Detection of Gas Production from Glucose by Heterofermentative Lactic Acid Bacteria

By A. C. HAYWARD

Department of Bacteriology, University of Birmingham

SUMMARY: Several modifications of the Durham tube method for detecting gas production have been tested with a variety of heterofermentative lactobacilli, some of which had been described in the literature as failing to produce gas from glucose by this method. Gas production in Durham tubes was detected consistently when the surface of a tomato juice broth medium containing 0.5 % or 2.5 % (w/v) glucose was sealed with liquid paraffin or with washed agar, but less consistently when the surface of the medium was unsealed. A minority of the strains examined grew weakly in a peptone + yeast extract medium containing glucose and produced very little titratable acidity or detectable gas. These produced large quantities of gas in the same medium when maltose replaced glucose.

Several methods may be used to differentiate between heterofermentative and homofermentative lactic acid bacteria. Analysis of the acid end products produced during the fermentation of hexoses shows that the former, including the genus *Leuconostoc* and some species of *Lactobacillus*, produce characteristic proportions of lactic and acetic acids, whereas the homofermentative species produce mainly lactic acid (Pederson, 1952). However, these two types may, for the most part, be differentiated by the production of gas from the fermentation of carbohydrates, which is confined to the heterofermentative organisms. This method, although simple in application, depends upon the reliability of the methods used for the detection of such gas (Davis, 1955*a*).

Many quantitative and qualitative methods of detecting gas production by lactic acid bacteria have been described (Gibson & Abd-el-Malek, 1945); qualitative tests have been carried out in liquid, solid or semi-solid media. The results of tests in liquid medium by the conventional Durham tube method, which is adequate for detecting gas production by Gram-negative bacteria, have led to erroneous conclusions. For example, Strålfors (1950) concluded that there were no gas-producing lactobacilli in the mouth, a conclusion at variance with the observations of Rogosa, Wiseman, Mitchell, Disraely & Beauman (1953) and Davis (1955*a*). The surface of the fermentation medium used by Strålfors was not sealed. The semi-solid medium of Gibson & Abd-el-Malek (1945) was sealed with a plug of nutrient agar, the displacement of which indicated gas production. Rogosa *et al.* (1953) detected gas production by the appearance of cracks in a 2 % (w/v) agar medium containing glucose + lactose; this solid medium was sealed with 2 % agar, the surface of which carried a thin layer of heavy mineral oil to prevent the escape of gas. Rosen, Ragheb, Hunt & Hoppert (1956) used a method similar to that described by Rogosa *et al.* (1953), in order to detect gas production by oral lactobacilli from rat. Although growth was observed in the medium, gas was produced in very small

quantities only in a few tubes. They thus concluded that most of the strains isolated were homofermentative, but their method clearly did not give unequivocal results.

Moore & Rainbow (1955), Camien, Dunn & Salle (1947) and Camien & Dunn (1955) described heterofermentative species of the genus *Lactobacillus* which fermented glucose very weakly, producing small quantities of titratable acidity, but which grew vigorously on a medium containing a pentose as energy source. Similarly, Cunningham & Smith (1940) isolated a group of lactobacilli from silage which gave low yields of fermentation products on glucose + yeast extract + tryptone broth. Although these organisms gave the low ratio of lactic to acetic acid characteristic of heterofermentative lactobacilli, the quantities of carbon dioxide produced were small, and in some cases comparable with the amounts yielded by homofermentative species. During the systematic study of numerous lactobacilli from diverse saprophytic and parasitic sources I have isolated several lactobacilli of this type which grew weakly or not at all upon a medium containing glucose as major source of energy. These observations led to the following re-investigation of the factors which affect the detection by the Durham tube method of gas production by lactic acid bacteria and to a search for a suitable fermentable substrate for gas production by those lactobacilli which ferment glucose weakly. Two experiments are described in the following paper: (i) gas production by known heterofermentative lactobacilli was compared in tomato juice (TJ) broth containing different concentrations of glucose with the surface of the medium sealed or unsealed, and also in TJ agar medium; (ii) gas production was compared in a medium containing various carbohydrates but no tomato juice.

METHODS

Strains L3, L4, L5 and L6, studied by Moore & Rainbow (1955), were supplied by courtesy of Dr C. F. Rainbow, Department of Applied Biochemistry, University of Birmingham. The remaining cultures were fresh isolates or were obtained from the National Collection of Industrial Bacteria, Teddington, Middlesex; these are listed in Table 1.

Freshly isolated strains were identified according to a simplified form of the classification described by Davis (1955*b*). Those listed without specific names did not belong to any recognizable species.

Media. In the first experiment the tomato juice (TJ) medium of Davis, Bisset & Hale (1955) was used. Separate portions of this medium contained 0.5 % or 2.5 % (w/v) glucose; a third portion containing 2.5 % glucose and 0.5 ml./l. of a 2.0 % (w/v) solution of bromocresol green was solidified with 1.2 % (w/v) New Zealand agar. These media were dispensed in 3.0 ml. quantities in $\frac{1}{2} \times 5$ in. tubes. The tubes containing liquid media were supplied with Durham tubes, and were sterilized by autoclaving at 120°/15 min. The liquid media were inoculated with one drop of culture from a Pasteur pipette giving about 40 drops/ml. The molten and cooled agar medium was heavily inoculated with a Pasteur pipette and rotated to distribute the organisms evenly. The 2.5 %

glucose TJ liquid medium was inoculated with each strain in triplicate; one of the tubes was sealed with about 2.0 ml. of sterile liquid paraffin, one with a similar quantity of molten 10 % (w/v) washed Japanese agar, and the third tube was not sealed. The 0.5 % glucose TJ liquid medium was inoculated in

Table 1. *Classification and names of lactobacilli used*

Species and strain no.		Source
<i>Lactobacillus</i> sp.	L3, L4, L5, L6	Moore & Rainbow (1955)
<i>Lactobacillus</i> sp.	60C	Saliva
<i>Lactobacillus</i> sp.	68C4	Saliva
<i>Lactobacillus</i> sp.	S42/2	Wasp
<i>L. brevis</i>	C/2/1	New Zealand Cheddar
<i>L. brevis</i>	E2/2	Red Cheshire cheese
<i>L. brevis</i>	K/2/2	Tilsit cheese
<i>L. brevis</i>	I/4	Saint Paulin cheese
<i>L. brevis</i>	J/2	Canadian Cheddar cheese
<i>L. brevis</i>	23F/2	Saliva
<i>L. brevis</i>	S48/3	Dried yeast
<i>L. brevis</i> var. <i>rudensis</i>	4617	NCIB
<i>L. buchneri</i>	8037	NCIB
<i>L. fermenti</i>	V28	Human vagina
<i>L. fermenti</i>	74C4A	Saliva
<i>L. fermenti</i>	V8S	Human vagina
<i>L. parvus</i>	8516 (Russell & Walker, 1953 <i>b</i>)	NCIB
<i>L. malefermentans</i>	8517 (Russell & Walker, 1953 <i>a</i>)	NCIB
<i>L. frigidus</i>	8518 (Bhandari & Walker, 1953)	NCIB
<i>L. plantarum</i>	12C	Homofermentative species used as controls
<i>L. plantarum</i>	51C/1	
<i>L. casei</i>	15C/1A	
<i>L. casei</i>	76CF	
<i>Leuconostoc</i> sp.	N/2/1	Leicester cheese
<i>Leuconostoc</i> sp.	G/4	Dutch Edam cheese

duplicate; one tube was sealed with liquid paraffin, the other was not sealed. The following strains were incubated at 28° after inoculation with samples of 24 hr. TJ broth cultures: 8516, 8517, 8518, 8037, 4617, L3, L4, L5 and L6. All other strains were incubated at 37° after inoculation with 14 hr. cultures.

In the second experiment the following basal medium was used: Peptone (Oxoid) 0.5 % (w/v); yeast extract (Difco) 0.3 % (w/v); salts A 0.5 % (v/v); salts B 0.5 % (v/v); Tween 80 0.1 % (v/v); sodium acetate (hydrated) 0.5 % (w/v). The constituents were diluted in glass-distilled water and the pH value of the medium adjusted to 6.8–7.0. The salt solutions A and B were those used by Davis *et al.* (1955). Solution A contained 10 g. KH_2PO_4 and 10 g. K_2HPO_4 in 100 ml. of distilled water; solution B contained 11.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.4 g. $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ and 0.68 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml. of distilled water. (Note: These formulae are given in full because Davis *et al.*, in stating the composition of this medium, attributed both solutions to Rogosa *et al.* (1953), whereas the latter workers used only solution B).

Portions of the basal medium were supplemented with 2.5 % (w/v) of glucose, lactose, sucrose, fructose or maltose. About 3.0 ml. quantities were added to 0.5 in. $\times$ 5 in. tubes containing Durham tubes (*c.* 1.2 in. $\times$ 0.25 in.).

Sterilization was effected by autoclaving, momentarily, at 15 lb./sq.in. Tubes were inoculated with one drop of a 24 hr. TJ broth culture from a standard pipette. According to Moore & Rainbow (1955) their strains grew well at 32–35° in malt wort and so L3, L4, L5 and L6 were incubated at 34°, the remaining cultures at 36–37°. In sets of experiments tubes were incubated for 6 days without shaking, and examined daily for gas production.

RESULTS

The results of the two experiments are shown in Tables 2 and 3. In the unsealed tubes (Table 2), gas production was usually greatest after 2–3 days of incubation, the volume decreasing later presumably because of loss of gas to the

Table 2. *Expt. 1: gas production detected by the Durham tube method in tomato juice (TJ) media*

Gas production in liquid medium was recorded as the degree to which the Durham tube was filled with gas, as follows: 0 = Absence of gas in Durham tube; 1 = bubble of gas at apex of Durham tube; 2 = c. one-third of Durham tube filled with gas; 3 = c. two-thirds of Durham tube filled with gas; 4 = whole of Durham tube filled with gas. Gas production in agar was recorded as follows: ++ = transverse cracks in agar, agar split apart and forced up the tube; + = cracks in agar, but medium not forced apart; – = medium entire. N.I. = not inoculated. The numbers in brackets indicate the day of incubation at which maximum gas production was observed.

Strain no.	2.5 % glucose TJ broth			0.5 % glucose TJ broth		2.5 % glucose TJ agar
	Agar seal	Paraffin seal	Unsealed	Paraffin seal	Unsealed	
8516	4 (2)	4 (3)	4 (2)	> 2 (2)	> 2 (2)	++ (1)
8517	4 (3)	4 (3)	3 (3)	4 (6)	> 2 (3)	++ (2)
8518	4 (1)	4 (2)	4 (2)	> 3 (5)	3 (1)	++ (1)
4617	4 (1)	4 (1)	4 (1)	4 (3)	3 (1)	++ (1)
8037	4 (3)	4 (3)	> 3 (4)	4 (4)	0	++ (2)
L3	4 (2)	4 (2)	> 3 (3)	4 (3)	1 (2)	+(2)
L4	4 (2)	4 (3)	3 (2)	> 3 (5)	> 1 (3)	+(4)
L5	0	1 (3)	0	1 (3)	0	–
L6	1 (6)	1 (6)	0	1 (4)	0	–
V28	4 (2)	4 (3)	> 2 (3)	4 (4)	0	–
74C4A	4 (6)	> 2 (6)	0	1 (5)	0	–
68C4	4 (2)	4 (3)	3 (3)	4 (6)	0	+(5)
23F/2	4 (2)	4 (2)	4 (2)	4 (2)	> 2 (2)	+(4)
C/2/1	4 (2)	4 (2)	4 (2)	4 (3)	> 2 (2)	+(2)
E2/2	4 (1)	4 (1)	4 (1)	4 (2)	3 (2)	++ (1)
K/2/2	4 (2)	4 (1)	4 (2)	4 (3)	< 3 (2)	++ (1)
I/4	4 (1)	4 (2)	> 3 (2)	4 (3)	> 3 (2)	++ (1)
N/2/1	4 (3)	4 (3)	< 3 (2)	< 4 (3)	< 3 (2)	++ (1)
60C	4 (2)	4 (2)	4 (2)	> 3 (3)	> 2 (2)	++ (2)
G/4	4 (2)	4 (2)	> 3 (2)	4 (3)	> 1 (2)	++ (1)
J/2	4 (1)	4 (2)	4 (2)	4 (3)	> 2 (2)	++ (1)
15C/1A	0	0	1 (6)	0	1 (1)	–

atmosphere. Durham tubes filled with gas were forced into the liquid paraffin seal or agar plug, the latter being forced up the tube by the pressure of gas not collecting in the Durham tube.

In Expt. 1 (Table 2) homofermentative strains 12C, 51C/1 and 76CF, and in Expt. 2 strain 51C/1, did not produce detectable gas. As shown in Table 2. *Lactobacillus casei* (15C/1A) produced a very small quantity of gas in TJ broth, comparable with that produced by L5 and L6 in the same type of medium. In Expt. 2 (Table 3), however, L5 and L6 produced gas in large quantities in maltose medium. It has been observed in routine examinations that some strains of the homofermentative species *L. casei* and *L. plantarum* occasionally produce a bubble of gas at the apex of the Durham tube, but never in quantities comparable with those produced by heterofermentative species.

Table 3. *Expt. 2: gas production by lactic acid bacteria from various carbohydrates in a peptone + yeast extract medium*

The degree of gas production is recorded as in Table 2.

Strain no.	Glucose		Fructose		Maltose		Lactose		Sucrose	
	Agar seal	Paraffin seal	Agar seal	Paraffin seal	Agar seal	Paraffin seal	Agar seal	Para-fin seal	Agar seal	Paraffin seal
L3	4 (4)	4 (3)	3 (3)	3 (5)	4 (2)	4 (2)	0	0	0	0
L4	> 1 (5)	> 1 (6)	3 (3)	< 3 (3)	4 (2)	4 (2)	0	0	0	0
L5	0	0	3 (5)	> 2 (3)	4 (2)	4 (2)	0	0	0	0
L6	0	0	> 3 (4)	> 2 (3)	4 (2)	4 (2)	0	0	0	0
V28	4 (4)	4 (4)	4 (2)	4 (3)	4 (2)	4 (3)	0	0	4 (3)	4 (3)
V8S	4 (2)	4 (2)	< 3 (4)	> 2 (2)	4 (1)	4 (1)	4 (1)	4 (1)	4 (1)	4 (1)
S42/2	4 (2)	4 (2)	0	0	4 (2)	4 (2)	0	0	0	0
68C4	0	0	< 4 (4)	4 (5)	4 (2)	4 (3)	0	0	4 (2)	4 (3)
4617	4 (2)	4 (2)	3 (4)	3 (4)	4 (1)	4 (1)	0	0	4 (3)	4 (4)
S48/3	4 (3)	4 (3)	> 2 (2)	< 3 (3)	4 (2)	4 (2)	0	0	N.I.	N.I.
8518	4 (2)	4 (2)	4 (3)	4 (2)	4 (2)	4 (2)	0	0	4 (2)	4 (2)
8037	4 (5)	4 (4)	4 (2)	4 (2)	4 (3)	4 (4)	0	0	4 (3)	4 (3)

Strain 68C4, which in previous studies had failed to produce visible turbidity or titrable acidity in media containing glucose, also produced gas in a medium containing maltose, which according to Rogosa *et al.* (1953) is fermented by all heterofermentative lactobacilli. *Lactobacillus fermenti* (V8S) alone produced detectable gas in lactose medium. Gas production in fructose medium was generally less than in the other media.

Tables 2 and 3 show that very similar results were obtained in media sealed with agar or liquid paraffin; but in unsealed media gas accumulated in smaller quantities and was sometimes not detected. Results of tests in the agar medium used were frequently equivocal.

DISCUSSION

It is apparent that the Durham tube method may be used for the detection of gas production by lactobacilli provided that the surface of the medium be sealed, thus preventing the escape of gas, presumably mainly carbon dioxide. A minority of the tubes of unsealed media containing Durham tubes failed to accumulate gas. Evidence in the literature (for example, Strålfors, 1950)

suggests that in other hands all such tubes were free of gas; a possible explanation of this difference is that wider test tubes than those used in my experiments have been generally employed in gas production tests, with the consequent exposure of a larger surface area of medium to the atmosphere.

The use of tomato juice in gas-production media may give misleading results, bearing in mind the observations of Chatfield & Adams (1931), that tomatoes contain an average of 3.4 % total sugars: gas may be produced from carbohydrates present in the tomato juice, but probably not from glucose (compare the results for strain 68C4). It is noteworthy that the gas-production medium used by Gibson & Abd-el-Malek (1945) included skimmed milk, tomato juice and 5.0 % (w/v) glucose, while that of Rogosa *et al.* (1953) included tomato juice, 0.2 % (w/v) lactose and 0.3 % (w/v) glucose. Although such media are adequate for the detection of gas production by most heterofermentative lactobacilli, it is concluded from this study that some strains require differentiation from non-gas-producing lactobacilli upon a medium containing maltose, in which all the heterofermentative lactobacilli examined were more readily defined as such than in any other medium.

REFERENCES

- BHANDARI, R. R. & WALKER, T. K. (1953). *Lactobacillus frigidus* n.sp. isolated from brewery yeast. *J. gen. Microbiol.* **8**, 330.
- CAMIEN, M. N. & DUNN, M. S. (1955). Pentose dependence of *Lactobacillus gayonii* and related species for early growth. *Proc. Soc. exp. Biol., N.Y.* **90**, 183.
- CAMIEN, M. N., DUNN, M. S. & SALLE, A. J. (1947). The effect of carbohydrates on acid production by 24 lactic acid bacteria. *J. biol. Chem.* **168**, 33.
- CHATFIELD, C. & ADAMS, G. (1931). *Circ. U.S. Dep. Agric.* no. 146. (Quoted by Camien *et al.* (1947). *J. biol. Chem.* **168**, 33.)
- CUNNINGHAM, A. & SMITH, A. M. (1940). The microbiology of silage made by the addition of mineral acids to crops rich in protein. II. The microflora. *J. Dairy Res.* **11**, 243.
- DAVIS, G. H. G. (1955a). *The Classification of Lactobacillus Strains of Oral Origin*. Ph.D. Thesis, University of Birmingham.
- DAVIS, G. H. G. (1955b). The classification of lactobacilli from the human mouth. *J. gen. Microbiol.* **13**, 481.
- DAVIS, G. H. G., BISSET, K. A. & HALE, C. M. F. (1955). Correlation between morphological and physiological characters in the classification of members of the genus *Lactobacillus*. *J. gen. Microbiol.* **13**, 68.
- GIBSON, T. & ABD-EL-MALEK, Y. (1945). The formation of CO₂ by lactic acid bacteria and *Bacillus licheniformis* and a cultural method of detecting the process. *J. Dairy Res.* **14**, 35.
- MOORE, W. B. & RAINBOW, C. (1955). Nutritional requirements and biochemical activities of brewery lactobacilli. *J. gen. Microbiol.* **13**, 190.
- PEDERSON, C. S. (1952). In 'A symposium on the lactic acid bacteria'. *Bact. Rev.* **16**, 227.
- ROGOSA, M., WISEMAN, R. F., MITCHELL, J. A., DISRAELY, M. N. & BEAUMAN, A. J. (1953). Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius* nov.spec. and *Lactobacillus cellobiosus* nov.spec. *J. Bact.* **65**, 681.
- ROSEN, S., RAGHEB, H. S., HUNT, H. R. & HOPPERT, C. A. (1956). A comparative study of oral lactobacilli isolated on two different media from the Hunt-Hoppert rats. *J. dent. Res.* **35**, 291.

- RUSSELL, C. & WALKER, T. K. (1953*a*). *Lactobacillus malefermentans* n.sp., isolated from beer. *J. gen. Microbiol.* 8, 160.
- RUSSELL, C. & WALKER, T. K. (1953*b*). *Lactobacillus parvus* n.sp., isolated from beer. *J. gen. Microbiol.* 8, 310.
- STRÅLFORS, A. (1950). Investigations into the bacterial chemistry of dental plaques. *Odont. Tidskr.* 58, 155.

(Received 15 June 1956)