Electrophoresis of Glucose-6-phosphate Dehydrogenase, Cell Wall Composition and the Taxonomy of Heterofermentative Lactobacilli

By R. A. D. WILLIAMS AND SHIRLEY A. SADLER

*Department of Biochemistry and Department of Dental Bacteriology and Biochemistry,
London Hospital Medical College, Turner Street, London E. 1*

(Accepted for publication 18 January 1971)

SUMMARY

Thirty-one strains of heterofermentative lactobacilli have been examined by routine physiological tests, starch-gel electrophoresis of glucose-6-phosphate dehydrogenase and by qualitative analysis of cell wall amino acids. Two major subdivisions are apparent, corresponding to *Lactobacillus fermenti* with *L. cellobiosus* on one hand, and *L. brevis* with *L. buchneri* and *L. viridescens* on the other.

INTRODUCTION

The heterofermentative lactobacilli (Orla-Jensen's subgenus *Betabacterium*) lack the glycolytic enzyme aldolase (Buyze, Van den Hamer & de Haan, 1957). They ferment glucose, producing a mixture which consists largely of lactic and acetic acids with carbon dioxide. The key enzyme in this pathway is phosphoketolase (Heath, Hurwitz, Horecker & Ginsburg, 1958). The production of carbon dioxide is a readily observable feature of heterofermenters, which correlates well with the ability to deaminate arginine under defined conditions (Sharpe, Fryer & Smith, 1966).

The identification of lactic acid bacteria has been described by Sharpe *et al.* (1966) and the American Type Culture Collection have published the properties of type strains of lactobacilli (Hansen, 1968). Heterofermentative strains are usually divided into five species. *Lactobacillus fermenti* has a distinct antigen, is reported to grow at 45° but not at 15°, and is usually unable to ferment pentoses, or to hydrolyse aesculin or hippurate. Those strains capable of growing at 15° have been allotted to four species. *Lactobacillus brevis* and *L. buchneri* share the same antigen but are differentiated by the inability of the former to ferment melezitose and raffinose. No specific antigens have been described for *L. cellobiosus* or *L. viridescens*, but the former is able to ferment cellobiose (Rogosa, Wiseman, Mitchell, Disraeli & Beaman, 1953) while the latter has very little fermentative capacity (Rogosa & Sharpe, 1959). Many studies have involved a simpler subdivision of this group, for example Davis (1955) allotted all heterofermentative strains from the human mouth to the two species *L. fermenti* and *L. brevis*. Seyfried's (1968) numerical analysis indicated that all strains described as *L. fermenti*, *L. brevis* and *L. viridescens* formed one group. This confirms the overall similarity of these heterofermentative cultures. However, the similarity within this group was lower than in the other groups of lactic bacteria examined by Seyfried. No strains described as *L. cellobiosus* or *L. buchneri* were included in this study.

Buoyant density analysis of DNA (Gasser & Mandel, 1968) indicates two major

subdivisions amongst heterofermenters. *Lactobacillus fermenti* (49.5 to 50.1 % G+C) together with *L. cellobiosus* (49.8 to 52.5 % G+C) are clearly distinguished from *L. viridescens* (37.9 to 41.0 % G+C), *L. brevis* (43.2 to 43.9 % G+C) and *L. buchneri* (42.0 to 42.6 % G+C).

Cell wall analysis of *Lactobacillus fermenti* and *L. brevis* (Cummins & Harris, 1956) revealed similar amino acid components (aspartate, glutamate, alanine and lysine) in both strains. Differentiation of the two was possible on carbohydrate components, as *L. brevis* contained glucose alone but *L. fermenti* contained glucose and galactose. Ikawa & Snell (1960) confirmed the amino acid composition reported by Cummins & Harris (1956) for *L. fermenti*, but did not analyse any other heterofermentative strains. Plapp & Kandler (1967) found that ornithine replaced lysine in the murein of *L. cellobiosus*. *Lactobacillus viridescens* contained lysine as the basic amino acid, but serine instead of aspartate was a major component in this species (Plapp, Schleifer & Kandler, 1967). Cell wall analyses such as these have often been carried out on single strains of the species concerned and their taxonomic significance is therefore open to doubt. Other chemical techniques useful in taxonomy have been applied to the heterofermentative lactobacilli without success. These include electrophoresis of esterases (Morichi, Sharpe & Reiter, 1968) paper chromatography of intracellular amino acids (Cheeseman, 1959) and infrared spectrophotometry of whole cells (Goulden & Sharpe, 1958).

Electrophoresis of dehydrogenase enzymes was found to correlate well with physiological tests (Williams & Bowden, 1968) and with murein structure (Kandler, Schleifer & Dandl, 1968) in the group D streptococci. Electrophoretic patterns of glucose-6-phosphate dehydrogenase and qualitative amino acid content of cell walls have therefore been examined in 31 strains of heterofermentative lactobacilli to determine their value in the taxonomy of the group.

METHODS

Strains used. The strains and their sources are listed in Table 1. All were cultured and checked for purity on receipt and multiple samples freeze-dried for subsequent use.

Growth conditions. All cultures were grown at 37° under 5 % CO₂ and 95 % H₂, except for gas production from maltose.

Media. The MRS medium of de Man, Rogosa & Sharpe (1960), and modifications of it, were used as follows: bulk cultures, growth at 15° and 45° and inocula for fermentation tests, MRS with 0.5 % glucose; fermentation tests, MRS at pH 6.7 with 2 % substrate (sterilized by filtration) in place of glucose; deamination of arginine, MRS at pH 7.4 with 2 % glucose and 0.3 % arginine hydrochloride but without ammonium citrate; hydrolysis of hippurate, MRS at pH 6.8 with 0.5 % glucose and 1 % sodium hippurate. Gas production was tested by the method of Hayward (1957) using 2.5 % maltose as substrate. For cellular and colonial morphology, broth and plates were prepared as described by Rogosa, Mitchell & Wiseman (1951) and the method of Davis (1956) and the staining technique of Davis & Baird-Parker (1959) were used for the examinations.

Electrophoresis and enzyme staining. Cells were harvested, washed and disintegrated, and cell-free extracts prepared and electrophoresis and staining carried out as described previously (Williams & Bowden, 1968). Phenol red was employed as a standard

and the electrophoresis was stopped when the front of the dye spot had migrated 6 cm. from the origin.

Preparation and examination of cell walls. The methods used were those described by Bowden (1969). In addition, lysine and ornithine in hydrolysates were differentiated by high voltage electrophoresis (1000 V for 2 h.) in pH 5.2 pyridine acetic acid buffer (Atfield & Morris, 1961).

Table 1. *Species and strains used, and their source*

Species	Strain no.	Source*
<i>Lactobacillus fermenti</i>	8830, 6991, 2797, 8828, 8829	NCIB
	14931	ATCC
	F1, F15, F25	MES
<i>L. brevis</i>	8038, 4617, 4036, 8561	NCIB
	14869	ATCC
	X1, X3, X7, X9	MES
<i>L. buchneri</i>	8838, 8007, 8839, 8518, 8837	NCIB
	BC5	MES
	4037	NCIB
<i>L. cellobiosus</i>	11739	ATCC
	G3, G4	MES
	8965	NCIB
<i>L. viridescens</i>	E1, E7	MES

* NCIB = National Collection of Industrial Bacteria; ATCC = American Type Culture Collection; MES = Dr M. E. Sharpe. ATCC 14869 is the type strain of *Lactobacillus brevis*; ATCC 14931 is a neotype strain of *L. fermenti*; ATCC 11739 is the type strain of *L. cellobiosus*.

RESULTS

Physiological tests (Table 2). All strains were named according to their designation on receipt. All strains produced gas from glucose and ammonia from arginine. In our hands growth at 15° and 45° did not allow *Lactobacillus fermenti* to be distinguished from the other heterofermenters. The most clearly identifiable group is *L. viridescens* in spite of the fact that all three strains used attacked arabinose. Individual strains showed some variations from the results regarded as typical of the five species (Sharpe *et al.* 1966). Apparently anomalous results include fermentation of pentoses by *L. viridescens* and by some *L. fermenti* strains, of arabinose and melezitose amongst strains of *L. cellobiosus*, of trehalose by some of the *L. brevis* and the *L. buchneri* groups, and an almost universal capacity to attack raffinose amongst *L. brevis* strains.

Colonial and cellular morphology. All the strains examined produced smooth, white or cream, convex colonies with entire margins. Well isolated colonies varied from 2 to 3 mm. to less than 1 mm. in diameter. The cells were generally short to medium square-ended rods. Size and shape variations did not appear related to species of the strains.

Electrophoresis of glucose-6-phosphate dehydrogenase (G6PD) (Fig. 1). Two major enzyme patterns are evident in the 31 strains. *Lactobacillus fermenti* and all but one strain of *L. cellobiosus* have a simple enzyme pattern consisting of a single strongly staining band. Prolonged staining or a very concentrated extract revealed a second band just behind the major band. This second band is not shown in Fig. 1. Cultures designated *L. brevis*, *L. buchneri* and *L. viridescens* usually had a more complex enzyme pattern, including one band migrating more rapidly and several bands migrating more slowly than that of *L. fermenti*. In most strains of *L. buchneri* and *L. brevis*

Table 2. Results of physiological tests

Fermentation of carbohydrates	Xylose	+	+						+	+						+	+		+	+	+	+	+	+
	Trehalose					+	+		+		+	+	+		+	+			+	+	+			
	Raffinose	+	+	+	+	+				+	+	+	+	+	+	+	+	+	+	+	+		+	
	Melibiose	+	+	+	+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Melezitose											+	+	+	+	+								
	Cellobiose								+	+	+	+												
	Arabinose				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Growth at 45°	+	+	+	+	+	+				+	+		+			+		+		+		+	
	Growth at 15°	+	+	+	+	+	+	+	+		+		+	+	+	+	+	+	+	+	+	+	+	+
	Arginine deamination	+	+	+	+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Aesculin hydrolysis	+					+			+	+	+	+	+	+	+	+			+	+	+	+	+
	Hippurate hydrolysis								+				+	+	+					+	+	+	+	+
	Gas production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Species designation	Strain no.
<i>Lactobacillus fermenti</i>	8830
	6991, 2797
	14931, F1, F15, F25
	8828
	8829
<i>L. viridescens</i>	E1
	E7
	8965
<i>L. cellobiosus</i>	4037
	G3
	11739
	G4
<i>L. buchneri</i>	8838
	8839, 8518
	BC5
	8837
	8007
<i>L. brevis</i>	8561, X9
	X7
	4617
	8038
	X3, 4036
	X1
	14869

the major bands were those migrating slowly, and in about half of these strains the fastest bands were not detected. *Lactobacillus viridescens* 8965 was unusual in that only the very fastest band was detectable. Two strains showed anomalous electrophoretic patterns: strain NCIB 4037 (designated *L. cellobiosus* on receipt) had the G6PD pattern of the *L. buchneri*-*L. brevis* type, while *L. buchneri* 8837 had the single band typical of *L. fermenti* strains.

Fig. 1. Electrophoretic mobility of glucose-6-phosphate dehydrogenase in 31 strains of heterofermentative lactobacilli.

Cell wall amino acids (Table 3). The most important finding was that the dibasic amino acid of the cell wall of all *Lactobacillus fermenti* strains is ornithine, not lysine. In this respect, as in G6PD pattern, *L. fermenti* is like *L. cellobiosus*. Lysine is found in the cell walls of the other three species. A secondary feature of the *L. fermenti* and *L. cellobiosus* group is the absence of serine and usually of glycine from the cell wall.

The exceptions to these results are the two strains which have atypical enzyme patterns. Thus *L. cellobiosus* 4037 contains lysine and *L. buchneri* 8837 contains ornithine as the basic amino acid.

Table 3. *Results of cell wall analysis for amino acids*

Species designation	Strain no.	Amino acid						
		Lys	Orn	Asp	Glu	Gly	Ala	Ser
<i>Lactobacillus fermenti</i>	8830, 6991, 2797,	—	+	+	+	—	+	—
	14931, F1, F15,							
	F25, 8828, 8829 }							
<i>L. cellobiosus</i>	G3, G4, 11739	—	+	+	+	—	+	—
	4037	+	—	+	+	+	+	—
<i>L. viridescens</i>	E1, E7	+	—	+	+	+	+	+
	8965	+	—	—	+	—	+	+
<i>L. buchneri</i>	8837	—	+	+	+	—	+	—
	8007	+	—	+	+	—	+	—
	8838, 8839 }	+	—	+	+	+	+	—
	BC5, 8837 }							
<i>L. brevis</i>	8561	+	—	+	+	+	+	—
	X1, X3, X7, X9 }	+	—	+	+	+	+	+
	4036, 4617							
	8038, 14869 }							

DISCUSSION

The material used in this study was 31 strains of heterofermentative lactobacilli obtained from Dr M. E. Sharpe or from culture collections. All had been previously allotted to one of five species. In our hands routine physiological tests for the identification of these five species showed some variation from the results regarded as typical (Sharpe *et al.* 1966). However, our results with three type or neotype strains compare well with those reported by the Taxonomic Subcommittee on Lactobacilli for the same cultures (Hansen, 1968). Thus for *Lactobacillus brevis* ATCC 14869, *L. cellobiosus* ATCC 11739 and *L. fermenti* ATCC 14931 there is complete agreement, including the positive arabinose fermentation by strain 11739 which was reported negative for *L. cellobiosus* by Sharpe *et al.* (1966).

Electrophoresis of G6PD indicates two basic types of pattern. The *Lactobacillus fermenti*–*L. cellobiosus* type is remarkably constant. The complex patterns of slow migrating bands found in *L. brevis* and *L. buchneri* show some interstrain variation, but none would cause any confusion with typical strains of *L. fermenti* or *L. cellobiosus*. Variation is greater amongst *L. viridescens*, *L. brevis* and *L. buchneri*. The typical pattern of four bands is observed in eight of the 18 strains including examples of each species. The rest differ from the typical pattern in mobility and number of the bands.

Cell wall analysis indicates a common composition for *Lactobacillus cellobiosus* and *L. fermenti*, both of which contain ornithine. On the other hand, *L. viridescens*, *L. brevis* and *L. buchneri* all have a cell wall containing lysine. The occurrence of ornithine in the cell wall of *L. cellobiosus* is already well documented (Plapp & Kandler, 1967; Plapp, Schleifer & Kandler, 1967). However, earlier reports of the occurrence of lysine in the wall of *L. fermenti* are presumably due to mistaken identification of

chromatographic spots (Cummins & Harris, 1956) and are not substantiated by high voltage electrophoresis. Ikawa & Snell (1960) examined the cell wall components of 12 strains of lactobacilli by qualitative and quantitative means including microbiological assay for lysine. Unfortunately the 13th strain they studied, *L. fermenti*, was only examined qualitatively by paper chromatography because of shortage of material. Thus the mistaken identification was perpetuated.

There seems to be little reason to distinguish *Lactobacillus brevis* from *L. buchneri*. The species have a common antigen and are similar in physiological tests except for the fermentation of melezitose. The similarity of these two species, especially with regard to DNA composition, has been discussed by Gasser & Mandel (1968). Cell wall composition and enzyme electrophoresis would support the amalgamation of these species. With regard to *L. fermenti* and *L. cellobiosus* only the fermentation of aesculin and cellobiose separate the species. Cell wall amino acids, glucose-6-phosphate dehydrogenase patterns and DNA base ratios (Gasser & Mandel, 1968) are all identical. Apparently no type F antigen has been demonstrated in *L. cellobiosus* (Sharpe *et al.* 1966). The significance of this difference in the face of the aforementioned similarities would depend on the nature of the F antigen. *Lactobacillus viridescens* is clearly distinguished from the other species by its overall lack of fermentative ability. *Lactobacillus fermenti* and *L. brevis* were closer to each other in overall similarity (80 %) than either was to *L. viridescens* (75 % according to Seyfried, 1968). However, this study was based solely on morphological and physiological properties, and the low fermentative potentialities of *L. viridescens* seem sufficient to explain this result. In terms of cell wall amino acids and G6PD electrophoresis *L. viridescens* closely resembles *L. brevis* and *L. buchneri*. In spite of interstrain differences in % G+C (Gasser & Mandel, 1968) the DNA composition of this species is also closer to that of *L. brevis* and *L. buchneri* than to *L. fermenti* and *L. cellobiosus*. The difference in DNA composition permits *L. viridescens* to be clearly differentiated from *L. fermenti*. Similarities in DNA base composition, while perhaps indicative, are not as significant as differences. However, taken together, the DNA composition, cell wall amino acids and G6PD pattern indicate that *L. viridescens* is probably related to *L. brevis* and *L. buchneri* and definitely different from *L. cellobiosus* and *L. fermenti*.

All strains have been described in this study by the names given by their suppliers. On the basis of the results presented, only two strains appear to be anomalous. *Lactobacillus cellobiosus* NCIB 4037 has the G6PD pattern and cell wall composition typical of *L. buchneri* or *L. brevis*. By contrast, *L. buchneri* 8837 is identical with *L. cellobiosus* and *L. fermenti* in these respects. The true status of these strains might be determined by analysing the DNA base composition of each and by examining them for the E and F antigen respectively.

REFERENCES

- ATFIELD, G. N. & MORRIS, C. J. O. R. (1961). Analytical separations by high-voltage paper electrophoresis. Amino acids in protein hydrolysates. *Biochemical Journal* **81**, 606-614.
- BOWDEN, G. H. (1969). The components of the cell walls and extracellular slime of four strains of *Staphylococcus salivarius* isolated from human dental plaque. *Archives of Oral Biology* **14**, 685-697.
- BUYZE, G., VAN DEN HAMER, C. J. A. & DE HAAN, P. G. (1957). Correlation between hexose monophosphate shunt, glycolytic system and fermentation type in lactobacilli. *Antonie van Leeuwenhoek* **23**, 345-350.

- CHEESEMAN, G. C. (1959). The application of chromatographic techniques for the identification of species and strains of lactobacilli. *Journal of Applied Bacteriology* **22**, 341-349.
- CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. *Journal of General Microbiology* **14**, 583-600.
- DAVIS, G. H. G. (1955). The classification of lactobacilli from the human mouth. *Journal of General Microbiology* **13**, 481-493.
- DAVIS, G. H. G. (1956). The cellular and colonial morphology of oral lactobacilli. *British Dental Journal* **101**, 235-239.
- DAVIS, G. H. G. & BAIRD-PARKER, A. C. (1959). The bacterial elements of materia alba. *British Dental Journal* **106**, 142.
- DE MAN, J. C., ROGOSA, M. & SHARPE, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology* **23**, 130-135.
- GASSER, F. & MANDEL, M. (1968). Deoxyribosenucleic acid base composition of the genus *Lactobacillus*. *Journal of Bacteriology* **96**, 580-588.
- GOULDEN, J. D. S. & SHARPE, M. E. (1958). The infrared absorption spectra of lactobacilli. *Journal of General Microbiology* **19**, 76-86.
- HANSEN, P. A. (1968). *Type Strains of Lactobacillus Species. A Report by the Taxonomic Subcommittee on Lactobacilli and Closely Related Organisms*. American Type Culture Collection, Rockville, Maryland.
- HAYWARD, A. C. (1957). Detection of gas production from glucose by heterofermentative lactic acid bacteria. *Journal of General Microbiology* **16**, 9-15.
- HEATH, E. C., HURWITZ, J., HORECKER, B. L. & GINSBURG, A. (1958). Pentose fermentation by *Lactobacillus plantarum*. I. The cleavage of xylulose-5-phosphate by phosphoketolase. *Journal of Biological Chemistry* **231**, 1009-1029.
- IKAWA, M. & SNELL, E. E. (1960). Cell wall composition of lactic acid bacteria. *Journal of Biological Chemistry* **235**, 1376-1382.
- KANDLER, O., SCHLEIFER, K. H. & DANDL, R. (1968). Differentiation of *Streptococcus faecalis* Andrewes & Horder and *Streptococcus faecium* Orla-Jensen based on the amino acid composition of their murein. *Journal of Bacteriology* **96**, 1935-1939.
- MORICHI, T., SHARPE, M. E. & REITER, B. (1968). Esterases and other soluble proteins of some lactic acid bacteria. *Journal of General Microbiology* **53**, 405-414.
- PLAPP, R. & KANDLER, O. (1967). Die Aminosäuresequenz der asparaginsäure enthaltenden Mureins von *Lactobacillus coryniformis* und *Lactobacillus cellobiosus*. *Zeitschrift für Naturforschung* **22B**, 1062-1067.
- PLAPP, R., SCHLEIFER, K. H. & KANDLER, O. (1967). The amino acid sequence of the mureins of lactic acid bacteria. *Folia microbiologica (Praha)* **12**, 205-213.
- ROGOSA, M., MITCHELL, J. A. & WISEMAN, R. F. (1951). A selective medium for the isolation and enumeration of oral fecal lactobacilli. *Journal of Bacteriology* **62**, 32.
- ROGOSA, M. & SHARPE, M. E. (1959). An approach to the classification of the lactobacilli. *Journal of Applied Bacteriology* **22**, 329-340.
- ROGOSA, M., WISEMAN, R. F., MITCHELL, J. A., DISRAELI, M. N. & BEAMAN, A. J. (1953). Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius* nov. spec. and *Lactobacillus cellobiosus* nov. spec. *Journal of Bacteriology* **65**, 681-699.
- SEYFRIED, P. L. (1968). An approach to the classification of lactobacilli using computer-aided numerical analysis. *Canadian Journal of Microbiology* **14**, 313-318.
- SHARPE, M. E., FRYER, T. F. & SMITH, D. G. (1966). Identification of the lactic acid bacteria. In *Identification Methods for Microbiologists*, p. 65. Edited by B. M. Gibbs & F. A. Skinner. London: Academic Press.
- WILLIAMS, R. A. D. & BOWDEN, E. (1968). The starch-gel electrophoresis of glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus faecalis*, *S. faecium* and *S. durans*. *Journal of General Microbiology* **50**, 329-336.