***Lactobacillus arizonensis* sp. nov., isolated from jojoba meal**

J. L. Swezey, L. K. Nakamura, T. P. Abbott and R. E. Peterson

Author for correspondence: L. K. Nakamura. Tel: +1 309 681 6395. Fax: +1 309 681 6672. e-mail: lnakamura@sunca.ncaur.usda.gov

Microbial Properties
Research Unit, National
Center for Agricultural
Utilization Research, 1815
North University Street,
Agricultural Research
Service, US Department of
Agriculture, Peoria, IL 61604,
USA

Five strains of simmondsin-degrading, lactic-acid-producing bacteria were isolated from fermented jojoba meal. These isolates were facultatively anaerobic, Gram-positive, non-motile, non-spore-forming, homofermentative, rod-shaped organisms. They grew singly and in short chains, produced lactic acid but no gas from glucose, and did not exhibit catalase activity. Growth occurred at 15 and 45 °C. All strains fermented cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, melibiose, D-ribose, salicin, D-sorbitol, sucrose and trehalose. Some strains fermented L-(–)-arabinose and L-rhamnose. D-Xylose was not fermented and starch was not hydrolysed. The mean G+C content of the DNA was 48 mol%. Phylogenetic analyses of 16S rDNA established that the isolates were members of the genus *Lactobacillus*. DNA reassociation of 45% or less was obtained between the new isolates and the reference strains of species with G+C contents of about 48 mol%. The isolates were differentiated from other homofermentative *Lactobacillus* spp. on the basis of 16S rDNA sequence divergence, DNA relatedness, stereoisomerism of the lactic acid produced, growth temperature and carbohydrate fermentation. The data support the conclusion that these organisms represent strains of a new species, for which the name *Lactobacillus arizonensis* is proposed. The type strain of *L. arizonensis* is NRRL B-14768^T (= DSM 13273^T).

Keywords: new species, taxonomy, simmondsin degrader, jojoba, *Lactobacillus arizonensis*

INTRODUCTION

Jojoba (*Simmondsia chinensis*) is an important commercial seed crop grown in the desert southwestern United States. Up to 60% of the seed weight consists of a unique oil that is used in the cosmetic and skin care industry. After hexane extraction of the oil, the remaining meal contains 25–30% crude protein, is high in fibre and could be a valuable cattle feed supplement. However, this meal is not currently fed to animals because it contains 5-demethyl simmondsin, 4,5-didemethylsimmondsin, simmondsin and simmondsin 2'-ferulate (Elliger *et al.*, 1973, 1974a, b; Verbiscar & Banigan, 1978). These substances are cyanogenic toxins that have been implicated in weight

loss of ruminants (Manos *et al.*, 1986) and in the death of mice (Weber *et al.*, 1983).

Studies by Verbiscar *et al.* (1981) indicated that *Lactobacillus* spp. attacked simmondsin in autoclaved meal but not when used as a sole carbon source in culture media. As the result of a screening effort, Abbott *et al.* (1990) obtained isolates of *Stenotrophomonas maltophilia*, '*Flavobacterium aurantiacum*' and *Fusarium moniliforme* that attacked simmondsin when used as a sole carbon source; however, only *F. moniliforme* degraded simmondsin effectively in the meal. Microbiological evaluation of large batches of meal inoculated with *F. moniliforme* and incubated out of doors revealed the involvement of bacteria as well as fungi in the simmondsin-degrading process.

The purpose of this study was to identify the simmondsin-degrading bacteria isolated from fermented jojoba meal. The results indicate that the simmondsin-de-

The GenBank accession number for the 16S rDNA sequence of *Lactobacillus arizonensis* sp. nov. is AF093757.

grading isolates represent strains of a new *Lactobacillus* species.

METHODS

Bacterial strains. The strains were isolated from sterile jojoba meal that had been inoculated with *F. moniliforme* and fermented for 20 d in the open air of Hyden Valley Desert in Arizona. One gram samples of meal were suspended in 100 ml 0.85% (w/v) sterile saline and 0.2 ml serial dilutions were spread on modified Rogosa SL agar in which the dextrose, L-(+)-arabinose and sucrose was replaced by 0.5% (w/v) simmondsin. Incubation was at 37 °C in an anaerobic jar having a H₂+CO₂ environment generated with a BBL Gas Pak (Becton Dickinson Microbiology Systems). Small, white, opaque, raised colonies appearing after 48 h were streaked onto MRS agar for purification.

The reference strains used in the DNA reassociation studies were *Lactobacillus amylophilus* NRRL B-4437^T, *Lactobacillus coryniformis* NRRL B-4391^T, *Lactobacillus plantarum* NRRL B-4496^T, *Lactobacillus paracasei* subsp. *paracasei* NRRL B-4560, *Lactobacillus rhamnosus* NRRL B-176^T, *Lactobacillus casei* subsp. *casei* NRRL B-1922^T, *Lactobacillus delbrueckii* subsp. *delbrueckii* NRRL B-763^T and *Lactobacillus pentosus* NRRL B-473^T. These strains are maintained at the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research, Peoria, IL, USA.

Characterization tests. Carbohydrate fermentation tests were conducted in modified MRS medium (Kandler & Weiss, 1986) with 0.0016% (w/v) bromocresol purple as an indicator. Inverted Durham tubes were placed in the broth tubes to detect gas production. Starch utilization was determined by growing micro-organisms on MRS agar plates containing 0.5% (w/v) soluble starch. The hydrolysis of starch was detected by flooding the plates with 1.0 ml iodine solution (0.2 g I₂ and 2.0 g KI in 100 ml distilled water). To determine growth at different temperatures (10, 15, 20, 25, 30, 35, 40, 45 and 50 °C), preincubated sterile medium in 15 × 150 mm screw-capped test tubes was inoculated and incubated in a Control Environment Incubator (New Brunswick Scientific).

Inocula for the characterization tests were prepared from cultures grown for 24 h at 37 °C in MRS broth. Cells were harvested by centrifugation at 4 °C, washed in sterile 0.85% saline and resuspended in sterile saline solution. One-tenth millilitre aliquots of inoculum were added to each test tube containing 6 ml medium. All tests were run in duplicate and incubated at 37 °C in an anaerobic jar with the screw caps loosened; readings were taken at 1, 5 and 10 d.

Simmondsin degradation was determined by growing the isolates in deep liver medium (McClung & McCoy, 1934), which was modified to contain 5 g l⁻¹ simmondsin instead of glucose as the carbohydrate source. Samples of the fermented broth were filtered through a 0.45 µm pore size Millipore membrane and frozen. Ten microlitre aliquots of the sample were injected into a HPLC system consisting of an SP8800 ternary pump (Spectra-Physics), ISCO V⁴ absorbance detector (Isco), Econosphere C-18 3 µm 100 × 4.6 mm i.d. column (Alltech) and a central ModComp 32/85 computer system (Modular Computer Systems). A linear 5–100% aqueous methanol gradient over 10 min at 0.75 ml min⁻¹ was used for all determinations. Column effluents were monitored at 220 nm. Analysis time per

sample, including re-equilibration, was typically 20 min. Peak areas in samples of fermentation media with no toxin (i.e. media blanks) were subtracted from sample peak areas. Toxin peak area in fermented samples (after media subtraction) was divided by toxin peak area in unfermented samples to determine the fraction of toxin that was not degraded. The retention time of simmondsin was 8.9 min.

Analyses of metabolic acid products. The concentration of lactic acid and other organic acids was measured by HPLC of cell-free supernatant of cultures incubated for 1 and 6 d in deep liver medium. The organic acids and other medium components were resolved on a HPX-87H column (Bio-Rad) and monitored with a refractive index detector (Shimizu *et al.*, 1989).

To determine the stereoisomerism of the lactic acid products, cultures were grown for 48 h at 37 °C in 1 l MRS broth modified to contain 1% (w/v) glucose. To prepare samples for assay, 15 ml culture liquor was centrifuged at 10 000 g for 15 min at 4 °C, and the resulting supernatant was deproteinized by titration with concentrated NaOH to pH 7.6 in the presence of 2.5% (w/v) ZnSO₄. The precipitate was removed by centrifugation at 4 °C and the supernatant was analysed. Beef heart L-(+)-lactate dehydrogenase (EC 1.1.1.27) and *Lactobacillus leichmannii* D-(−)-lactate dehydrogenase (EC 1.1.1.28) were used for assessing the stereoisomerism of the lactic acid produced by the simmondsin-degrading isolates. Enzymes were purchased from Boehringer Mannheim. The method used was that of Latorre-Guzman *et al.* (1977). The assay mixture consisted of 0.1 ml (50 µg) enzyme solution, 2.0 ml glycine/hydrazine sulfate buffer [7.5% (w/v) glycine, 5.2% (w/v) hydrazine sulfate, 0.2% (w/v) sodium EDTA and 4.0% (w/v) NaOH; pH 9.5], 0.1 ml 56 mM NAD solution, 0.1 ml of appropriately diluted sample and 0.7 ml distilled water. Uninoculated broths with and without L-(+)- and D-(−)-lithium lactate were used as controls. The above-mentioned enzymes showed specificity for their respective substrates. Substrate utilization, indicated by NAD reduction, was monitored at 340 nm in a UV-VIS Thermal Analyzer System (Gilford Systems).

DNA base composition and reassociation. DNA was extracted and purified by a modification of the method of Marmur (1961). The modification involved the use of hydroxyapatite (Bio-Gel HTP; Bio-Rad) chromatography (Markov & Ivanov, 1974) to produce highly purified DNA preparations. The purity and quality of each DNA preparation were checked by monitoring the absorbance ratio at 260/280 nm (1.8–1.9) and at 260/230 nm (2.0–2.3) (according to Marmur, 1961) and by the melting curves, which showed hyperchromicity values from 38 to 40% (Mandel & Marmur, 1968).

The G+C content of the DNA was estimated by the buoyant density method (Schildkraut *et al.*, 1962). Buoyant density of the DNA was measured by CsCl₂ density gradient centrifugation in a Beckman Instrument model E ultracentrifuge. '*Micrococcus lysodeikticus*' DNA served as an internal standard.

DNA reassociation values were obtained by a spectrophotometric method described previously (Nakamura & Swezey, 1983). Percentage relatedness values were calculated by the equation of DeLey *et al.* (1970). All DNA reassociation determinations were repeated twice.

16S rDNA sequencing. A 16S rDNA fragment of strain NRRL B-14768^T that corresponds to positions 9–1510 of

Table 1. Characteristics useful for differentiating the simmondsin degraders (SD) from other *Lactobacillus* species

+, Positive; –, negative; d, strain variation.

Character	Organism:								
	SD	<i>L. plant-arum</i> *	<i>L. pentosus</i> †	<i>L. para-casei</i> *	<i>L. coryni-formis</i> *	<i>L. amylo-philus</i> *	<i>L. del-brueckii</i> *	<i>L. para-casei</i> *	<i>L. casei</i> *
G + C (mol %)	48	45	47	46	45	45	50	46	46
Stereoisomer of lactic acid	DL	DL	DL	DL	D-(–)	L-(+)	D-(–)	L-(+)	L-(+)
Growth at 15 °C	+	+	+	+	+	+	–	+	+
Growth at 45 °C	+	–	–	–	–	–	+	+	–
Simmondsin degradation (%)	90	2	20	0	0	0	0	70	0
Fermentation of:									
L-(–)-Arabinose	d	d	d	–	–	–	–	d	–
Mannitol	+	+	+	+	+	–	–	+	+
Melibiose	+	+	+	–	d	–	–	–	–
Raffinose	+	+	+	–	d	–	–	–	–
L-Rhamnose	d	–	d	–	+	–	–	+	–
D-Ribose	+	+	+	+	–	–	–	+	+
Sorbitol	+	+	+	+	d	–	–	+	+
Starch	–	–	–	–	–	+	d	–	–
Trehalose	+	+	+	+	–	–	–	+	+
D-Xylose	–	d	+	–	–	–	–	–	–

* According to Kandler & Weiss (1986).

† According to Zanoni *et al.* (1987).

Escherichia coli 16S rRNA was amplified by PCR, using purified DNA and a primer combination consisting of forward primer 27f and reverse primer 1492r (Lane, 1991). After purification with the GENE-CLEAN II kit (Bio101), the amplification products were sequenced with a Prism ABI dideoxy terminator cycle sequencing kit manufactured by Applied Biosystems. The manufacturer's recommended protocols were followed. Sequences were determined with the automated Applied Biosystems DNA sequencer. The following primers cited by Lane (1991) were used for sequence analyses: 27f, 530f, 1114f, 1406f, 109r1, 342r, 685r3, 907r, 1100r and 1492r. In addition, two other primers, designated 304f (5'-GTAGCCGACCTGAGAGG-3') and 801f (5'-AACAGGATTAGATACCC-3'), were designed to obtain unambiguous results. The CLUSTAL V (Higgins *et al.*, 1992) program was used to align the 16S rRNA gene sequence generated with sequences of selected members of the family *Lactobacillaceae* obtained from GenBank. A similarity matrix was constructed from the aligned sequences. Genetic distances were computed by using the two-correction model (Kimura, 1980) and used for neighbour-joining analysis.

Confidence limits of the groupings were estimated by bootstrap analysis (500 replications). Phylogenetic trees were constructed using the neighbour-joining and maximum-parsimony methods provided by PAUP 4.0b1 (Sinauer Associates, Sunderland, MA, USA); both methods produced trees with similar topologies.

The GenBank accession number for the 16S rRNA gene sequence of NRRL B-14768^T is AF093757.

RESULTS

Morphology and physiology

The five simmondsin-degrading isolates (NRRL B-14768^T, NRRL B-14769, NRRL B-14770, NRRL B-14771 and NRRL B-14772) were Gram-positive, non-motile rods that occurred singly or in short chains. Phase-contrast microscopic examinations of broth or agar cultures grown aerobically or anaerobically revealed no spores after 2 weeks incubation at 37 °C. Granules were noted in some microscopically observed Gram-stained cells. Mean cell measurements were 1 × 4–5 µm. Colonies on MRS agar were white, smooth, circular, convex and opaque. The isolates grew at both 15 and 45 °C but not at 10 or 50 °C, and optimally at 35–40 °C (based on visual inspection). They were catalase-negative and failed to hydrolyse starch. The isolates grew anaerobically and at initial pH values that ranged from 4.5 to 8.0; the pH was adjusted with 1 and 5 M HCl or NaOH. Growth was not detected in MRS medium that lacked a metabolizable sugar. All isolates degraded approximately 90 % of the simmondsin after 6 d. By comparison, 0–70 % degradation was recorded from the other *Lactobacillus* reference strains examined (Table 1). The fermentation pattern of the carbohydrates listed in Table 1 was useful for differentiating the isolates from the recognized species. Mannitol, melibiose, raffinose, D-ribose,

Fig. 1. Phylogenetic tree showing the position of *Lactobacillus arizonensis* NRRL B-14768^T among selected bacteria belonging to the *Lactobacillaceae*. The tree, which is rooted by using *Bacillus subtilis* as the outgroup, was generated by the neighbour-joining method. Bootstrap values based on 500 replications are given at the nodes. Trees generated by the neighbour-joining method and by maximum-parsimony analysis had similar topologies. GenBank accession numbers are given in parentheses. The bar represents 10% sequence divergence.

sorbitol and trehalose were fermented by all the isolates; L-arabinose and L-rhamnose were fermented only by NRRL B-14768^T and NRRL B-14769. Starch and D-xylose were not fermented. Other sugars fermented (not shown) were cellobiose, D-fructose, D-galactose, D-glucose (no gas produced), lactose, maltose, D-mannose, salicin and sucrose.

Analysis revealed that the isolates apparently produced only lactic acid (0.046 mol) from glucose

(0.028 mol) in 6 d, and therefore the organisms are homofermentative. Theoretically, homofermentative conversion should yield 2 mol lactic acid for each mol glucose consumed. Therefore, the isolates converted approximately 80% of the glucose to lactic acid. Under optimum conditions *L. delbrueckii* (Schopmeyer, 1954) fermentations have given 95% yields. Determination with specific enzymes demonstrated that both D-(–)- and L-(+)-lactic acids were produced.

Table 2. DNA relatedness among the simmondsin degraders (SD) and *Lactobacillus* reference strains

Reassociation values are means of two determinations; the maximum difference found between determinations was 5 %. The values in parentheses indicate that, by definition, the reassociation value was 100 %.

Strain	Reassociation (%) to DNA from SD strains:				
	NRRL B-14768 ^T	NRRL B-14769	NRRL B-14770	NRRL B-14771	NRRL B-14772
NRRL B-14768 ^T	(100)				
NRRL B-14769	71	(100)			
NRRL B-14770	88	95	(100)		
NRRL B-14771	96	84	100	(100)	
NRRL B-14772	87	70	86	89	(100)
<i>L. coryniformis</i> NRRL B-4391 ^T	39	37	30	31	29
<i>L. plantarum</i> NRRL B-4496 ^T	42	40	38	39	42
<i>L. paracasei</i> subsp. <i>paracasei</i> NRRL B-4560	25	21	18	24	26
<i>L. rhamnosus</i> NRRL B-176 ^T	7	5	13	12	5
<i>L. casei</i> subsp. <i>casei</i> NRRL B-1922 ^T	21	17	14	19	24
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i> NRRL B-763 ^T	28	36	24	26	31
<i>L. pentosus</i> NRRL B-473 ^T	39	31	29	33	36

16S rDNA sequence

Similarity values (not shown) of 95 % established that NRRL B-14768^T was most closely related to members of the genus *Lactobacillus*. Values of 80–87 % suggested a more distant relationship between NRRL B-14768^T and the genus *Carnobacterium*. Data searches using the BLAST system (Altschul *et al.*, 1990) corroborated the positioning of NRRL B-14768^T within the genus *Lactobacillus*, the closest relatives being *L. plantarum* and *L. pentosus*.

In a neighbour-joining tree (Fig. 1) based on sequences obtained in this study and from the GenBank database, NRRL B-14768^T is positioned in the *L. casei*/*Pediococcus* cluster (Collins *et al.*, 1991). Thus, the data demonstrate that NRRL B-14768^T clearly belongs to the genus *Lactobacillus* and is closely related to *L. plantarum* and *L. pentosus*. This relationship was supported by the bootstrap analyses at a confidence level of 100 %.

DNA base composition and reassociation

The buoyant densities of the DNAs for the isolates ranged from 1.7069 to 1.7073 g ml⁻¹, which corresponds to G + C contents of 47.8–48.3 mol %. These G + C values are within the range reported for *Lactobacillus* (Kandler & Weiss, 1986).

Table 2 shows that DNA relatedness ranging from 5 to 45 % was obtained between the simmondsin-degrading strains and homofermentative *Lactobacillus* species having G + C values of 45–50 mol %. DNA relatedness of 70–100 % was measured among all simmondsin-degrading strains. Studies have shown that microorganisms with DNA relatedness of 70 % or higher belong to the same species (Johnson, 1973).

DISCUSSION

The simmondsin-degrading strains were isolated from fermenting jojoba meal on modified acetate agar (Rogosa *et al.*, 1951), a selective medium for *Lactobacillus* species. These isolates were homofermentative, facultative, anaerobic or microaerophilic, Gram-positive, non-motile, non-spore-forming, rod-shaped cells that grew singly or in short chains and did not produce gas from glucose, were catalase-negative, and produced substantial amounts of lactic acid. These characteristics are typical of *Lactobacillus* species (Kandler & Weiss, 1986; Rogosa & Sharpe, 1960). Several characteristics eliminated the other 'regular, nonsporing, Gram-positive rods' as possible placements for the isolates. *Carnobacterium* species do not grow on acetate agar (Collins *et al.*, 1987). *Erysipelothrix* Rosenbach 1909 (Jones, 1986), *Brochothrix* Sneath & Jones 1976 (Sneath & Jones, 1986) and *Listeria* Pirie 1940 (Seeliger & Jones, 1986) often form filaments and have a G + C range of 36–40 mol %; the latter two genera are also catalase-positive. *Kurthia* Trevisan 1885 (Keddie & Shaw, 1986), *Caryophanon* Peshkoff 1939 (Trentini, 1986) and *Renibacterium* Sanders & Fryer 1980 (Sanders & Fryer, 1986) are strictly aerobic organisms. The granules revealed by Gram-staining are common in homofermentative *Lactobacillus* spp. (Kandler & Weiss, 1986). The new isolates distinctly differ from the facultative anaerobic, lactic-acid-producing genera *Staphylococcus* Rosenbach 1884 (Kloos & Schleifer, 1986), *Streptococcus* Rosenbach 1884 (Hardie, 1986), *Leuconostoc* van Tieghem 1878 (Garvie, 1986a) and *Pediococcus* Claussen 1903 (Garvie, 1986b), which exhibit definite coccoidal morphology. Also, classification of the new isolates as *Bifidobacter* was ruled out because this genus is strictly anaerobic, produces acetic acid as well as lactic acid,

and has a G+C range of 59–70 mol% (Scardovi, 1986).

Phylogenetic analysis in the present study established that the isolates are members of the genus *Lactobacillus*. It is generally agreed that organisms exhibiting differences in G+C contents no greater than $\pm 2\%$ might be members of the same species (Johnson, 1973). According to Kandler & Weiss (1986) *L. plantarum*, *L. paracasei*, *L. casei*, *L. coryniformis*, *L. delbrueckii*, *L. pentosus* and *L. rhamnosus* are homofermentative *Lactobacillus* species with G+C contents within 2% of the 48 mol% values of the new isolates. *L. amylophilus* is another species that meets this criterion (Nakamura & Crowell, 1979). The DNA relatedness data in Table 2 indicate that the simmondsin-degrading isolates were unrelated to the reference strains of these established species. The new isolates are homofermenters and differ from the other homofermenters in a number of ways (Table 1). Whilst both simmondsin-degraders and *L. rhamnosus* grow at 15 and 45 °C, the latter species produces only L-(+)-lactic acid and does not ferment melibiose or raffinose. Additionally, in contrast to the new isolates, *L. amylophilus*, *L. casei* subsp. *casei*, *L. delbrueckii* and *L. coryniformis* do not produce both D-(–)- and L-(+)-lactic acid and, except for *L. coryniformis*, do not ferment melibiose and raffinose. Furthermore, *L. amylophilus* hydrolyses starch, a trait not shown by the simmondsin-degraders. Unlike the new isolates, *L. plantarum*, *L. pentosus*, *L. coryniformis*, *L. amylophilus*, *L. casei* and *L. paracasei* do not grow at 45 °C. The inability to ferment D-xylose further distinguishes the simmondsin-degraders from *L. pentosus*. In contrast to the new isolates, *L. paracasei* does not ferment melibiose or raffinose. Furthermore, *L. plantarum* does not ferment L-rhamnose, an activity exhibited by some of the new isolates.

The evidence presented indicates that the simmondsin-degrading *Lactobacillus* strains are members of a new species, for which the name *Lactobacillus arizonensis* is proposed. In spite of some minor differences in sugar fermentation patterns among the isolates, their consistently high DNA relatedness suggest that all isolates are members of single species.

Description of *Lactobacillus arizonensis* sp. nov.

Lactobacillus arizonensis (ar.i.zon'en.sis. L. gen. n. *arizonensis* named after the state in the United States where it was isolated).

Cells are rods, $1 \times 4.0\text{--}5.0\ \mu\text{m}$ in size, which occur singly and in short chains. Gram-positive and non-motile. Endospores are not produced. Colonies on agar are white, convex, smooth, circular and opaque. In broth, growth causes turbidity which clears after a few days due to settling of cells. Acid is produced from cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, D-ribose, salicin, D-sorbitol, sucrose and trehalose.

Some strains may ferment L-(–)-arabinose and rhamnose, but D-xylose is not fermented. No gas is produced from D-glucose. L-(+)- and D-(–)-lactic acids are the only organic acids produced. Catalase is not produced. Facultatively anaerobic. Temperature relations are as follows: optimum, 35–40 °C; minimum, 10–15 °C; maximum, 45–50 °C. G+C content is 48 mol%. Isolated from jojoba meal fermentations. The type strain is NRRL B-14768^T (= DSM 13273^T).

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