E. coli* numbering system) that differed from the sequences at the same positions in OND 32ᵀ and YAM 1 16S rRNA sequences (Table 3). However, OND 32ᵀ and YAM 1 share the same sequence signature in this region. This shows that both strains OND 32ᵀ and YAM 1 are more closely related to each other than they are to *L. casei*. A minor structural difference between *L. casei*

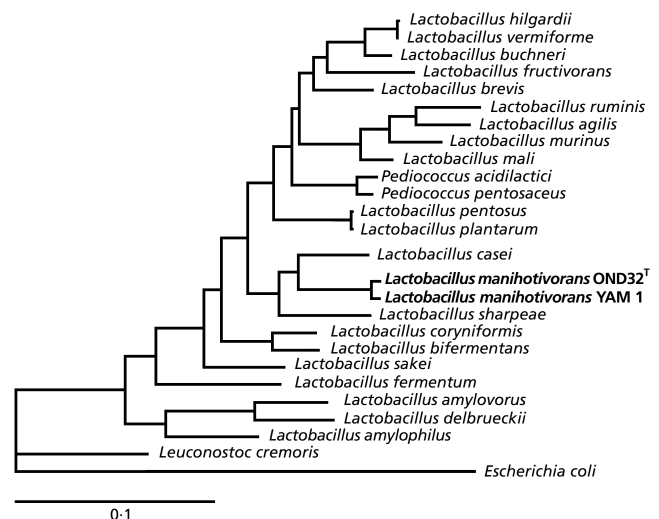

Fig. 2. Phylogenetic dendrogram derived from 16S rDNA sequence analysis showing the relationships of strains OND 32ᵀ and YAM 1 to other lactic acid bacteria and *E. coli*. The dendrogram was generated by applying a maximum-likelihood approach of bootstrapped data sets. Scale bar, 10 % estimated sequence divergence.

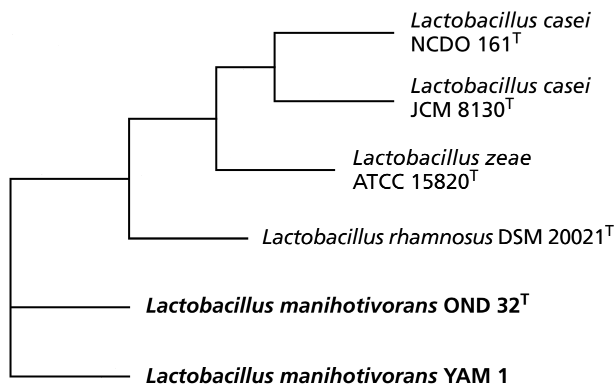

Fig. 3. Unrooted tree derived from 16S rDNA sequence analysis and showing the phylogenetic relationships of strains OND 32^{T} and YAM 1 and *L. casei*-related taxa. The dendrogram was generated by applying a maximum-likelihood approach of bootstrapped data sets. Scale bar, 10% nucleotide substitution.

and strains OND 32^{T} and YAM 1 was also found in regions susceptible to stem–loop formation [positions 199–226 and 286–296 (*E. coli* numbering system)].

Alternative tree-making programs available in the PHYLIP package (Felsenstein, 1993) produced trees that were topologically similar to the tree shown in Fig. 2. This dendrogram (Fig. 2) shows the phylogenetic position of OND 32^{T} and YAM 1 compared to the *L. casei/Pediococcus* cluster, as well as to other reference species scattered outside this cluster. Treeing analysis confirmed that OND 32^{T} and YAM 1 are closely related to members of *L. casei/Pediococcus* cluster. The dendrogram in Fig. 3 shows a detailed position of OND 32^{T} and YAM 1 within the *L. casei* cluster and strongly suggests that OND 32^{T} and YAM 1 form a distinct group within the cluster.

DISCUSSION

Strains OND 32^{T} and YAM 1, isolated from a traditional Colombian cassava fermentation process and used to produce sour starch, are Gram-positive, non-spore-forming, mesophilic, homolactic, facultatively anaerobic bacteria. Like lactic acid bacteria, they are non-spore-forming, chemo-organotrophic and catalase-negative. The type of metabolism and low DNA G+C content (48·4±0·2 mol%) of strains OND 32^{T} and YAM 1, together with their morphological and physiological characteristics, are consistent with the description of the genus *Lactobacillus* (Dellaglio *et al.*, 1994; Hammes & Vogel, 1995).

Since strains OND 32^{T} and YAM 1 produce only lactic acid and do not ferment pentose or gluconate they should be considered to be obligately homofermentative and should be included in lactobacilli Group I as proposed by Kandler & Weiss (1986) or classified either in phylogenetic Cluster 1 (the *Lactobacillus delbrueckii* group, which contains obligately homofermentative species) or in Cluster 2 (the *L. casei/Pediococcus* group, which contains a limited number of obligately homofermentative as well as a majority of heterofermentative species) (Collins *et al.*, 1991). For the sake of simplicity, the term Group I lactobacilli is used in this report to cover both the obligately homofermentative lactobacilli described by Kandler & Weiss (1986), and the obligately homofermentative species of Cluster 2 of Collins *et al.* (1991).

Differential characteristics of strains OND 32^{T} and YAM 1 with obligately homofermentative *Lactobacillus* species are shown in Table 4. Among starch degraders which belong to Group I, the ability of OND 32^{T} and YAM 1 to produce L(+)lactic acid only is a characteristic which differentiates them from *L. amylovorus* (DL-lactic acid producer). Unlike strains OND 32^{T} and YAM 1, *L. amylovorus* is also unable to ferment lactose, melibiose, raffinose and does not grow at 15 °C (Hammes & Vogel, 1995). When compared to *L. amylophilus* [Group I, L(+)lactic acid producer], strains OND 32^{T} and YAM 1 differ in the use of cellobiose, lactose, melibiose, raffinose, sucrose, amygdalin and aesculin. Based on these criteria, strains OND 32^{T} and YAM 1 are clearly different from previously described ALAB of Group I.

Characteristics of strains OND 32^{T} and YAM 1 which differentiate them from non-amylolytic species belonging to Group I are listed in Table 4, and encompass the fermentation of amygdalin, melibiose, raffinose, trehalose, cellobiose and sorbitol, lactic acid isomer, growth at 15 °C, and G+C content. Among the L(+)lactic acid producers of Group I, *Lactobacillus animalis* and *Lactobacillus ruminis* closely resemble strains OND 32^{T} and YAM 1 (Table 4), but both species do not use trehalose and do not grow at 15 °C. Strains OND 32^{T} and YAM 1 are similar to species belonging to the *L. delbrueckii* group (Hammes & Vogel, 1995), such as *Lactobacillus johnsonii* and *Lactobacillus gallinarum* (Table 4) but differ from *L. johnsonii* in that they use melibiose and from *L. gallinarum* in that they use of trehalose and have a very different G+C content. Strains OND 32^{T} and YAM 1 differ from *Lactobacillus mali* by their G+C content.

Based on the analysis of protein electrophoretic patterns, a first group of reference strains consisted of 20 strains belonging to the *L. casei* group comprising *Lactobacillus* (*para*)*casei*, *L. rhamnosus* and *L. zeae*. As expected, the *L. casei* cluster (Dicks *et al.*, 1996) contains the type strain of *Lactobacillus paracasei* (Collins *et al.*, 1989). The three species can clearly be discriminated. The type strain of *L. casei* subsp. *tolerans* occupies a separate position in the *L. casei* cluster with a correlation value of 73·9%. The remaining members of this cluster show homologies above 88%.

A second group of reference strains consisted of 28 reference strains of the *Lactobacillus acidophilus* complex belonging to the *L. delbrueckii* group (Hammes & Vogel, 1995). Representative strains of seven species (*L. gallinarum*, *L. amylovorus*, *Lactobacillus helveticus*,

Table 4. Main differential characteristics between strains OND 32^T and YAM 1 and closely related obligately homofermentative *Lactobacillus* spp. (Group I)

+, 90 % or more strains positive; −, 90 % or more strains negative; d, 11–89 % strains positive; ND, not done; $+_w$, positive to weak reaction.

Lactobacillus species (mol % G+C)	Acid production from:						Lactic acid isomers*	Growth at 15 °C
	Starch	Amygdalin	Cellobiose	Melibiose	Raffinose	Trehalose		
OND 32^T and YAM 1 (48·4)	+	+	+	+	+	+	L	+
L. acidophilus† (34–37)	d	+	+	d	d	d	DL	−
L. kefirgranum‡ (34·3–38·6)	ND	−	d	+	+	d	D(L)	$+_w$
L. kefiranofaciens‡, § (34–35)	ND	−	−	+	+	−	D(L)	−
L. johnsonii‡, § (35–38)	ND	+	+	−	d	d	L‡	+
L. gallinarum‡, § (36–37)	d	+	+	+	+	−	L‡	+
L. amylophilus (44–46)	+	−	−	−	−	−	L	+
L. amylovorus (40–41)	+	$+_w$	+	−	−	+	DL	−
L. animalis (41–44)	ND	d	+	+	+	−	L	−
L. crispatus (35–38)	d	+	+	−	−	−	DL	−
L. farciminis (34–36)	−	+	+	−	−	+	L(D)	+
L. gasseri (33–35)	d	+	+	d	d	d	DL	−
L. helveticus (38–40)	−	−	−	−	−	d	DL	−
L. jensenii (35–37)	−	+	+	−	−	+	D	−
L. ruminis (44–47)		+	+	+	+	−	L	−
L. salivarius (34–36)	−	−	−	+	+	+	L	−
L. sharpeae (53)	ND	+	+	−	−	−	L	+
L. vitulinus (34–37)	ND	+	+	+	+	d	D	−
L. mali§ (*L. yamanashiensis*†) (32–34)	ND	+	+	d	d	+	L or DL	+

* Based on the content of L(+)lactic acid: D, 0–20 %; L, 80–100 %; L(D), the D isomer makes up 15–20 % total lactic acid (data from Kandler & Weiss, 1986); D(L), the L isomer makes up 20–40 % total lactic acid (data from Takizawa *et al.*, 1994).

† Data from Kandler & Weiss (1986).

‡ Data from Takizawa *et al.* (1994).

§ Data from Dellaglio *et al.* (1994).

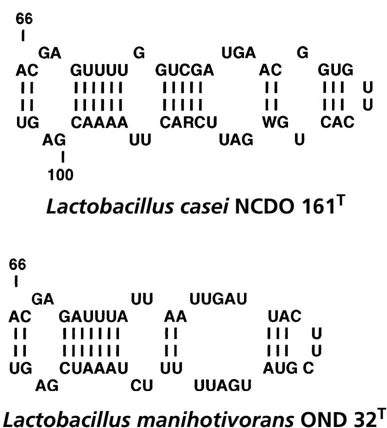

Fig. 4. Secondary structures of a 16S rRNA region (positions 66–103 of the *E. coli* numbering system) of strain OND 32^T and *Lactobacillus casei.*

L. acidophilus, *L. johnsonii*, *Lactobacillus gasseri* and *Lactobacillus crispatus*, Fig. 1) form separate clusters with correlation values between 94·7 (*L. amylovorus*) and 89·4 % (*L. johnsonii*). The relationship between these species and *L. manihotivorans* is less than 57 % (Fig. 1).

To determine the evolutionary and taxonomic position of OND 32^T and YAM 1, the sequences of their 16S rDNAs were compared. This appears to be one of the most appropriate methods for determining taxonomic relationships (Fox *et al.*, 1980; Woese, 1987). Several types of distance matrix and tree-generating programs (Felsenstein, 1993) were used to analyse the sequence data. All the results obtained showed that OND 32^T and YAM 1 belong to the same species and that they form a distinct cluster.

This phylogenetic analysis of 16S rRNA sequences was consistent with the DNA G+C content of strain OND 32^T (48·4 mol %), situating the strain between *L. casei* (46 mol %) and *Lactobacillus sharpeae* (53 mol %). The patterns of variation in the secondary structure of the hypervariable region of the 16S rRNAs (positions 66–103 of the *E. coli* numbering system; Fig. 4) also support the hypothesis that OND 32^T and YAM 1 should be differentiated and separated from other members of the *L. casei* group.

In view of the presented phylogenetic evidence (16S rRNA), protein pattern differences measured by SDS-PAGE and the phenotypic distinctiveness of OND 32^T and YAM 1, it is proposed that these strains belong to a new species *Lactobacillus manihotivorans* sp. nov.

Description of *Lactobacillus manihotivorans* sp. nov.

Lactobacillus manihotivorans (ma.ni.ho.ti.vo′rans. L. n. *manihot* cassava; L. v. *vorare* to devour; M.L. adj. *manihotivorans* cassava-devouring).

Cells are Gram-positive, non-motile, non-spore-forming rods which occur in short chains or occasionally as single cells. Grows at 15 and 45 °C. Optimal temperature and pH for growth are about 30 °C and 6·0, respectively. Grows at NaCl concentrations of 20–65 g l^{-1} and does not grow at 100 g l^{-1} NaCl. Facultatively anaerobic. Catalase-negative. Obligately homofermentative, produces L(+)lactic acid. Starch is utilized. Strain OND 32^T has an extracellular amylase activity and strain YAM 1 shows cell-linked amylase activity. Acid is produced from cellobiose, mannose, sucrose, lactose, raffinose, trehalose, aesculin, salicin, amygdalin, methyl α-D-glucoside, β-gentobiose and *N*-acetylglucosamine. Pentoses are not used. The DNA G+C content of the type strain is $48{\cdot}4 \pm 0{\cdot}2$%. The type strain is strain OND 32^T (=LMG 18010^T).

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