

THE CHARACTERISTICS OF LACTATE-FERMENTING SPOREFORMING ANAEROBES FROM SILAGE

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In studies on the sporeforming anaerobic bacteria in orchard grass silages, two groups were found to predominate on the basis of spore counts (Bryant *et al.*, 1952). Several proteolytic strains isolated were similar to *Clostridium sporogenes*, but the majority of isolates were lactate-fermenting anaerobes which were similar to *Clostridium tyrobutyricum* (van Beynum and Pette, 1935).

Since the work of van Beynum and Pette (1935, 1936), lactate-fermenting, sporeforming anaerobes have been isolated from silage by Martos (1941) and Rosenberger (1951), but descriptions of additional characteristics of these organisms were not given.

The current edition of Bergey's Manual (Breed *et al.*, 1948) does not recognize *C. tyrobutyricum* as a separate species but places it in the appendix to the species *C. butyricum*, even though it ferments lactate and ferments few carbohydrates, while *C. butyricum* ferments many carbohydrates and supposedly not lactate.

Bhat and Barker (1947) showed that the fermentation of lactate by a sporeforming anaerobe from soil, *Clostridium lacto-acetophilum*, was dependent on the presence of acetate in the medium. These organisms were identical with *C. butyricum* (Breed *et al.*, 1948) except for the fermentation of lactate. They did not test known strains of *C. butyricum* for fermentation of lactate under their conditions.

The purpose of the present work is to determine the characteristics of the lactate-fermenting clostridia in grass silage to obtain a better understanding of the species involved.

EXPERIMENTAL METHODS

The medium for primary isolation was prepared and used under carbon dioxide using the anaerobic procedure of Hungate (1950) as modified by Bryant and Burkey (1953). The medium contained agar, 6 g; tryptone, 3 g; yeast extract, 3 g;

glucose, 0.3 g; tomato juice, 60 ml; resazurin, 0.3 mg; Na_2CO_3 , 1.2 g; cysteine-HCl, 0.15 g; and distilled water to make 300 ml. Sterile solutions of cysteine-HCl and Na_2CO_3 were added after other ingredients of the medium were adjusted to pH 6.8 and autoclaved.

Samples of silage were collected and diluted in physiological saline solution as described by Kroulik *et al.* (1955), and tubes of the medium were inoculated with 0.1 ml of appropriate dilutions that had been heated at 80 C for 10 min. Other tubes of medium were inoculated from unheated dilutions. Cultures were incubated at 30 C for 48 hr before strains were isolated from representative colonies.

The strains of lactate-fermenting anaerobes studied were isolated from silages with more than 10,000 spores per g to lessen the possibility of isolating anaerobes present in the silage but not multiplying therein. Most strains were isolated from culture tubes representing 10^{-5} g or less of silage. Sixty-two strains were isolated from 10 orchard grass silages that represented three crops in 3 successive years. Twelve strains were isolated from two alfalfa silages prepared from the same crop. Eight of the strains from orchard grass were isolated from unheated dilutions of silage. All other strains were isolated from cultures prepared from heated dilutions. Silages from which strains were isolated varied in age from 12 to 210 days, but most strains came from silages between 30 and 60 days of age.

Strains ATCC 6014, 6015, 8260, and 859 of *C. butyricum* and strains ATCC 6013, 7040, and 7041 of *Clostridium pasteurianum* were included in the study as controls and to determine if any of these cultures would ferment lactate if acetate was included in the medium.

All isolates were cultured in Trypticase Soy Agar deeps (Baltimore Biological Laboratory, Inc.) supplemented with cysteine-HCl, 0.05 per cent; glucose, 0.2 per cent; and resazurin, 0.0001 per cent to determine their relations to oxygen.

The basal medium used to determine the carbohydrates fermented (medium 1) had the following percentage composition: trypticase, 1.5; yeast extract, 0.5; cysteine-HCl, 0.05; reazurin, 0.0001; pH, 7. Soluble carbohydrates were sterilized in 10 per cent aqueous solution by filtration before addition to the sterile basal medium. Salicin and soluble starch were autoclaved in 5 per cent suspensions. The final concentration of carbohydrates in the basal medium was 0.5 per cent. Controls consisted of the inoculated medium minus carbohydrate and uninoculated media containing each carbohydrate. Acid production was detected by adding brom thymol blue solution to the culture tubes after 1 week of incubation.

The lactate medium consisted of medium 1 plus lactic acid, 0.5 per cent; and sodium acetate·3H₂O, 0.5 per cent. Lactate fermentation was detected by determining the final pH with a glass electrode pH meter. This fermentation was accompanied by a sharp rise in pH from 7 to about 8 or higher.

Hydrogen sulfide production was determined in medium 1 supplemented with agar, 0.5 per cent; glucose, 0.1 per cent; and ferric ammonium citrate, 0.05 per cent. Gelatin liquefaction and action in milk were determined with the methods of Spray (1936) except that 0.05 per cent of cysteine-HCl was used in place of iron wire and resazurin was added.

Indol production and nitrate reduction were

determined after 3 and 7 days of incubation in Indol-Nitrite medium (Baltimore Biological Laboratory, Inc.) supplemented with cysteine-HCl and resazurin. Both nitrite and residual nitrate tests were made.

In early work all media for physiological studies were prepared in cotton-plugged culture tubes and liquid media contained 0.25 per cent of agar according to the method of Spray (1936). Later, the agar was omitted and rubber-stoppered tubes with a gaseous phase of nitrogen were used, as in the anaerobic procedure of Hungate (1950).

Products of the fermentation of lactate were determined in a medium of the following percentage composition: lactic acid, 1.0; sodium acetate, 0.8; cysteine-HCl, 0.05; yeast extract, 0.5; and the mineral solution of Bryant and Burkey (1953). Fermentation products were determined with essentially the same methods as formerly described (Bryant, 1952) except that the volatile acids were determined chromatographically without subsequent Duclaux distillation.

RESULTS AND DISCUSSION

All strains of lactate-fermenting anaerobes from silage were rather large (0.8–1.2 by 2–15 μ), usually short, motile rods. They were gram positive in young cultures. Spores were oval and subterminal, and produced slight but definite swelling of the sporangium. The organisms

TABLE 1

*Physiological characteristics of lactate-fermenting anaerobes from silage and strains of Clostridium butyricum and Clostridium pasteurianum obtained from the American Type Culture Collection**

Number of Strains	Xylose	Galactose	Maltose	Lactose	Sucrose	Raffinose	Salicin	Inulin	Starch	Mannitol	Glycerol	Lactate	Milk	Source	Species Designation
13	—	—	—	—	—	—	—	—	—	—	w†	+	—	Silage	<i>C. tyrobutyricum</i>
54	—	—	—	—	—	—	—	—	—	—	w	+	—	Silage	<i>C. tyrobutyricum</i>
1	+	—	—	—	—	—	—	—	—	—	w	+	—	Silage	<i>C. tyrobutyricum</i>
2	+	+	—	—	—	—	—	—	—	—	w	+	—	Silage	<i>C. tyrobutyricum</i>
1	+	+	—	+	—	—	—	—	—	—	w	+	—	Silage	<i>C. tyrobutyricum</i>
3	+	+	—	+	—	—	—	—	—	—	w	+	—	Silage	<i>C. tyrobutyricum</i>
3	+	+	+	+	+	+	+	+	+	+	w	+	cg†	ATCC 6014, 6015, 8260	<i>C. butyricum</i>
1	+	+	+	+	+	+	+	+	+	+	+	+	cg	ATCC 859	<i>C. butyricum</i>
2	—	+	+	—	+	+	+	+	—	+	+	—	—	ATCC 6013, 7041	<i>C. pasteurianum</i>
1	—	+	+	—	+	+	—	—	—	+	+	—	—	ATCC 7040	<i>C. pasteurianum</i>

* All strains fermented glucose and fructose. None produced catalase, indol, or hydrogen sulfide or liquefied gelatin. Strains varied in ability to reduce nitrate but most did not.

† w indicates a weak reaction.

‡ cg indicates curd and gas.

formed lenticular deep colonies and entire, opaque, slightly convex, cream-colored, surface colonies about 2 to 3 μ in diameter after incubation for 48 hr in the tomato juice medium. Colonies often contained gas bubbles. Only a slight amount of turbidity occurred in medium 1 with no carbohydrate added.

Table 1 shows the physiological characteristics of the lactate-fermenting organisms from silage as compared to the known strains of *C. butyricum* and *C. pasteurianum*.

Five of the silage strains were found to grow well at 30 and 37 C and more slowly at 22 C; no growth was observed at 45 C. The final pH of these strains in the glucose medium at 30 C was 4.3 to 4.7.

As in the lactate fermentation by other spore-forming anaerobes (Bhat and Barker, 1947; Tabachnick and Vaughn, 1948), silage strains failed to produce an appreciable fermentation of lactate in a medium with small amounts of yeast extract unless acetate was added. Five of the strains were placed in lactate medium similar to medium 1 of Bhat and Barker, except that 0.1 per cent of yeast extract was added in place of yeast autolyzate. This medium did not support appreciable growth of the organisms but, when 0.8 per cent of sodium acetate was added, growth was good.

The products formed from the fermentation of lactate by one strain of the type that fermented only glucose, fructose, and glycerol were the following in moles per 100 moles of lactate fermented: acetate, -37; butyrate, 63; carbon dioxide, 97; and hydrogen, 53. The carbon recovery was 93 per cent. These products and the amounts produced are very similar to those reported for *C. lacto-acetophilum* (Bhat and Barker, 1947).

Thirteen of the silage strains appeared to be identical with *C. tyrobutyricum* as described by van Beynum and Pette (1935). Fifty-four strains differed from their description only in the fermentation of mannitol. The remaining seven strains differed from their description in the fermentation of xylose and lactose or mannitol. The difference between van Beynum and Pette's organisms and the present strains in the fermentation of glycerol is probably more apparent than real. Bhat and Barker (1947) showed that other lactate-fermenting sporeforming anaerobes fer-

mented glycerol vigorously only if the medium contained acetate.

Richard (1948) observed that many of the morphological, physiological and cultural characteristics of single strains of anaerobic butyric acid bacteria varied from time to time. The characteristics of three of the present isolates from silage were determined a second time 2 years after they were first studied. They were found to maintain the same characteristics over this period.

We have concluded that the present silage strains should be identified as *C. tyrobutyricum*. This species should be considered distinct from *C. butyricum* because of the large differences in carbohydrates fermented (table 1).

Data in table 1 show that, under the conditions established by Bhat and Barker, *C. butyricum* ferments lactate. This finding invalidates the species *C. lacto-acetophilum* which was described as differing from *C. butyricum* only in the fermentation of lactate. The three strains of *C. pasteurianum* did not ferment lactate. This suggests an additional characteristic for the separation of this species from *C. butyricum*.

The present studies support the conclusion of van Beynum and Pette (1936) that *C. tyrobutyricum* is the important lactate-fermenting spore-forming anaerobe in grass silage.

Strain R of Tabachnick and Vaughn (1948) appears to be identical to most of the lactate-fermenters of the present study which fermented glucose, fructose, and mannitol. This indicates that these organisms are not peculiar to the geographical location or the experimental conditions of the present study.

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SUMMARY

A study was made of the characteristics of 74 strains of lactate-fermenting sporeforming anaerobes isolated from ten orchard grass silages prepared from three different crops and two alfalfa silages from the same crop. All strains were similar in characteristics except for differences in the fermentation of a few carbohydrates.

Thirteen strains fermented only glucose and fructose and 54 strains fermented only glucose, fructose and mannitol. Seven strains differed from these in the fermentation of xylose, galactose and lactose.

It was concluded that all strains should be identified as *Clostridium tyrobutyricum*. This species should be considered distinct from *Clostridium butyricum* on the basis of carbohydrates fermented.

Contrary to the description in *Bergey's Manual* (Breed *et al.*, 1948), strains of *C. butyricum* from the American Type Culture Collection were found to ferment lactate.

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