Two New Species of *Lactobacillus* Isolated from the Bovine Rumen, *Lactobacillus ruminis* sp.nov. and *Lactobacillus vitulinus* sp.nov.

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SUMMARY

Two new anaerobic species of *Lactobacillus* from the bovine rumen are described. They belong to the subgenus *Thermobacterium* and contain meso diamino-pimelic acid in the wall peptidoglycan. The species differ from each other in being motile and non-motile, in the type of lactic acid produced and in % base composition of their DNA. They have been named *Lactobacillus ruminis* and *L. vitulinus*.

INTRODUCTION

During an investigation of the effect of high-carbohydrate diets on the microflora of the bovine rumen (Latham, Sharpe & Sutton, 1971; J. D. Sutton, unpublished), motile, anaerobic strains of Gram-positive rods were occasionally isolated. When present these organisms occurred in high dilutions of rumen contents and constituted part of the majority flora of the rumen at the time of sampling. As motile lactobacilli have been described previously (Harrison & Hansen, 1950; Mann & Oxford, 1954; Thornley & Sharpe, 1959; Langston & Bouma, 1960; Gemmell & Hodgkiss, 1964; Reuter, 1970) the rumen strains were further investigated to determine their relationships to some of these and to other known species of *Lactobacillus*. Also, two non-motile strains of anaerobic Gram-positive rods, isolated as part of the majority flora from the rumen of a 6-month-old steer on a hay and concentrates diet, were examined and compared with two strains of anaerobic lactobacilli previously isolated and described by Bryant, Small, Bouma & Robinson (1958).

METHODS

Bacteria. The six rumen strains were isolated from samples of rumen contents of animals on the diets shown in Table 1. Cows A, B and C were non-lactating, rumen-fistulated animals; animal D was a 6-month-old steer and the rumen sample was obtained by stomach tube. Strains were isolated using the anaerobic techniques of Hungate (1950) on Medium 10 of Caldwell & Bryant (1966), modified as described by Latham & Sharpe (1971). The sources of laboratory and collection strains are described in Table 1.

For all cultural work the eight rumen isolates were grown in a McIntosh and Fildes anaerobic jar under an atmosphere of 90 % H₂ + 10 % CO₂ at 30 °C, and the four other strains were grown in air at 37 °C.

Detection of motility. Motility was tested after 18 h of incubation in the fluid of syneresis of Medium 10 agar slopes, and after 6 h of incubation in sloppy Medium 10 agar (0.25 %, w/v,

Table 1. *Strains of Lactobacillus examined*

Strain and laboratory number			Source	Diet of animal	Obtained from:
<i>Lactobacillus</i> sp.	RF1		Rumen, cow A	Flaked maize	Isolated at N.I.R.D.
	RF2	}	Rumen, cow B	Rolled barley and	
	RF3			hay	
	RF4	Rumen, cow C	Flaked maize		
	CL1	Rumen, 6-month-old steer D	Hay and concen- trates		
	CL13				
B62	RL1	Rumen, 3-week-old calf	Hay, grain, milk	M. P. Bryant, U.S A	
T185	RL2	Rumen, 6-week-old calf	Hay, grain, milk		
153/1F	H1	}	Meat product	.	G. Reuter, Germany
23/a II	H2				
<i>Lactobacillus plantarum</i> var. <i>mobilis</i> 20-23	T1		Caecum of turkey	.	A. P. Harrison, U.S.A.
<i>L. salivarius</i> var. <i>salivarius</i> ATCC 11741			Human saliva	.	M. Rogosa, U.S.A.

agar), stabs and MRS broth (de Man, Rogosa & Sharpe, 1960) modified to contain only 0.05 % (w/v) glucose.

Flagella staining and electron microscopy. Bacteria were stained using the method of Rhodes (1958) and observed with the light microscope. For electron microscopy, droplets of bacterial suspension were placed on formvar-coated grids and shadowed with gold palladium (40/60 w/w) at an angle of 22°. The grids were examined in a Siemens Elstrop 1 electron microscope.

Physiological tests for identification. The methods of Sharpe, Fryer & Smith (1966) and Rogosa (1970) were used, 0.02 % (w/v) cysteine HCl being added to all media.

Growth in selective lactobacillus medium. Some strains were inoculated into the acetate medium of Rogosa, Mitchell & Wiseman (1951) which is selective for lactobacilli, pediococci and some leuconostocs.

Fermentation products. To determine free fatty acids, strains were grown for 48 h in MRS broth, with acetate omitted and 0.02 % (w/v) cysteine HCl added. After centrifuging, a portion (5 ml) of the supernatant was analysed on a silicic acid column, by the method of Harper, Schwartz & El-Hagarawy (1956). To determine lactic acids the strains were grown for 48 h in MRS broth, with meat extract omitted and 0.02 % (w/v) cysteine HCl added, and the supernatants tested. Or washed cell suspensions were allowed to ferment glucose in phosphate buffer at pH 5.6 (Abo Elnaga & Kandler, 1965) and the supernatants tested. The method of Bergmeyer (1962) with L-lactate (EC. 1.1.1.27) and D-lactate (EC. 1.1.1.28) dehydrogenases (Boehringer, Mannheim) was used to determine L(+) and D(-) lactic acids.

Determination of lactate dehydrogenases in strain RF1. The lactate dehydrogenase-linked oxidation of NADH and reduction of NAD, and electrophoresis of the enzymes were studied by the methods of Garvie (1969), except that electrophoresis was carried out in cylindrical gels and sucrose (final concentration 5 to 10 %, w/v) added to the crude enzyme preparation before layering it on to the gel surface.

To convert pyruvate to lactate, one drop of concentrated, crude, bacterial extract was added to a reaction mixture consisting of 1.0 ml tris (0.2 M) + maleate buffer at the required pH values of 6.3, 7.0, 7.5 or 8.3, 1.1 mg sodium pyruvate, 7.0 mg NADH and 1.0 ml water. After incubation at 22 °C for 5 h, preparations were treated with acid-washed charcoal

(Sutcliffe, Speakman no. 5) to remove NAD and residual NADH, and centrifuged. The supernatants were held at 100 °C for 5 min and deep frozen until tested for total and L(+)-lactic acid (Garvie, 1967), D(-)-lactic acid being estimated by difference.

Preparation and analysis of walls. Bacteria were disrupted by shaking with glass beads and the crude wall preparation treated by tryptic digestion as described by Schleifer & Kandler (1967). The walls were hydrolysed in 4 N-HCl for 14 h at 100 °C and the qualitative and quantitative amino acid composition of the total hydrolysate determined using an amino acid analyser (Beckman 120c, Beckman Instruments). The amino acid sequence of the peptidoglycan was checked by two-dimensional paper chromatography of partial hydrolysates of the wall (Schleifer & Kandler, 1967; 1970). The resulting 'finger prints' were compared with those from previously investigated organisms which possessed walls of the same amino acid composition. When the pattern of the oligopeptides was identical it was assumed that the amino acid sequence was also identical.

For the detection of sugar components, walls were hydrolysed in 2 N-HCl for 2 h and the hydrolysate was analysed by two dimensional paper chromatography using the following solvent systems: *n*-butanol + pyridine + water + acetic acid (60:40:30:3); water saturated phenol + 1M-Na₂EDTA + acetic acid (1000:1:10). Chromatograms were sprayed with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950).

Teichoic acid was extracted from the walls by incubating with 10 % (w/v) TCA at 4 °C for 3 days, centrifuging, adding 2 vol. of ethanol to the supernatant and harvesting the precipitated teichoic acid by centrifugation. After hydrolysis with 2N-HCl for 1 h at 100 °C sugar components were determined by chromatography as described above, and phosphate by the method of Fiske & Subbarow (1925).

Deoxyribonucleic acid base composition. The method of Marmur & Doty (1962) was used.

Serological tests. The general methods for agglutination tests were those described by Sharpe, Latham & Reiter (1969). An antiserum was prepared against strain RFI, which was grown for 18 h in MRS broth + 0.02 % (w/v) cysteine HCl and then suspended in 0.2 % (v/v) formaldehyde in 0.15 M-NaCl. Formolized suspensions of organisms for agglutination tests were prepared in the same way, and tests performed using doubling dilutions of antiserum.

RESULTS

The four strains, RFI to RF4, from cows A, B and C were among those picked from roll tubes at a dilution of 10⁻⁸ g rumen contents. With steer D, in which the viable count was only 3.2 × 10⁸/g, colonies were picked from the 10⁻⁷ dilution, where strains morphologically similar to CLI and CLI3 comprised 50 % of the majority flora of the rumen contents. Strains RFI–RF4 were Gram-positive, non-sporing, regular rods. Strains CLI and CLI3 were Gram-positive, club-shaped or irregular rods, morphologically similar to bifidobacteria. As all six strains grew well in boiled MRS broth + 0.02 % (w/v) cysteine HCl incubated in a conventional anaerobic jar, the Hungate techniques were unnecessary for cultivation. Strains RL1 and RL2 also grew under conventional anaerobic conditions in MRS broth + 0.02 % (w/v) cysteine HCl. In MRS broth under aerobic conditions strains CLI, CLI3, RL1, RL2 did not grow at all, and RFI to RF4, which on first isolation did not grow in this medium, grew only scantily after laboratory cultivation. Strains H1, H2, T1 and ATCC 11741 grew aerobically in MRS broth but were cultured in MRS broth + 0.02 % (w/v) cysteine to conform to the medium used for the other strains.

Motility. Strains RFI to RF4 were originally observed to be motile in the fluid of syneresis of Medium 10 agar slopes. These strains exhibited motility more consistently in Medium 10,

Fig. 1. Electron micrograph of *Lactobacillus ruminis* RFI, showing peritrichous flagella.

but strains H1 and T1 were more frequently observed to be motile in the MRS broth with low glucose content. Motility was not always easy to demonstrate and was often only sluggish. However, strains CL1, CL13, RL1, RL2, H2 and ATCC 11741 were consistently non-motile. ATCC 11741 was described as motile by Gemmell & Hodgkiss (1964), and H2 was motile on first isolation, but this characteristic was later lost (Reuter, 1970). This loss may occur on laboratory cultivation, particularly in media containing high carbohydrate levels, customary for lactobacilli, which discourage motility. The motility of the rumen micro-organism *Butyrivibrio fibrisolvens* may be temporarily suppressed if the carbohydrate concentration in the medium exceeds 0.1 % (Bryant & Small, 1956).

Flagella staining and electron microscopy. Stained preparations of RFI, RF2, RF3 and RF4 showed the presence of peritrichous flagella, and this was confirmed by electron microscopy of strain RFI (Fig. 1). Previous workers with motile lactobacilli (Harrison & Hansen, 1950; Gemmell & Hodgkiss, 1964) also demonstrated peritrichous flagella.

Physiological tests. All strains tested were homofermentative and catalase-negative. By our method of testing RL1 did not produce gas from glucose, although Bryant *et al* (1958) observed slight gas production. As several homofermentative strains of lactobacilli have been observed previously to produce small amounts of gas on occasion this was not regarded as significant. All eight rumen strains had similar fermentation patterns (Table 2) except that strains CL1, CL13, and RL2 fermented inulin weakly, RL2 fermented sorbitol and RL1 fermented trehalose. Strains T1 and ATCC 11741 differed from the rumen strains in that they fermented mannitol and grew well aerobically. They were differentiated from each other,

Table 2. *Physiological characteristics of twelve strains of Lactobacillus*

	RF1	RF2	RF3	RF4	CL1	CL13	RL1	RL2	T1	H1	H2	ATCC 11741
Growth at 15 °C	—	—	—	—	—	—	—	—	—	+	+	—
45 °C	+	+	+	.	+	+	+	+	+	—	—	+
Motility	+	+	+	+	—	—	—	—	+	+	—	—
Aerobic growth	—	—	—	—	—	—	Slight	—	+	+	+	+
pH in MRS broth	4.1	3.9	4.3	.	4.5	4.4	4.2	4.3	3.8	4.2	4.0	3.7
Fermentation of:												
Ribose	—	—	—	.	—	—	—	—	+	+	+	—
Rhamnose	—	—	—	.	—	—	—	—	—	—	—	+ w
Sucrose	+	+	+	+	+	+	+	+	+	+	—	+
Trehalose	—	—	—	—	—	—	+	—	+	+	—	+
Cellobiose	+	+	+	.	+	+	+	+	+	+	—	—
Melibiose	+	+	+	+	+	+	+	+	+	—	—	+
Raffinose	+	+	+	+	+	+	+	+	+	—	—	+
Inulin	—	—	—	—	+ w	+ w	—	+ w	—	—	—	—
Lactose	+	—	—	—	+	+	+	+	+	+	+	+
Mannitol	—	—	—	—	—	—	—	—	+	—	—	+
Sorbitol	—	—	—	—	—	—	—	+	+	—	—	+
Salicin	+	+	+	+	+	+	+	+	+	+	+	—
Aesculin	+	+	+	+	+	+	+	+	+	+	+	—
Lactic acid:												
% D(—)	5	3	5	5	100	100	100	100	8	45	50	1
% L(+)	95	97	95	95	0	0	0	0	92	55	50	99

All strains fermented fructose, glucose, galactose, mannose, maltose, amygdalin. None fermented xylose, arabinose, melezitose, glycogen, glycerol, inositol. None produced gas from glucose or from gluconate. None hydrolysed arginine.

+, Strong fermentation; + w, weak fermentation; —, no fermentation; ., no test.

as T1 fermented ribose and cellobiose and hydrolysed aesculin and salicin whilst ATCC 11741 did not. Strains H1 and H2 differed from the others in growing at 15 °C, in failing to ferment melibiose and raffinose, and from all but T1 in fermenting ribose.

Growth on acetate media. Strains RF1, RF2, RF3, CL1, CL13 and T1 grew well under anaerobic conditions in the selective, acetate broth medium of Rogosa, Mitchell & Wiseman (1951).

Fermentation products. No acetic, propionic, butyric or higher volatile fatty acids were detected in the broth supernatants of the strains tested (RF1, RF2, RF3, CL1 and CL13). Lactic acid was produced by all twelve strains. The lactic acid produced by RF1 to RF4 was almost entirely L(+), with only about 5 % D(—), whilst CL1, CL13, RL1 and RL2 produced only D(—)-lactic acid (Table 2). Strains T1 and ATCC 11741 also produced almost entirely L(+)-lactic acid, whilst H1 and H2 differed from all the others by producing equal amounts of D(—) and L(+).

Lactate dehydrogenases of strain RF1. Oxidation of NADH and reduction of NAD, were both followed at 340 nm and the latter also using nitroblue tetrazolium and phenazine methosulphate as reduction links. The results showed that RF1 had two NAD-linked lactate dehydrogenases (EC. 1.1.1.27, 28). No NAD non-linked ones (EC. 1.1.1.99, —) were detected. The enzyme forming L(+)-lactic acid had an optimum pH of about 5.5 and the reaction was almost irreversible, whilst the enzyme forming D(—)-lactic acid had a reaction reversible above pH 7.0. On electrophoresis the D(—)-enzyme, which migrated faster than the L(+)-enzyme, moved to a position close to the enzyme of *Lactobacillus lactis* NCDO 280. The L(+)-enzyme was close to the L(+)-enzyme of *L. plantarum* NCDO 82. Fructose-1,6-diphosphate did not influence the activity of either the D(—)- or L(+)-enzyme.

Table 3. *Wall composition of strains of lactobacilli*

	Amino acids, NH ₃ ; molar ratios						PO ₄ content %	Sugar components					
	Asp	Mur	Glu	Ala	DAP	Gluc NH ₃		Lys	NH ₃	Glucose	Glucos- amine	Rham- nose	Galac- tose
RF1	—	0.88	1.00	1.51	1.04	0.70	—	1.98	+	—	(+)	+	—
RF2	—	0.92	1.00	1.46	1.05	0.85	—	1.54	+	—	(+)	+	—
RF3	—	0.80	1.00	1.54	1.04	0.70	—	1.65	+	—	+	+	+
RF4	—	0.73	1.00	1.46	0.97	0.74	—	1.46	+	—	(+)	+	—
T1	—	0.97	1.00	1.51	1.03	0.68	—	1.86	+	—	—	+	—
CL13	—	0.80	1.00	1.52	1.08	0.64	—	0.59	.	.	.	.	.
RL1	—	0.27	1.00	1.68	1.05	0.56	—	0.41	+	—	+	—	+
RL2	—	0.42	1.00	1.62	0.94	1.06	—	0.60	+	—	—	—	—
H1	0.37	0.77	1.00	1.73	—	1.43	0.34	1.91	+	+	+	—	—
H2	0.35	0.72	1.00	1.67	—	1.94	0.33	1.56	+	+	+	—	—
ATCC 11741	0.29	0.82	1.00	1.64	—	1.04	0.37	1.97	+	—	—	+	—

., Not tested; (+), only trace amounts present; —, not detected.
 Serine, glutine and ornithine were not detected in the walls of any strain.

Table 4. *Differentiating characteristics of Lactobacillus ruminis, L. vitulinus, some previously isolated motile lactobacilli and L. jensenii, L. salivarius, L. plantarum and L. lactis*

Species	<i>L. ruminis</i>	<i>L. plantarum</i> <i>var. mobilis</i>	<i>L. vitulinus</i>	Strepto- bacteria	<i>L. jensenii</i> ⁱⁱ	<i>L. salivarius</i>	<i>L. plantarum</i> [§]	<i>L. lactis</i> [§]
Laboratory number	RF1, RF2, RF3, RF4	T1	CL1, CL13 RL1, RL2	HI1, H2				
Characteristics								
Type of lactic acid produced	L(+) 95 % D(-) 5 %	L(+) 92 % D(-) 8 %	D(-)	DL	D(-)	L(+) 1	DL	D(-)
Amino acids in peptidoglycan:								
Meso-DAP	+	+	+	-	-	-	+	-
D-Asp	-	-	-	-	+	+	-	+
L-Lys	-	-	-	-	+	+	-	+
Wall teichoic acid	Glycerol*	.	Glycerol†	ND	ND	ND	Usually ribitol [¶] , glycerol [¶]	
Fermentation of:								
Ribose	-	+	-	+	-	-	+	-
Mannitol	-	+	-	-	-	+	+	-
Sorbitol	-	+	+	-	-	+	+	-
Trehalose	-	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	-
Cellobiose	+	+	+	+	+	-	+	-
Gas from gluconate	-	-	-	-	-	-	+	-
Growth at 15 °C	-	-	-	+	-	-	+	-
DNA composition	43.7* 43.8	42.4	34.9, 34.4, 37.1	ND	36.1 ⁱⁱ	34.7 [‡]	45.0 [‡]	50.3 [‡]

* Only two strains tested

† Only one strain tested

¶ Gasser, Mandel & Rogosa (1970).

ii Sharpe (1970)

ND, No data.

The growing culture of RF1 formed very little D(–)-lactic acid in the medium but, when reacted with pyruvate, bacterial extracts formed a higher proportion of D(–)-lactic acid. Using bacterial extracts at pH 8.3, 40 % of the lactic acid formed was the D(–)-isomer, but this dropped to 10 to 18 % at pH 7.0 or lower.

Wall composition. The amino acid composition of the wall peptidoglycans divided the ten strains examined into three groups (Table 3). The first group included strains RF1 to RF4 and T1. These all contained a *meso*-diaminopimelic acid (DAP)-type peptidoglycan similar to that of *Lactobacillus plantarum* (Weiss, Plapp & Kandler, 1967), with no D-aspartic acid or L-lysine. The molar ratios of the amino acids, as well as the pattern of peptides in two dimensional chromatograms of partial acid hydrolysates, were almost identical in these strains. Between 1.5 and 2.0 mol of ammonia were found, indicating that both carboxyl groups α -carboxyl of glutamic acid and ω -carboxyl of DAP were amidated. The molar ratio of alanine was 1.5, which is also the same as that of *L. plantarum* (Weiss *et al.* 1967). The second group included the three non-motile strains CLI3, RLI and RL2 (CLI was not examined); these strains also contained *meso*-DAP type peptidoglycans and no D-aspartic acid or L-lysine. However only about 0.5 mol of ammonia was present, suggesting that the carboxyl groups of glutamic acid and DAP were not amidated. The third group included strains ATCC 11741, H1 and H2 which contained L-lysine and D-aspartic acid but no *meso*-DAP. As shown by the molar ratios of amino acids and the pattern of peptides in the acid partial hydrolysate, the peptidoglycan present in these strains was of the L-Lys-D-Asp-type found in most other lactobacilli (Kandler, 1970).

The composition of the wall carbohydrates was more variable (Table 3). All strains in the first group except T1 contained glucose, galactose and rhamnose. The two strains RLI and RL2 in the second group differed from each other in that rhamnose, galactose and galactosamine were all absent from RL2. Strains CLI and CLI3 were not examined. Strains H1 and H2 were identical to each other and differed from all the other strains in containing glucosamine. They also differed from ATCC 11741.

The low phosphate content of some preparations (1 % or less) indicated that these walls contained little teichoic acid, although other strains contained significantly more phosphate in the walls. Strains RF1, RF2 and RLI, which showed the highest phosphate content, were examined in some detail. Glycerol, but no ribitol, was found in the walls of all three strains.

Deoxyribonucleic acid base composition. Strains RF1, RF2, T1, CLI3, RLI and RL2 were examined. The % GC ratios were 43.8, 43.7, 42.4, 37.1, 34.9 and 34.4 respectively (Table 4). The two RF strains and T1, therefore, had % GC ratios within the range of *Lactobacillus plantarum*, whilst that of CLI3, RLI and RL2 were within the range for *L. salivarius* (Gasser & Mandel, 1968).

Agglutination tests. When suspensions of the 10 strains RF1 to 4, T1, RLI, RL2, H1, H2 and ATCC 11741 were tested against antiserum RF1 diluted 1/20, agglutination occurred only with strains RF1, RF2, RF3 and T1. The agglutination titres of these strains were 640, 320, 320 and 20 respectively. There was no agglutination reaction with strain RF4. Whilst RF1, RF2 and RF3 appeared to have a common agglutinating antigen, this was not present in strain RF4. The reaction with T1 occurred at too low a titre to be significant.

DISCUSSION

The six fresh rumen isolates have the morphological characteristics of lactobacilli. They produce lactic acid but not acetic acid or CO₂ from glucose. They grow well at 45 °C but not at 15 °C. They fail to ferment ribose or to produce gas from gluconate. Thus they fall

into the subgenus *Thermobacterium* (Rogosa, 1970). Anaerobic lactobacilli, containing meso-DAP and belonging to the subgenus *Thermobacterium*, have not previously been described. However, these organisms conform to the characteristics of the genus *Lactobacillus* and there seems no reason at present to assign them elsewhere.

The four motile strains differ from other motile lactobacilli previously described in requiring anaerobic conditions. In addition motile strains from silage (Langston & Bouma, 1960; Gemmell & Hodgkiss, 1964), from chicken meat (Thornley & Sharpe, 1959), and from meat products (strains H1 and H2 examined here) were all low temperature strains with physiological characteristics different from those of the rumen isolates. The spore-forming lactobacillus *Sporolactobacillus inulinus* (Kitahara & Suzuki, 1963) is motile and also contains the meso-DAP type peptidoglycan (Kandler, 1970). However, this organism grows well aerobically, forms D(–)-lactic acid, ferments mannitol and sorbitol but not cellobiose or salicin, and also forms spores.

The most closely related motile lactobacilli are *Lactobacillus plantarum* var. *mobile* (designated T1 in our work) and strain 678 of Mann & Oxford (1954), isolated from the rumen of a 33-day-old, aureomycin-fed calf; the latter strain had the same fermentation reactions and produced the same type of lactic acid as T1, and was considered by Mann & Oxford to be very similar to this organism. Our strains are similar to T1 in having a meso-DAP type peptidoglycan, in forming the same proportion of L(+)– and D(–)-lactic acids and in having a similar GC ratio (Table 4). However, our organisms require anaerobic conditions, and do not ferment ribose, sorbitol, mannitol or trehalose. Ribose and mannitol are regarded as useful criteria for subgeneric and species differentiation. Indeed, Rogosa (1970) suggested that one of the characteristics dividing lactobacilli into subgroups thermobacteria, streptobacteria and betabacteria is the inability of the thermobacteria to ferment ribose. The most closely related non-motile lactobacillus is *L. plantarum* which also has the meso-DAP type peptidoglycan and a similar GC ratio (Table 4). However, *L. plantarum* produces DL-lactic acid and is a member of the streptobacteria. We therefore consider our strains to be a new species of *Thermobacterium*, to be named *Lactobacillus ruminis* sp. nov.

The four non-motile lactobacilli CL1, CL13, RL1 and RL2 appear to belong to yet another group in the subgenus *Thermobacterium*, also having the meso-DAP type wall peptidoglycan and not the L-Lys-D-Asp-type, but differing from *Lactobacillus ruminis* in lacking motility, in having no amide groups in the wall peptidoglycan (Table 3), forming D(–)-lactic acid and having a different GC ratio (35.7%). They appear to resemble the newly described species *L. jensenii* (Gasser, Mandel & Rogosa, 1970) which also forms D(–)-lactic acid and has a GC ratio of 36.1% and the same fermentation pattern (Table 4). However, *L. jensenii* has the D-Asp-L-Lys-type wall peptidoglycan, grows aerobically and hydrolyses arginine.

Strains RL1 and RL2, strains B62 and T185 of Bryant *et al.* 1958, were regarded by these workers as belonging to two different groups on the basis of morphology and production of traces of gas, ability to grow at 22 °C and fermentation of trehalose but not inulin by the RL1 group. As RL1 was identical in fermentation reactions, type of lactic acid produced and growth temperatures, to an anaerobic lactobacillus from the rumen of a calf, previously isolated and tentatively identified by Mann & Oxford (1954) as an atypical strain of *Lactobacillus lactis*, RL1 was also considered by Bryant *et al.* (1958) to be *L. lactis*. However our more detailed examination shows that RL1 differs from *L. lactis* in having a meso-DAP type of wall peptidoglycan, in % GC and in fermenting amygdalin and cellobiose (Table 4).

Although RL1 and RL2 differ from each other as referred to above, and in the wall carbohydrate composition (Table 3), they are similar enough to assign them at the present time

to the same species, although they are recognized as different varieties. The two fresh isolates CLI and CLI3 resemble RL2 in their club-shaped morphology, weak fermentation of inulin, and lack of fermentation of trehalose (Table 2). These four organisms differ sufficiently from other species of thermobacteria to be designated as a new species, and are assigned the name *Lactobacillus vitulinus* sp. nov. Strain RL2 (T185) was one of seven similar strains examined by Bryant *et al.* (1958) and was the only one to ferment sorbitol. However, as it is the earliest strain extant on which further work has now been done, this should be the type strain.

Strains RLI and RL2 and Mann & Oxford's strain 123, which probably was also a strain of *Lactobacillus vitulinus*, were isolated from young calves. However, CLI and CLI3 came from a 6-month-old steer, and Bryant (personal communication) has isolated from adult cows strains similar to RL2 except that they did not ferment lactose. Such lactobacilli are therefore likely to be present in adult animals and may be isolated when the animal is on a suitable diet.

These DAP-containing lactobacilli, if present in large numbers in the rumen, may affect estimations of microbial nitrogen. The amount of DAP present in the digesta of sheep has been used to determine bacterial N in the presence of other N passing from the rumen, through the intestine, to the faeces (Mason, 1969; Smith, 1969; Mason & White, 1970). On high-roughage, or mixed, rations the largely Gram-negative bacterial species in the rumen will generally contain DAP in the wall (Work, 1970), and this measurement may be relevant. However, on a high-carbohydrate ration the DAP content may not reflect the bacterial population: streptococci, lactobacilli and bifidobacteria may constitute a high proportion of the bacterial flora (Latham *et al.* 1971) and none of these bacteria except *Lactobacillus plantarum* contain DAP (Synge, 1953; Gooder, 1970; Kandler, 1970). Large numbers of the DAP-containing lactobacilli in the rumen of a cow on a high-carbohydrate ration would result in a higher estimation of microbial nitrogen than that found when other lactic acid bacteria were present, unless estimations were made of the DAP content of the rumen flora in the experimental animal at the time of estimation of intestinal microbial nitrogen.

Motile lactobacilli may be common inhabitants of the digestive tract, as Sharpe *et al.* (1969) demonstrated the presence of agglutinating antibodies to RFI not only in the serum of the cow from which it was isolated but also in that of other ruminants and a horse.

The lactate dehydrogenases of Lactobacillaceae are useful species characteristics for differentiation (Garvie, 1969; Gasser, 1970). The L(+)-lactate dehydrogenase in strain RFI appears to be similar to the enzyme in *Lactobacillus jugurt* (Gasser, Doudoroff & Contopoulos, 1970), in that it has a low optimum pH and is only weakly reversible. The D(-)-enzyme has similar characteristics to the D(-)-enzymes of most other lactobacilli and it is not clear why the L(+)-enzyme is dominant in the growing culture and in crude enzyme preparations. As *L. casei* also forms predominantly L(+)-lactic acid it seemed possible that the lactate dehydrogenases of RFI might be similar. However, this was not so, as the L(+)-lactate dehydrogenase of *L. casei* requires fructose-1,6-diphosphate and the D(-)-enzyme is active with pyruvate, but not lactate, as substrate (E. I. Garvie, unpublished).

Lactobacillus ruminis sp. nov.*Description*

Homofermentative, producing mostly L(+) and about 5% D(-)-lactic acid.

DNA base composition: 43.7 ± 0.1 mol % G + C.

Growth at 45 °C but not at 15 °C.

Motile, with peritrichous flagella.

Amino acid type in wall peptidoglycan: *meso*-DAP.

Fructose, glucose, mannose, galactose, sucrose, maltose, cellobiose, melibiose, raffinose, salicin, amygdalin and aesculin fermented; xylose, arabinose, ribose, rhamnose, melezitose, inulin, trehalose, mannitol, sorbitol, glycerol, inositol not fermented; lactose fermented by some strains.

Arginine not hydrolysed.

The type strain RFI has been deposited in the National Collection of Dairy Organisms.

Lactobacillus vitulinus sp. nov.*Description*

Homofermentative, producing D(-)-lactic acid.

DNA base composition: 35.7 ± 1.3 mol % G + C.

Growth at 45 °C but not at 15 °C.

Non-motile.

Amino acid type in wall peptidoglycan: *meso*-DAP.

Fructose, glucose, mannose, galactose, sucrose, maltose, cellobiose, melibiose, lactose, raffinose, salicin, amygdalin and aesculin fermented; xylose, arabinose, ribose, rhamnose, melezitose, mannitol, glycerol, inositol not fermented; inulin, trehalose, sorbitol may be fermented.

Arginine not hydrolysed.

The type strain T185 has been deposited in the National Collection of Dairy Organisms.

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REFERENCES

- ABO ELNAGA, J. & KANDLER, O. (1965). Zur Taxonomie der Gattung *Lactobacillus* Beijerinck. I. Das Subgenus *Streptobacterium* Orla Jensen. *Zentralblatt für Bakteriologie, Parasitenkunde Infektionskrankheiten und Hygiene (Abteilung II)* **119**, 1–36.
- BERGMAYER, M. U. (1962). Methoden der enzymatischen Analyse. *Weinheim Verlag Chemie*.
- BRYANT, M. P. & SMALL, S. N. (1956). The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. *Journal of Bacteriology* **72**, 16–26.
- BRYANT, M. P., SMALL, S. N., BOUMA, C. & ROBINSON, I. (1958). Studies on the composition of the ruminal flora and fauna of young calves. *Journal of Dairy Science* **41**, 1747–1767.
- CALDWELL, D. R. & BRYANT, M. P. (1966). Medium without rumen fluid for non-selective enumeration and isolation of rumen bacteria. *Applied Microbiology* **14**, 794–80.
- FISKE, C. M. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorus. *Journal of Biological Chemistry* **66**, 375–377.
- GARVIE, E. I. (1967). The production of L(+) and D(-) lactic acid in culture of some lactic acid bacteria with a special study of *Lactobacillus acidophilus* NCDO 2. *Journal of Dairy Research* **34**, 31–38.
- GARVIE, E. I. (1969). Lactic dehydrogenases of strains of the genus *Leuconostoc*. *Journal of General Microbiology* **58**, 85–94.

- GASSER, F. (1970). Electrophoretic characterization of lactic dehydrogenases in the genus *Lactobacillus*. *Journal of General Microbiology* **62**, 223-239.
- GASSER, F., DOUDOROFF, M. & CONTOPOULOS, R. (1970). Purification and properties of NAD-dependent lactic dehydrogenases of different species of *Lactobacillus*. *Journal of General Microbiology* **62**, 241-250.
- GASSER, F. & MANDEL, M. (1968). Deoxyribonucleic acid base composition of the genus *Lactobacillus*. *Journal of Bacteriology* **96**, 580-588.
- GASSER, F., MANDEL, M. & ROGOSA, M. (1970). *Lactobacillus jensenii* sp. nov., a new representative of the subgenus *Thermobacterium*. *Journal of General Microbiology* **62**, 219-222.
- GEMMELL, M. & HODGKISS, W. (1964). The physiological characters and flagellar arrangement of motile homofermentative lactobacilli. *Journal of General Microbiology* **35**, 519-526.
- GOODER, H. (1970). Cell wall composition in the classification of streptococci. *International Journal of Systematic Bacteriology* **20**, 475-482.
- HARPER, W. J., SCHWARTZ, D. P. & EL-HAGARAWY, I. S. (1956). A rapid silica gel method for measuring total free fatty acids in milk. *Journal of Dairy Science* **39**, 46-50.
- HARRISON, A. P., JUN. & HANSEN, A. P. (1950). A motile lactobacillus from the cecal feces of turkeys. *Journal of Bacteriology* **59**, 444-446.
- HUNGATE, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bacteriological Reviews* **14**, 1-49.
- KANDLER, O. (1970). Amino acid sequence of the murein and taxonomy of the genera *Lactobacillus*, *Bifidobacterium*, *Leuconostoc* and *Pediococcus*. *International Journal of Systematic Bacteriology* **20**, 491-508.
- KITAHARA, K. & SUZUKI, J. (1963). *Sporolactobacillus* nov. subgen. *Journal of General and Applied Microbiology* **9**, 59-71.
- LANGSTON, C. W. & BOUMA, C. (1960). A study of the microorganisms from grass silage. II. The lactobacilli. *Applied Microbiology* **8**, 223-234.
- LATHAM, M. J. & SHARPE, M. E. (1971). The isolation of anaerobic organisms from the bovine rumen. In *Isolation of Anaerobes*, p. 133. Edited by D. A. Shapton & R. G. Board. London: Academic Press.
- LATHAM, M. J., SHARPE, M. E. & SUTTON, J. D. (1971). The microbial flora of the rumen of cows fed hay and high cereal rations and its relationship to rumen fermentation. *Journal of Applied Bacteriology* **34**, 425-435.
- DE MAN, J. C., ROGOSA, M. & SHARPE, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology* **23**, 130-135.
- MANN, S. O. & OXFORD, A. E. (1954). Studies of some presumptive lactobacilli isolated from the rumens of young calves. *Journal of General Microbiology* **11**, 83-90.
- MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *Journal of Molecular Biology* **5**, 109-118.
- MASON, V. C. (1969). Some observations on the distribution and origin of nitrogen in sheep faeces. *Journal of Agricultural Science* **73**, 99-112.
- MASON, V. C. & WHITE, F. (1970). Some observations on the metabolism of -E diaminopimelic acid in sheep. *Proceedings of the Nutrition Society* **30**, 13A.
- REUTER, G. (1970). Laktobazillen und eng verwandte Mikroorganismen in Fleisch und Fleischerzeugnissen. *Fleischwirtschaft* **50**, 954-962.
- RHODES, M. (1958). The cytology of *Pseudomonas* spp. as revealed by a silver plated staining method. *Journal of General Microbiology* **18**, 639-648.
- ROGOSA, M. (1970). Characters used in the classification of lactobacilli. *International Journal of Systematic Bacteriology* **20**, 519-534.
- ROGOSA, M., MITCHELL, J. A. & WISEMAN, R. F. (1951). A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *Journal of Bacteriology* **62**, 132.
- SCHLEIFER, K. H. & KANDLER, O. (1967). Die Aminosäuresequenz des Mureins von *Streptococcus thermophilus* und *Str. faecalis*. *Archiv für Microbiologie* **57**, 335-364.
- SCHLEIFER, K. H. & KANDLER, O. (1970). Amino acid sequence of the murein of *Planococcus* and other Micrococcaceae. *Journal of Bacteriology* **103**, 387-392.
- SHARPE, M. E. (1970). Cell wall and cell membrane antigens used in the classification of lactobacilli. *International Journal of Systematic Bacteriology* **20**, 509-518.
- SHARPE, M. E., FRYER, T. F. & SMITH, D. G. (1966). In *Identification Methods for Microbiologists*. Edited by B. M. Gibbs and F. A. Skinner. London and New York: Academic Press.
- SHARPE, M. E., LATHAM, M. J. & REITER, B. (1969). The occurrence of natural antibodies to rumen bacteria. *Journal of General Microbiology* **56**, 353-364.
- SMITH, R. H. (1969). Reviews of the progress of Dairy Science Section G. General. Nitrogen metabolism and the rumen. *Journal of Dairy Research* **36**, 313-331.

- SYNGE, R. L. M. (1953). Note on the occurrence of diaminopimelic acid in some intestinal micro-organisms from farm animals. *Journal of General Microbiology* **9**, 407-409.
- THORNLEY, M. J. & SHARPE, M. E. (1959). Microorganisms from chicken meat related to both lactobacilli and aerobic sporeformers. *Journal of Applied Bacteriology* **22**, 368-376.
- TREVELYAN, W. E., PROCTER, D. D. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, London* **166**, 444-448.
- WEISS, N., PLAPP, R. & KANDLER, O. (1967). Die Amino-Säuresequenz des DAP-haltigen Mureins von *Lactobacillus plantarum* und *Lactobacillus inulinus*. *Archiv für Mikrobiologie* **58**, 313-323.
- WORK, E. (1970). The distribution of diamino acids in cell walls and its significance in bacterial taxonomy. *International Journal of Systematic Bacteriology* **20**, 425-434.