***Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage**

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Inoculation of maize silage with *Lactobacillus buchneri* (5×10^5 c.f.u. g⁻¹ of maize silage) prior to ensiling results in the formation of aerobically stable silage. After 9 months, lactic acid bacterium counts are approximately 10^{10} c.f.u. g⁻¹ in these treated silages. An important subpopulation (5.9×10^7 c.f.u. g⁻¹) is able to degrade 1,2-propanediol, a fermentation product of *L. buchneri*, under anoxic conditions to 1-propanol and propionic acid. From this group of 1,2-propanediol-fermenting, facultatively anaerobic, heterofermentative lactobacilli, two rod-shaped isolates were purified and characterized. Comparative 16S rDNA sequence analysis revealed that the newly isolated bacteria have identical 16S rDNA sequences and belong phylogenetically to the *L. buchneri* group. DNA–DNA hybridizations, whole-cell protein fingerprinting and examination of phenotypic properties indicated that these two isolates represent a novel species, for which the name *Lactobacillus diolivorans* sp. nov. is proposed. The type strain is LMG 19667^T (= DSM 14421^T).

Keywords: lactic acid bacteria, 1,2-propanediol degradation, 1-propanol, 16S rDNA, identification

INTRODUCTION

Fermentative treatment of forage crops with lactic acid bacteria is a common procedure to preserve cattle feed of high nutritional value in many countries all over the world. This ensiling procedure is based on lactic acid fermentation of water-soluble carbohydrates by lactic acid bacteria, which are common members of the natural epiphytic microflora of freshly harvested crops (Bolsen *et al.*, 1996; McDonald *et al.*, 1991). As a result of their intensive fermentative activity, a rapid increase takes place in the lactic acid

concentration with a concomitant decrease in pH, eventually yielding a stable feed product of high nutritive value (Driehuis & Oude Elferink, 2000; McDonald *et al.*, 1991). If the production of lactic acid does not result in a sufficiently rapid drop in the pH, growth of anaerobic ‘spoilage’ organisms such as saccharolytic and proteolytic clostridia will occur. However, even when satisfactory preservation under anoxic conditions has been attained, exposure to air, particularly during feed-out, may result in aerobic growth of yeasts and fungi at the expense of lactic acid (aerobic spoilage), which results in dramatic losses of nutritional value (Courtin & Spoelstra, 1990; Woolford, 1984).

Biological additives such as bacterial inoculants have been used widely to improve the silage process (reviewed by Bolsen *et al.*, 1996; Weinberg & Muck, 1996), primarily to increase the extent and rate of lactic acid production. Unfortunately, these procedures do not necessarily improve the aerobic stability of silage

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Abbreviation: MRS-MOD medium, modified MRS medium.

The GenBank/EMBL/DBJ accession numbers for the 16S rDNA sequences of strain LMG 19667^T, *Lactobacillus parakefiri* LMG 15133^T, *Lactobacillus parabuchneri* LMG 11457^T and strain LMG 19668 are respectively AF264701 and AY026750–AY026752.

(Driehuis *et al.*, 1999). However, when Driehuis *et al.* (1999) used the heterofermentative lactic acid bacterium *Lactobacillus buchneri* as an inoculant, a 20-fold increase in aerobic stability was observed. These authors demonstrated a decrease in lactic acid concentration and an increase in acetic acid concentration in silages treated in this way. Further investigations demonstrated that *L. buchneri*, as well as *Lactobacillus parabuchneri*, was able to degrade 1 mol lactic acid under anoxic conditions, without requiring an external electron acceptor, to approximately 0.5 mol acetic acid, 0.5 mol 1,2-propanediol, 1 mol CO₂ and traces of ethanol (Oude Elferink *et al.*, 2001). Nevertheless, Driehuis *et al.* (1999) detected predominately 1-propanol, with low levels of propionic acid and no 1,2-propanediol, as a predominant fermentation product in maize silages inoculated with *L. buchneri*. Since *L. buchneri* is unable to degrade 1,2-propanediol either in pure culture or in the experimental maize silages, the involvement of other bacteria in the subsequent conversion of 1,2-propanediol is likely. It was hypothesized that certain members of the epiphytic microflora are involved in the conversion of 1,2-propanediol produced by *L. buchneri*, resulting in the accumulation of 1-propanol and propionic acid as end-products.

In this paper, we support this hypothesis by showing that a significant part of the epiphytic flora is capable of degrading 1,2-propanediol to 1-propanol and propionic acid. Subsequent enrichments resulted in the isolation of two bacterial isolates that are able to use 1,2-propanediol as a sole carbon source under anoxic conditions. These isolates were subjected to physiological and biochemical characterization. The two strains were isolated from two different aerobically stable maize silages, both inoculated with *L. buchneri*. Comparative sequence analysis of 16S rDNA showed that the two strains have identical 16S rRNA genes and belong phylogenetically to the *L. buchneri* group. Further genotypic and phenotypic analyses confirmed that these two isolates represent a novel species, for which the name *Lactobacillus diolivorans* sp. nov. is proposed.

METHODS

Isolation procedure. Samples for isolation of 1,2-propanediol-fermenting micro-organisms were obtained from two different whole-crop maize silages that had both been inoculated with *L. buchneri* (5×10^5 c.f.u. g⁻¹ of fresh maize) immediately before the onset of the ensiling process. After 9 months of ensiling, a sample was taken from each of these silages and used as an inoculum in dilution series (MPN) for selective enrichment and enumeration of anaerobic, 1,2-propanediol-degrading bacteria.

The two inocula were prepared by suspending 17 g (dry weight) from each silage in 100 ml 50 mM phosphate buffer (pH 4.7) and shaking for 2 h in a rotary incubator, before use as an inoculum for serial dilutions. Serial dilutions of up to 10⁻¹⁰ were done with five replicates in Hungate tubes containing modified MRS (MRS-MOD) medium [containing l⁻¹ distilled water: 5.0 g Bacto peptone, 4.0 g 'lab lemco'

powder (Difco), 2.0 g yeast extract, 0.5 ml Tween 80, 1.0 g K₂HPO₄, 3.0 g NaH₂PO₄·H₂O, 0.6 g sodium acetate, 0.2 g MgSO₄·7H₂O and 0.04 g MnSO₄·H₂O; Oude Elferink *et al.*, 2001] supplemented with 50 mM 1,2-propanediol and 50 mM acetic acid and adjusted to pH 4.7. Tubes were incubated at 30 °C. From each separate dilution series, a sample was taken from the highest dilution (10⁻⁶ and 10⁻⁷) in which 1-propanol could be detected, and this was used to inoculate fresh medium. From these separate cultures, 0.1 ml was spread on agar plates containing the above-mentioned medium and plates were incubated at 30 °C under a N₂ atmosphere. Both plates showed similar off-white colonies, and one isolate was purified from each plate. The pure cultures obtained, strains JKD6^T and ACD7, have been deposited in the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium) as strains LMG 19667^T and LMG 19668, respectively. Strain JKD6^T was also deposited in the DSMZ (Braunschweig, Germany) as strain DSM 14421^T.

Bacterial strains and maintenance conditions. The reference strains used in this study were *Lactobacillus brevis* LMG 9606^T, *L. buchneri* LMG 6892^T, *Lactobacillus hilgardii* LMG 6895^T, *Lactobacillus kefir* LMG 9480^T, *L. parabuchneri* LMG 11457^T, *Lactobacillus parakefir* LMG 15133^T and *Lactobacillus reuteri* LMG 9213^T. These strains and strains LMG 19667^T and LMG 19668 were maintained on MRS agar medium (Oxoid) at 30 °C.

16S rRNA sequencing and phylogenetic analysis. High-molecular-mass native DNA was isolated from cells grown in MRS-MOD medium containing 50 mM glucose, pH 5.7, according to the method described by Lewington *et al.* (1987). The 16S rRNA genes were amplified by PCR using the forward primer 5'-AGAGTTTGATCMTGGCTCAG-3', hybridizing at positions 8-27, and the reverse primer 5'-TACGGYTACCTTGTTACGACTT-3', hybridizing at positions 1492-1511 (*Escherichia coli* sequence numbering). The double-stranded PCR fragments obtained were subjected to a sequencing PCR with fluorescent dye-labelled dideoxynucleotides and subsequently sequenced (*Taq* cycle sequencing) using an ABI 310 automated DNA sequencer (Perkin-Elmer) according to the manufacturer's guidelines. The sequencing primers have been described previously (Lane *et al.*, 1985). The closest relatives of the newly isolated bacterial strains were determined by performing sequence database searches (Ribosomal Database Project; RDP) and the sequences of the closest related strains were retrieved from the NCBI database and RDP. Evolutionary distances were computed by using the correction method of Jukes & Cantor (1969). A tree was constructed by neighbour-joining analysis of the distance matrix from the multiple alignment (CLUSTAL W; Thompson *et al.*, 1994).

PAGE of whole-cell proteins. Strains LMG 19667^T and LMG 19668 and related reference strains were grown for 24-48 h on MRS agar (Oxoid) and incubated aerobically at 30 °C. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot *et al.* (1994). Registration of the protein patterns, normalization of the densitometric traces, pattern storage, grouping of the strains using Pearson's product-moment correlation coefficient (*r*) and UPGMA cluster analysis were performed as described by Pot *et al.* (1994) using the GELCOMP software (Applied Maths). For numerical analysis of the patterns, positions 20-80 and 150-325 of the 400 points registered were taken into account, omitting the stacking gel/separation gel interface (positions 0-19), the zone with disturbing high-

density bands (positions 81–149) and the front of the electrophoretic profile (positions 326–400).

Duplicate protein extracts were prepared to check the reproducibility of the growth conditions and the preparation of the extracts. The correlation level for duplicate protein patterns was $r > 0.94$.

DNA base compositions. High-molecular-mass native DNA was prepared from strains LMG 19667^T and LMG 19668. Cells were cultivated in MRS broth (Oxoid) for 24 h at 30 °C. DNA was extracted from 0.75–1.25 g wet weight of cells using the protocol described by Pitcher *et al.* (1989) with the following modifications: the washed cell pellet was resuspended and lysed in a buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) containing RNase (200 µg ml⁻¹; Sigma), mutanolysin (100–500 U ml⁻¹; Sigma) and lysozyme (25 mg ml⁻¹; SERVA) for 1 h at 37 °C. Before addition of GES reagent, proteinase K (200 µl; Merck) was added to the mixture for 15 min.

For determination of the DNA base composition, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters SymmetryShield C8 column thermostatted at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

DNA–DNA hybridization experiments. High-molecular-mass native DNA was prepared as described above for determination of the DNA base composition. Type strains of the species *L. buchneri*, *L. parabuchneri*, *L. kefir* and *L. hilgardii* were included in the present analyses. Efforts to extract DNA from the type strain of *L. parakefir* were not successful.

DNA–DNA hybridizations were performed by a modification of the microplate method described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with single-stranded unlabelled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 37 °C in hybridization mixture (2 × SSC, 5 × Denhardt's solution, 2.5% dextran sulfate, 50% formamide and 100 µg denatured salmon sperm DNA ml⁻¹ with 1250 ng biotinylated DNA probe ml⁻¹).

Morphological, physiological and biochemical tests. Gram staining and morphology were examined after 24 h incubation on MRS agar according to Doetsch (1981). For preparation of electron micrographs, cells were treated following the procedure described by Van der Linden *et al.* (1992). Cells grown on 1,2-propanediol (40 mM) or glucose (50 mM) were fixed with 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at 0 °C. Post-fixation was performed in a 2:1 mixture of 1% OsO₄ and 5% K₂Cr₂O₇ in the same buffer. Subsequently, the material was stained overnight in 1% (w/v) uranyl acetate in distilled water, dehydrated in a graded series of ethanol and embedded in Epon resin.

Catalase activity and gas production were determined as described previously by Strohmair & Diekmann (1992). Tests for growth at pH 4.0 and 7.0 in 0, 2, 4 and 7.0% NaCl and at 12, 20, 30 and 42 °C were performed in duplicate in MRS broth (Oxoid). Results were recorded after 72 h.

Carbohydrate fermentation tests were carried out with the

API 50 CHL system for lactobacilli (bioMérieux) according to the manufacturer's instructions: reactions were determined after incubation at 30 °C for 48 h.

Additional substrate utilization tests were performed in MRS-MOD medium (pH 5.7) supplemented separately with 45 mM glucose, 1-propanol, 2-propanol, 1,2-propanediol, 1,3-propanediol, propionic acid, 2,3-butanediol and glycerol. All substrates used were added separately to the medium from filter-sterilized stock solutions. A 1 M HCl solution was used to adjust the medium to the appropriate pH. Bacterial growth was followed by measuring turbidity at 433 nm. The concentrations of organic acids, alcohols and sugars were determined by HPLC of cell-free supernatant. The organic acids, alcohols and sugars were resolved on a pre-packed cation-exchange resin column (Polyspher OA HY, 300 × 6.5 mm; Merck) at a temperature of 65 °C and a flow rate of 0.6 ml min⁻¹ with 0.005 M H₂SO₄ as the eluant. The injection volume was 25 µl and detection occurred on a refractometer.

The temperature and pH optima for growth of strain LMG 19667^T were determined on MRS-MOD medium supplemented with 40 mM 1,2-propanediol. Growth was followed and maximum growth rates were calculated at various temperatures (range 17.9–41.3 °C) at pH 5.7 and various pH values (range 3.8–6.7) at 30 °C. These experiments were done in triplicate.

RESULTS AND DISCUSSION

Lactic acid bacteria have been isolated from a variety of habitats, such as foods, vegetation and animals (Kandler & Weiss, 1986; Hammes *et al.*, 1992). In this study, we describe the selective enrichment and enumeration of anaerobic, 1,2-propanediol-fermenting lactobacilli from aerobically stable maize silage. Two 1-propanol-producing isolates were subsequently purified and identified using genotypic and phenotypic analyses.

Isolation of 1,2-propanediol-degrading strains

Using medium that resembles the conditions that prevail in silage inoculated with *L. buchneri* (Driehuis *et al.*, 1999), two separate samples were taken from two stable maize silages and subsequently used as inocula in dilution series containing 1,2-propanediol. The highest dilutions in which 1-propanol production could be detected revealed that the number of viable 1,2-propanediol-degrading bacteria was 5.9×10^7 g⁻¹ of maize silage (dry weight). From each of the two dilution series, the dominant 1,2-propanediol-metabolizing population was plated on MRS-MOD agar plates and revealed similar off-white colonies. A pure culture was isolated from each plate (LMG 19667^T and LMG 19668). Both strains are Gram-positive, non-motile and catalase-negative rods that occur singly or in pairs. Longer cells were observed on MRS-MOD medium with glucose as substrate than with 1,2-propanediol (Fig. 1a, b). A cell-wall thickness of about 25 nm was observed, characteristic for Gram-positive organisms (Fig. 1c). No capacity to form spores was

Fig. 1. Electron micrographs of *L. diolivorans* LMG 19667^T grown on glucose (a), on 1,2-propanediol (b) and a close-up of the cell-wall structure (c). Bars, 0.1 μm.

Fig. 2. Phylogenetic tree showing the relationships among *L. diolivorans* strains, related species of the *L. buchneri* group and species representing different lineages within the genus *Lactobacillus*. The tree was constructed by neighbour-joining analysis of a distance matrix from a multiple-sequence alignment. Bar, estimated evolutionary distance (a mean of 10 substitutions at any nucleotide position per 100 nucleotide positions).

observed either aerobically or anaerobically. These basic screening tests clearly confirmed that both isolates are lactobacilli.

Phylogenetic allocation of 1,2-propanediol-degrading strains

To establish the phylogenetic position of the newly isolated strains, the 16S rRNA genes of strains LMG 19667^T and LMG 19668 were amplified and sequence comparison revealed 100% similarity. Further comparison with deposited sequences available in the RDP (Maidak *et al.*, 1999) indicated that the strains are classified in the *Lactobacillus casei*–*Pediococcus* group and, more particularly, among the obligately heterofermentative species of the *L. buchneri* group (Schleifer & Ludwig, 1995a, b). Significant sequence similarities for possible relatedness at the species level (> 97%) were obtained with *L. buchneri* (98.2%), *L. hilgardii* (98.1%) and *L. kefir* (97.7%). Because no 16S rDNA

sequences were available for the phenotypically highly related species *L. parabuchneri* and *L. parakefiri*, the 16S rDNAs of the type strains of the latter two species were subsequently sequenced (Farrow *et al.*, 1986; Takizawa *et al.*, 1994). Comparison with the newly isolated strains LMG 19667^T and LMG 19668 yielded similarities of 97.5 and 98.1%, respectively. A phylogenetic tree was generated from evolutionary distances by the neighbour-joining method (Fig. 2).

Identification of 1,2-propanediol-degrading strains

The whole-cell protein profiles of the newly isolated strains were initially compared with all available patterns of reference strains of validly described species of the *L. buchneri* phylogenetic group (data not shown). Fig. 3 shows a summarizing dendrogram obtained after average-linkage cluster analysis of the two isolated strains and type strains of the species

Fig. 3. Protein profiles of *L. diolivorans* strains and the corresponding dendrogram derived from the UPGMA linkage of correlation coefficients r (expressed as percentages). The molecular mass markers (lane M) are (from left to right): lysozyme, 14500 Da; trypsin inhibitor, 20100; trypsinogen, 24000; carbonic anhydrase, 29000; glyceraldehyde-3-phosphate dehydrogenase, 36000; egg albumin, 45000; bovine serum albumin, 66000; and β -galactosidase, 116000.

Table 1. DNA relatedness among *L. diolivorans* and phylogenetically closely related *Lactobacillus* species

Strain	DNA–DNA reassociation (%) with:					
	1	2	3	4	5	6
1. <i>L. diolivorans</i> LMG 19667 ^T	100	–	–	–	–	–
2. <i>L. diolivorans</i> LMG 19668	69	100	–	–	–	–
3. <i>L. buchneri</i> LMG 6892 ^T	24	15	100	–	–	–
4. <i>L. hilgardii</i> LMG 6895 ^T	22	15	18	100	–	–
5. <i>L. kefir</i> LMG 9480 ^T	30	15	31	20	100	–
6. <i>L. parabuchneri</i> LMG 11457 ^T	26	14	24	16	26	100

L. brevis, *L. buchneri*, *L. parabuchneri*, *L. hilgardii*, *L. kefir* and *L. parakefir*. Strains LMG 19667^T and LMG 19668 grouped together at a level of $r > 0.96$, clearly separated from other reference strains. The two strains are easily differentiated from each other visually by the varying position of a dominant protein band (molecular mass ranging from 45 to 66 kDa; Fig. 3). The latter band may indicate the presence of an S-layer on the outside of the bacteria, as was demonstrated for species of the *Lactobacillus acidophilus* group (Boot *et al.*, 1996). All organisms from the present study contained an analogous strain-specific dense band. In order to deduce reliable taxonomic relationships at the species level, the zone containing this dense band (40–66 kDa) was omitted from the numerical analysis.

The G + C contents of strains LMG 19667^T and LMG 19668 were 39.6 and 40.2 mol%. DNA homology studies with strains LMG 19667^T and LMG 19668 show that the two strains are related at the species level (69% DNA binding; Table 1). No significant DNA relatedness (less than 30%) was measured between the newly isolated strains and the type strains of the species *L. buchneri*, *L. parabuchneri*, *L. kefir* and *L. hilgardii*, indicating that the two newly isolated strains represent a separate genomic species.

The carbohydrate fermentation patterns of strains LMG 19667^T and LMG 19668 and the type strains of *L. buchneri*, *L. hilgardii*, *L. kefir*, *L. parabuchneri* and *L. parakefir* were determined using the API 50CHL system (Table 2). Strains LMG 19667^T and LMG 19668 showed analogous phenotypic characteristics. The only variable feature is that D-raffinose was fermented weakly by strain LMG 19668 and not at all by strain LMG 19667^T. Furthermore, both strains were clearly differentiated from closely related *Lactobacillus* species by their ability to acidify D-arabitol, methyl α -D-glucoside and methyl β -xyloside. Data obtained in the present study for *L. buchneri*, *L. hilgardii*, *L. kefir*, *L. parabuchneri* and *L. parakefir* largely corroborate the characteristics presented previously (Farrow *et al.*, 1986; Kandler & Weiss, 1986; Takizawa *et al.*, 1994).

The abilities of *L. buchneri* LMG 6892^T and strains LMG 19667^T and LMG 19668 to grow at various temperatures (12, 20, 30 and 42 °C), at different NaCl concentrations (0, 2, 4 and 7%) and at pH 4.0 and 7.0 were determined. None of these lactobacilli showed growth at 12 or 42 °C. At 30 °C, all three strains were able to grow at 0 and 2% NaCl, at both pH 4.0 and pH 7.0. At 4% NaCl, only *L. buchneri* LMG 6892^T was

Table 2. Carbohydrate fermentation pattern of *L. diolivorans* and related taxa

Strains are listed as: 1, *L. buchneri* LMG 6892^T; 2, *L. hilgardii* LMG 6895^T; 3, *L. kefir* LMG 9480^T; 4, *L. parabuchneri* LMG 11457^T; 5, *L. parakefir* LMG 15133^T; 6, *L. diolivorans* LMG 19667^T; 7, *L. diolivorans* LMG 19668. Acid formation is given as: +, positive; −, negative; w, weakly positive. All strains acidify galactose, D-glucose, maltose, melibiose and ribose. None of the strains acidifies adonitol, amygdalin, D-arabinose, L-arabitol, arbutin, cellobiose, dulcitol, erythritol, aesculin, D-fucose, L-fucose, β-gentiobiose, 2-ketogluconate, N-acetylglucosamine, glycerol, glycogen, inositol, inulin, mannitol, D-mannose, methyl α-D-mannoside, D-lyxose, rhamnose, salicin, sorbitol, L-sorbose, starch, D-tagatose, trehalose, D-turanose, xylitol or L-xylulose as a sole carbon source.

Substrate	1	2	3	4	5	6	7
L-Arabinose	+	−	+	+	+	+	+
D-Arabitol	−	−	−	−	−	+	+
D-Fructose	+	+	+	+	−	+	+
Gluconate	±	w	w	w	−	+	+
5-Ketogluconate	+	w	−	w	−	w	+
Methyl α-D-glucoside	−	−	−	−	−	w	+
Lactose	−	+	+	+	+	−	−
Melezitose	+	−	−	+	−	−	−
D-Raffinose	−	−	−	+	−	−	w
Sucrose	+	−	−	+	−	−	−
D-Xylose	+	+	−	−	−	+	+
Methyl β-xyloside	−	−	−	−	−	+	+

able to grow and only at pH 4.0. At 20 °C, all three strains grew without additional NaCl and only *L. buchneri* LMG 6892^T showed growth at 2 % NaCl (pH 4.0 and 7.0).

Substrate-utilization tests with compounds related to 1,2-propanediol were performed for strains LMG 19667^T and LMG 19668, *L. buchneri* LMG 6892^T, *L. kefir* LMG 9480^T, *L. parabuchneri* LMG 11457^T and *L. reuteri* LMG 9213^T. Cells were grown in media supplemented with 1-propanol, 2-propanol, 1,2-propanediol, 1,3-propanediol, propionic acid, 2,3-butanediol, glycerol or glucose (as a positive control). As expected, all species were able to grow on glucose, resulting in increased concentrations of lactate and ethanol and traces of acetate. Only strains LMG 19667^T and LMG 19668 showed increased cell densities on 1,2-propanediol. Both strains could use 1,2-propanediol as a sole substrate under anoxic conditions and fermented 45 mM 1,2-propanediol stoichiometrically to 20 mM propionic acid and 23 mM 1-propanol. No growth was observed on any of the other substrates.

The genomic and biochemical data given above clearly support the conclusion that the two isolated strains represent a novel *Lactobacillus* species, for which the name *Lactobacillus diolivorans* sp. nov. is proposed.

Fig. 4. Influence of temperature (a) and pH (b) on the growth rate of *L. diolivorans* LMG 19667^T when degrading 40 mM 1,2-propanediol anaerobically at pH 5.7 (a) and 30 °C (b).

Fermentation of 1,2-propanediol by *L. diolivorans* LMG 19667^T

Anaerobic growth of *L. diolivorans* LMG 19667^T on 1,2-propanediol was studied in more detail in batch cultures. Optimal growth on 1,2-propanediol was obtained at 30–32 °C and at pH 5.7. The growth rate ($\mu_{\max} = 0.042 \text{ h}^{-1}$ at pH 5.7; Fig. 4a) on 40 mM 1,2-propanediol (30 °C) was affected only slightly by changes in the pH over the range 3.8–5.7 (Fig. 4b) whereas, at higher pH values, the growth rate dropped rapidly. A more significant effect of changes in pH was observed on the growth yield. When grown on 40 mM 1,2-propanediol, the final OD₄₃₃ dropped from 0.900 to 0.450 when the pH was decreased from 5.7 to 3.8 (not shown).

Fig. 5 shows the formation of fermentation products during growth on 38 mM 1,2-propanediol by strain LMG 19667^T (at pH 3.8). 1,2-Propanediol was fermented to approximately equimolar amounts of 1-propanol and propionic acid, according to the following equation:

Transient formation of propionaldehyde was detected in these cultures. No significant change in the rate of product formation or the ratio between fermentation

Fig. 5. Anaerobic growth of *L. diolivorans* LMG 19667^T on 40 mM 1,2-propanediol at pH 3.8. The substrate is degraded into 1-propanol and propionic acid, with the transient accumulation of propionaldehyde as an intermediate. ◇, 1,2-propanediol; ◆, propionaldehyde; ■, 1-propanol; □, propionic acid; ○, lactate; ▲, acetate. OD₄₃₃ is shown by the dotted line.

products was observed on 1,2-propanediol at pH 5.7. Similar results were obtained with strain LMG 19668 (not shown).

Although anaerobic degradation of alkanediols by lactic acid bacteria has been shown previously (Veiga-da-Cunha & Foster, 1992), anaerobic degradation of 1,2-propanediol has, to the best of our knowledge, not been described for this group of bacteria. Toraya *et al.* (1979) demonstrated the fermentation of 1,2-propanediol and 1,2-ethanediol to *n*-propanol and propionic acid by members of some genera of the *Enterobacteriaceae*, using the coenzyme B₁₂-dioldehydratase. Ouattara *et al.* (1992) described *Desulfovibrio* sp. strain HDv and *Desulfovibrio alcoholovorans*, both capable of anaerobic degradation of 1,2-propanediol. Walter *et al.* (1997) suggested that 1,2-propanediol could act as an electron sink for *Salmonella typhimurium*.

Based on the stoichiometric production of 1-propanol and propionic acid and the transient accumulation of propionaldehyde during 1,2-propanediol fermentation, we presume that 1,2-propanediol is degraded by the same pathway as proposed by Toraya *et al.* (1979). Hence, two moles of 1,2-propanediol may be degraded by a diol-dehydratase (Toraya, 2000) to produce propionaldehyde, partly being used as an electron

acceptor, yielding one mole of 1-propanol, and be oxidized further to produce one mole of propionic acid and one mole of ATP.

Environmental significance of *L. diolivorans* in maize silage

Moon (1983) demonstrated that growth of acid-tolerant yeasts was inhibited by mixtures of lactate, acetate and propionic acid and Driehuis *et al.* (1999) concluded that the main factor responsible for the inhibition of yeasts, and thus the increased aerobic stability in silages inoculated with *L. buchneri*, was probably the increased contents of acetic acid and propionic acid. As reported by Oude Elferink *et al.* (2001), *L. buchneri* is able to ferment lactate to acetate, 1,2-propanediol, CO₂ and traces of ethanol under anoxic conditions at low pH values (< 4). However, this lactic acid bacterium is unable to degrade 1,2-propanediol further to propionic acid. Based on the ability of *L. diolivorans* to ferment 1,2-propanediol to 1-propanol and propionic acid under conditions that prevail in silage and the observed absence of 1,2-propanediol in experimental and full-scale aerobically stable silages, *L. diolivorans* may play an important role in stabilizing maize silages.

Description of *Lactobacillus diolivorans* sp. nov.

Lactobacillus diolivorans (di.o.li.vo'rans. N.L. *diol* from 1,2-propanediol; *L. v. vorare* to devour; N.L. adj. *diolivorans* devouring diols).

Cells are Gram-positive, non-motile, non-spore-forming rods that occur as single cells, in pairs or occasionally in short chains. On MRS-MOD medium with 1,2-propanediol as substrate, cells are 1 µm in width and 2 µm in length whereas, on glucose, the cells are longer, up to 10 µm. Colonies are off-white on MRS medium. Facultatively anaerobic. Catalase-negative. Heterofermentative. Fermentative growth on 1,2-propanediol under anoxic conditions, producing 1-propanol and propionic acid. Optimal temperature and pH for growth on 1,2-propanediol are 30–32 °C and pH 5.7. No growth is obtained at 12 or 42 °C. Growth at NaCl concentrations of 2% (w/v) at 30 °C; no growth at 4%. Other phenotypic characteristics of *Lactobacillus diolivorans* LMG 19667^T are summarized in Table 2. The DNA G + C content is 40 mol%.

The type strain is strain JKD6^T (= LMG 19667^T = DSM 14421^T), which was isolated from maize silage in The Netherlands.

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REFERENCES

- Bolsen, K. K., Ashbell, G. & Weinberg, Z. G. (1996). Silage fermentation and silage additives. *Asian-Australas J Anim Sci* **9**, 483–493.
- Boot, H. J., Kolen, C. P. A. M., Pot, B., Kersters, K. & Pouwels, P. H. (1996). The presence of two S-layer-protein-encoding genes is conserved among species related to *Lactobacillus acidophilus*. *Microbiology* **142**, 2375–2384.
- Courtin, M. G. & Spoelstra, S. F. (1990). A simulation model of the microbiological and chemical changes accompanying the initial stage of aerobic deterioration of silage. *Grass Forage Sci* **45**, 153–165.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–22. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, W. E. Nester, W. A. Wood, N. R. Krieg & G. Briggs-Phillips. Washington, DC: American Society for Microbiology.
- Driehuis, F. & Oude Elferink, S. J. W. H. (2000). The impact of the quality of silage on animal health and food safety: a review. *Vet Q* **22**, 212–216.
- Driehuis, F., Oude Elferink, S. J. W. H. & Spoelstra, S. F. (1999). Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. *J Appl Microbiol* **87**, 583–594.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Farrow, J. A. E., Phillips, B. A. & Collins, M. D. (1986). Nucleic acid studies on some heterofermentative lactobacilli: description of *Lactobacillus malefermentans* sp. nov. and *Lactobacillus parabuchneri* sp. nov. *FEMS Microbiol Lett* **55**, 163–168.
- Hammes, W. P., Weiss, N. & Holzapfel, W. (1992). The genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes*, 2nd edn, pp. 1535–1594. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kandler, O. & Weiss, N. (1986). Genus *Lactobacillus* Beijerinck 1901. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1209–1234. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A* **82**, 6955–6959.
- Lewington, J., Greenaway, S. D. & Spillane, B. J. (1987). Rapid scale preparation of bacterial chromosomal DNA, suitable for cloning and hybridization analysis. *Lett Appl Microbiol* **5**, 51–53.
- McDonald, P., Henderson, A. R. & Heron, S. J. E. (1991). *The Biochemistry of Silage*. Marlow: Chalcombe Publications.
- Maidak, B. L., Cole, J. R., Parker, C. T., Jr & 11 other authors (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Res* **27**, 171–173.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Moon, N. J. (1983). Inhibition of growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. *J Appl Bacteriol* **55**, 453–460.
- Ouattara, A. S., Cuzin, N., Traore, A. S. & Garcia, J. L. (1992). Anaerobic degradation of 1,2-propanediol by a new *Desulfovibrio* strain and *D. alcoholovorans*. *Arch Microbiol* **158**, 218–225.
- Oude Elferink, S. J. H. W., Krooneman, J., Gottschal, J. C., Spoelstra, S. F., Faber, F. & Driehuis, F. (2001). Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Appl Environ Microbiol* **67**, 125–132.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidinium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.
- Schleifer, K. H. & Ludwig, W. (1995a). Phylogenetic relationships of lactic acid bacteria. In *The Genera of Lactic Acid Bacteria*, pp. 7–18. Edited by B. J. B. Wood & W. H. Holzapfel. London: Blackie Academic & Professional.
- Schleifer, K. H. & Ludwig, W. (1995b). Phylogeny of the genus *Lactobacillus* and related genera. *Syst Appl Microbiol* **18**, 461–467.
- Strohm, W. & Diekmann, H. (1992). Die Microbiologie eines Langzeit-Sauerteiger. *Z Lebensm Unters Forsch* **194**, 536–540.
- Takizawa, S., Kojima, S., Tamura, S., Fujinaga, S., Benno, Y. & Nakase, T. (1994). *Lactobacillus kefirgranum* sp. nov. and *Lactobacillus parakefir* sp. nov., two new species from kefir grains. *Int J Syst Bacteriol* **44**, 435–439.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Toraya, T. (2000). The structure and the mechanism of action of coenzyme B₁₂-dependent diol dehydratases. *J Mol Catal B Enzym* **10**, 87–106.
- Toraya, T., Honda, S. & Fukui, S. (1979). Fermentation of 1,2-propanediol and 1,2-ethanediol by some genera of *Enterobacteriaceae*, involving coenzyme B₁₂-dependent diol dehydratase. *J Bacteriol* **139**, 39–47.
- Van der Linden, M. P., Vonck, J., Sjollem, K. A. & Keck, W. (1992). In vivo crystal formation in *Escherichia coli* of an over-expressed soluble form of penicillin-binding protein 5. *FEMS Microbiol Lett* **78**, 117–123.
- Veiga-da-Cunha, M. & Foster, M. A. (1992). 1,3-Propanediol:NAD⁺ oxidoreductases of *Lactobacillus brevis* and *Lactobacillus buchneri*. *Appl Environ Microbiol* **58**, 2005–2010.
- Walter, D., Ailion, M. & Roth, J. (1997). Genetic characterization of the pdu operon: use of 1,2-propanediol in *Salmonella typhimurium*. *J Bacteriol* **179**, 1013–1022.
- Weinberg, Z. G. & Muck, R. E. (1996). New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiol Rev* **19**, 53–68.
- Woolford, M. K. (1984). The microbiology of silage. In *The Silage Fermentation*, pp. 35–42. New York: Marcel Dekker.