CHARACTERISTICS OF TARTRATE-FERMENTING SPECIES OF CLOSTRIDIUM

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While establishing methods for the control of spoilage in the tartrate recovery plants of California, Vaughn *et al.* (1943*a*,*b*; 1946) isolated a number of D-tartrate-decomposing microorganisms, including several mesophilic clostridia. These organisms, among the most active fermenters of D-tartrate, are of historical as well as economic interest, for it was in a tartrate medium that Pasteur (1863) encountered similar organisms in his study of anaerobiosis. This investigation was undertaken to determine the characteristics of a number of these D-tartrate-fermenting clostridia, for, despite the early observation of anaerobic tartrate decomposition, to the writers' knowledge a pure culture study of the tartrate-fermenting species of the genus *Clostridium* has never been reported.[1]

EXPERIMENTAL RESULTS

Isolations were made from 18 different sources. The majority of cultures were obtained from spoiled crude D-tartrate or tartrate recovery equipment.[2] Several strains were isolated from vineyard soil. One isolate was obtained from marine mud. Fifteen of the cultures were obtained by direct isolation from the source material placed on glucose agar. The eight remaining strains were obtained by an enrichment technique involving D-tartrate media. Fifty-four additional cultures representing twenty-five species of the genus *Clostridium* were used as controls. For the majority of the cultures in this latter collection the authors are indebted to Dr. Elizabeth McCoy, Dr. C. E. Clifton, Dr. L. S. McClung, Dr. F. W. Tanner, Dr. Sanford S. Elberg, Dr. K. W. Raper, and Dr. E. J. Cameron. The remaining control cultures were obtained from the American Type Culture Collection. Conventional techniques were used throughout the course of the study. References rather than detailed descriptions will serve to acquaint the reader with them.

Characteristics of the Bacteria

A majority of the cultures possessed similar morphological characteristics. They were large, motile rods with rounded ends, occurring singly, in pairs, in chains, and occasionally in long filaments. The cells were gram-positive in very young cultures, but became gram-negative from 5 to 10 hours after turbidity appeared in liver infusion broth. The cultures all gave typical granulose reactions with iodine.

[1] For a summary of the early literature concerning the microbial decomposition of tartrates, see Vaughn, Marsh, *et al.* (1943*b*, 1946).

[2] For an understanding of the tartrate recovery processes consult Marsh (1943).

The cultures varied in their ability to produce spores; some sporulated profusely, others sparingly. The spores were always elliptical and generally contained in a subterminal position inside the sporangium. Sporulation resulted in distinct swelling of the cells.

Typical colonies grown on tryptone glucose agar (3 days at 37 C) averaged 1.5 mm in diameter and were smooth, white, opaque, convex, glistening, and circular with irregular margins. On tryptone ammonium D-tartrate agar the colonies were minute and appeared translucent or transparent.

The differential characteristics of the 23 tartrate-fermenting isolates are shown in table 1. None of the cultures softened or digested coagulated egg albumen, blackened or digested brain medium, or liquefied gelatin. The bacteria did

TABLE 1

Characteristics of the tartrate-fermenting isolates

NUMBER OF CULTURES	DECOMPOSITION OF PROTEINACEOUS MATERIALS			FERMENTATION AS INDICATED BY GAS PRODUCTION AND CHANGE IN pH											TENTATIVE SPECIES ALLOCATION (AFTER SPRAY, 1948)
	Coagulated egg albumen	Brain medium	Gelatin liquefaction	D-Xylose	Glucose	Lactose	Maltose	Sucrose	Galactose	Glycerol	Mannitol	Salicin	Inulin	Starch*	
	Number of cultures showing positive reactions at 37 C														
12	0	0	0	12	12	12	12	12	12	10	12	12	7	12	*Clostridium butyricum*
8	0	0	0	8	8	8	8	8	8	8	8	8	2	0	*Clostridium beijerinckii*
1	0	0	0	1	1	1	1	1	1	0	0	1	0	0	*Clostridium pasteurianum* (Strain N)
1	0	0	0	1	1	0	1	1	0	0	0	0	0	0	? (Strain Q)
1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	? (Strain R)

* Soluble and insoluble starches were tested. Demonstration of extracellular amylase as well as fermentation with gas production and change in pH were criteria for decomposition.

not produce indole in tryptone medium even when galactose, D-xylose, or ammonium D-tartrate were substituted for glucose (Spray, 1936; Evans *et al.*, 1942). Hydrogen sulfide was not produced from the sulfur-containing constituents of tryptone. The organisms did not grow in tryptone broth in the absence of a fermentable substrate. Strain Q was the only isolate that reduced nitrate to nitrite.

The cultures all grew well at temperatures ranging from 30 to 37 C. They grew slowly at 25 C but did not grow at all at 40 C (temperature of the medium).

The minimum pH value for the growth of these bacteria in tartrate media was 4.7. The majority of the strains, however, could not grow at pH values below 5.1. The desired pH values of the tartrate media were obtained as described by Vaughn *et al.* (1946). The minimum pH value for growth was determined

by an adaptive transfer technique (Gililland and Vaughn, 1943). It was not determined whether the effect on growth was caused by the hydrogen ion activity or was in part the result of the toxicity of undissociated tartaric acid.

The D-tartrate-fermenting cultures are representative of the saccharolytic clostridia. The majority (12 isolates) closely resemble the type species *Clostridium butyricum* regardless of their ability to ferment glycerol.[3] The eight strains allocated to the species *Clostridium beijerinckii* possess the general characteristics of *Clostridium butyricum* but are separated from it by their inability to ferment starch.

The three remaining cultures, N, Q, and R, differ markedly from the other isolates studied. They are more difficult to grow and grow more slowly. Strain N has been allocated tentatively to the species *Clostridium pasteurianum* despite the fact that it ferments lactose. It will be noted that strain R ferments glucose and mannitol, whereas strain Q ferments D-xylose, glucose, maltose, and sucrose, although they both attack D-tartrate. On morphological grounds strain R is similar to the other identified isolates. Strain Q, however, characteristically forms surface colonies that are flat and circular, have myceloid edges, and are dull yellow in color. Because of these differences it is felt that specific designations for these two isolates are not warranted until further investigations have been made.

Some supporting evidence to strengthen the specific allocations for the majority of the strains (N, Q, and R excepted) was obtained by determination of the end products of unbuffered glucose fermentations by three representative isolates. The chief end products of the glucose fermentations by these three cultures were carbon dioxide, hydrogen, and butyric and acetic acids. The molar ratio of butyric to acetic acid was approximately 4 to 1.

The production of only very small amounts of isopropyl alcohol by one strain, and only trace quantities of neutral volatile products by the other two cultures, plus the absence of acetoin and 2,3-butanediol from the fermentation products of all three isolates, is considered additional evidence for placing the D-tartrate-fermenting cultures in the *Clostridium butyricum* group. (See McCoy *et al.*, 1930; Langlykke *et al.*, 1935; Osburn *et al.*, 1937.)

End Products of D-Tartrate Fermentation

Since earlier workers apparently had not used pure cultures in their studies of the anaerobic fermentation of tartrate, it was desirable to determine the end products formed from tartrate fermentations by several of the pure cultures just described. Four representative cultures (N, Q, and R excepted) were used. They were grown in a medium containing 0.2 per cent yeast extract (Difco), 0.1 per cent tryptone (Difco), 0.1 per cent dibasic potassium phosphate, and traces of ferrous, manganous, and magnesium salts, to which were added concentrations of ammonium D-tartrate consisting of 1.0, 1.5, 2.0, and 4.0 per cent,

[3] Species allocation follows Spray (1948) in *Bergey's Manual of Determinative Bacteriology*. Some authorities consider that glycerol fermentation is variable among the strains of *Clostridium butyricum*. See Spray (1936).

respectively. The reactions of the media containing the various concentrations of D-tartrate were between pH 6.8 and 7.0 after sterilization. The inoculated spores germinated and grew readily in the cultures containing from 1.0 to 2.0 per cent ammonium D-tartrate with complete utilization (96 per cent ± 5 per cent) of the tartrate in all cases within 12 to 18 hours after the first visible signs of growth. After 3 weeks' incubation at 30 C, only one of the four cultures tested grew in the medium containing 4.0 per cent ammonium D-tartrate. The final pH value of the fermented medium varied from 7.5 to 8.5.

The major end products formed from D-tartrate by these bacteria were carbon dioxide, hydrogen, and acetic acid. Butyric acid was formed but in much smaller amounts than acetic acid. The molar ratio of acetic to butyric acid was approximately 10 to 1. The fact that acetic acid is the principal acid formed in the tartrate fermentation, rather than butyric acid as in the fermentation of glucose, can be explained by the more oxidized state of tartrate (Johnson *et al.*, 1931). The ratio of carbon dioxide to hydrogen was approximately 2.3 to 1. These ratios were similar for the four isolates tested. Small amounts of ethyl alcohol and pyruvic acid also were detected. Pyruvic acid was identified as the 2,4-dinitrophenylhydrazine derivative (Campbell, 1936; Osburn *et al.*, 1937). Succinic acid (Moyle, 1924) was not detected in any of the D-tartrate fermentations. Neither acetone, isopropyl alcohol, butanol, acetoin, nor 2,3 butanediol could be recovered or demonstrated by qualitative means.

The failure to find succinic acid as one of the end products of the D-tartrate fermentation by these bacteria gives additional support to the belief that previous investigators who reported both succinic and butyric acids as end products of the anaerobic decomposition of tartrate were using mixed cultures. It has been known since 1899 (Grimbert) that the coliform bacteria ferment D-tartrate with the production of succinic acid. (Also see Barker, 1936; Sakaguchi and Tada, 1940.)

Two facts of significance in establishing the mechanism of the D-tartrate fermentation by these bacteria were obtained from these experiments: (1) Small amounts of pyruvic acid were recovered and identified among the end products. (2) The 2.3 to 1 ratio of carbon dioxide to hydrogen gas was found to be almost constant. Proposed steps in the mechanism for the anaerobic decomposition of tartrate by these bacteria, supported by additional quantitative data, will be discussed in a later paper.

Utilization of Tartrate by Other Clostridia

To determine whether the ability to ferment tartrate is a common characteristic of strains of different species of the genus *Clostridium*, cultures were obtained from sources other than those of the tartrate-fermenting isolates. These included 54 pure cultures representing 25 species of both saccharolytic and proteolytic types. After checking for viability and purity of suspected cultures, they were grown in liver infusion broth and then tested. The test medium contained 0.5 per cent ammonium D-tartrate, 0.3 per cent yeast extract (Difco), 0.5 per cent tryptone (Difco), 0.1 per cent dibasic potassium phosphate, and 0.05 per cent sodium thioglycolate in distilled water. The medium was

adjusted to pH 6.8 with sodium hydroxide, tubed in 200-by-20-mm test tubes filled to two-thirds capacity, sterilized in the autoclave, and inoculated. The inoculum consisted of 10 ml of a 4-day-old liver infusion broth culture of the test organism. The inoculated tubes were incubated at 30 C for 2 weeks and then analyzed for the quantity of unfermented tartrate as compared with uninoculated controls.

With the exception of four strains classified as *Clostridium multifermentans* by Gililland and Vaughn (1943), which actively fermented the tartrate, none of the cultures even caused partial decomposition of the tartrate. On the basis of this experiment it may be concluded that the ability to utilize tartrate readily is not a property common to the majority of strains of the genus *Clostridium*, even of the saccharolytic group.

The Adaptive Character of the Tartrate Fermentation

The realization that the majority of strains of the genus *Clostridium* do not possess the ability to ferment D-tartrate prompted experiments on the adaptive character of that fermentation.

Demonstration of an adaptive enzyme system. Adaptive enzymes in contrast to constitutive enzymes are those enzymes whose production is markedly increased by the presence of a specific substrate. To determine the type of enzyme system involved in the tartrate fermentation an experiment was conducted with an isolate identified as *Clostridium beijerinckii*. The organism was grown in glucose and ammonium D-tartrate media, respectively. The basal medium contained 1.0 per cent tryptone (Difco), 0.5 per cent yeast extract (Difco), and 0.2 per cent dibasic potassium phosphate in distilled water. The glucose medium contained 2.0 per cent glucose. The tartrate medium contained 1.0 per cent ammonium D-tartrate. The two media were autoclaved in glass-stoppered pyrex bottles, then inoculated and filled to capacity with additional sterile solution. After incubation for 20 hours at 30 C the bacteria in approximately 200 ml of the medium were removed by centrifugation, washed once with M/30 Sorensen phosphate buffer (pH 6.8) containing 0.02 per cent sodium sulfide to ensure the reduced conditions necessary for maintaining the activity of the vegetative cells, resuspended in the buffer, and then used.

The experiment was conducted in Thunberg tubes. To one set of tubes was added the same volume of 1.5 per cent sodium potassium D-tartrate solution. Each tube containing substrate or control solutions then received 2 ml of cell suspension. The bacterial suspension was added carefully and in the presence of carbon dioxide, which was blown into the tubes to prevent undue access of air. The tubes were then evacuated. Control tubes for zero time data were heated to kill the vegetative cells, and the remainder incubated at 37 C without shaking. On the termination of incubation (90 minutes) the contents of the Thunberg tubes were heated to destroy the vegetative cells. The cells then were removed from the solutions by centrifugation. The supernatant liquids were decanted and analyzed for glucose and D-tartrate. The results are shown in table 2.

The results show the enzymes involved in the fermentation of D-tartrate to

be adaptive in nature. Only those cells which have grown in the presence of D-tartrate could ferment it. Since tartrate alone was added to the washed cell suspensions, the experiment also demonstrates that utilization of D-tartrate by these organisms involves dissimilation of this substrate alone. The presence of another organic compound in the medium is not necessary to initiate the breakdown. It also is of interest that the D-tartrate was utilized at a much faster rate than the glucose.

Adaptation of species of Clostridium to the utilization of tartrate. In view of the adaptive nature of the enzymes involved in the D-tartrate fermentation an effort was made to adapt several related species to utilize tartrate. The species selected were three strains each of *Clostridium butyricum* and *Clostridium beijerinckii* and one strain each of *Clostridium pasteurianum*, *Clostridium multifermentans*, *Clostridium acetobutylicum*, and *Clostridium felsineum*. Two methods were used in the attempt to train the cultures to utilize tartrate. In the first effort the bacteria were inoculated into a semisolid tryptone medium containing

TABLE 2

Utilization of glucose and tartrate by washed cells of Clostridium beijerinckii (strain O)

SUBSTRATE FOR PRODUCTION OF CELLS	SUBSTRATE FOR WASHED CELLS	SUBSTRATE		SUBSTRATE UTILIZED	SUBSTRATE UTILIZED PER ML
		Initial	After 90 min		
		mg/ml	*mg/ml*	*mg*	*micromols*
Glucose	Glucose	4.97	3.57	1.40	7.78
	D-Tartrate	2.96*	3.27	0	0
D-Tartrate	Glucose	4.97	4.05	0.92	5.11
	D-Tartrate	3.02	0.19	2.83	19.1

* As tartrate ion.

0.5 per cent calcium D-tartrate and 0.5 per cent glucose. By repeated transfer the cultures were kept in active fermentation in this medium for 1 month. Periodically transfers were taken from the glucose-tartrate medium and placed in tryptone medium with 0.5 per cent calcium D-tartrate as the only substrate. The cultures failed to ferment the tartrate. The amount of glucose in the glucose-tartrate medium was reduced to 0.25 per cent and the same procedure repeated. Again, none of the cultures could be trained to utilize the tartrate although the tartrate-fermenting controls always actively fermented the tartrate in the medium. The second method involved serial transfers of the cultures in 0.5 per cent ammonium D-tartrate, tryptone, yeast extract medium containing decreasing amounts of glucose (0.3, 0.2, 0.1, 0.05, and 0.00 per cent). This method also failed.

None of the species tested could be trained to utilize D-tartrate in whole or in part although the cultures were kept in constant contact with calcium or ammonium D-tartrate for 6 to 8 months. The addition of acetate to the tartrate media did not affect these results (see below).

Utilization of Other Organic Acids

At best the task of determining species allocation of the D-tartrate-fermenting anaerobes was not easy. It was hoped therefore that useful taxonomic data might be obtained by the determination of the ability of these isolates to decompose other organic acids. Fifteen D-tartrate-fermenting cultures were tested for their ability to utilize lactic, succinic, fumaric, L-malic, L-tartaric, malonic, and citric acids in three different semisolid media. The various acids were added to the three basal media containing varying amounts of complex nitrogenous materials, after which the media were adjusted to pH 7.1 with sodium hydroxide and sterilized in the autoclave. Only L-malate was utilized in all three media by 8 of the 15 strains tested. Since D-tartrate is related to D-malate in structural configuration it was thought that D-malate might be utilized by those strains that could not utilize the L-isomer. However, upon inoculation of six of the isolates that did not ferment L-malate into a tryptone, yeast extract medium containing DL-malate, no fermentation was detected after 1 week of incubation.

In a medium rich in complex nitrogenous material qualitative evidence was obtained for the slow fermentation of malonate and other dicarboxylic acids. These studies are being continued.

Clostridium lacto-acetophilum, a species recently described by Bhat and Barker (1947), requires the presence of acetate before it can ferment lactate. Although this anaerobe does not ferment D-tartrate, its other characteristics are similar to those of the 12 tartrate-fermenting isolates identified as *Clostridium butyricum*. It was desirable therefore to determine the effect of acetate on the ability of the tartrate-fermenting strains to utilize lactate. Eight strains were tested in two media. One contained lactate alone; the other contained lactate and acetate. The basal medium contained 0.5 per cent yeast extract (Difco), 0.1 gram dibasic potassium phosphate, 0.3 per cent agar, and traces of ferrous, manganous, and magnesium salts. Lactate and acetate were added in concentrations of 0.5 per cent. The media were sterilized and inoculated as previously described.

The results of the experiment were clear-cut and uniform. In the medium containing lactate alone the bacteria showed a slight activity, whereas in the medium with lactate plus acetate a vigorous fermentation was observed. This type of fermentation may be one possible explanation of the positive results obtained with malonate, as mentioned above. Acetate or some other compound that can perform the same function as acetate in the fermentation of lactate by these clostridia may be present in the medium.

The addition of acetate, however, did not influence the ability of the tartrate-fermenting cultures to decompose the other dicarboxylic acids. Those strains that failed to ferment succinate, fumarate, and L-malate alone did not ferment these substrates in the presence of acetate.

REMARKS ON SPECIES ALLOCATION

As previously stressed, the D-tartrate-fermenting cultures in general had characters that related them to *Clostridium butyricum*. Some of the strains

differed in their ability to utilize one or more carbohydrates. By following Spray (1948) in *Bergey's Manual of Determinative Bacteriology* it was possible to allocate all but two of the isolates to one of three species: *Clostridium butyricum*, *Clostridium beijerinckii*, or *Clostridium pasteurianum*. Two of the isolates differed significantly from other well-described species and could not be classified.

If the isolates had been similar enough to have been identified as belonging to one species, it would have been possible to name a new species or subspecies based on their unique ability to ferment D-tartrate. The cultures, however, could be allocated to several recognized species. Therefore, creation of a new species or subspecies is not justified, even for the purpose of serving to identify or emphasize tartrate-fermenting butyric acid bacteria of the type described. To do so would give unwarranted prominence to the tartrate-fermenting ability of the bacteria and lead directly to further confusion in the taxonomy and nomenclature of the saccharolytic species of *Clostridium*.

Enrichment cultures containing as yet untried substrates, when inoculated with material from proper sources, undoubtedly will yield many more types of clostridia than now recognized. If the ability to utilize these untried substrates were given undue importance, as for example could be given to D-tartrate fermentation, in order to classify all of the isolates, it is obvious that an infinite number of new species and varieties soon would be created.

When confronted with a similar type of problem with the coliform bacteria, Levine (1918) chose those few differential characters that careful statistical analysis had shown would give good correlative relationships. This method has resulted in a significant reduction in the number of differential tests necessary to identify the individual species. Perhaps a similar approach to the taxonomy of the saccharolytic bacteria of the genus *Clostridium* would reduce the present number of species and simplify the identification and classification of new isolates.

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SUMMARY

The characteristics of 23 D-tartrate-fermenting butyric anaerobes isolated from spoiled tartrates, tartrate recovery equipment, and vineyard soils are given. All but two isolates were tentatively identified as representing previously described species closely related to or identical with *Clostridium butyricum*.

The major end products of D-tartrate fermentation by representative cultures included acetic acid, butyric acid, carbon dioxide, and hydrogen. The molar ratio of acetic acid to butyric acid formed was approximately 10:1. Small amounts of ethyl alcohol and pyruvic acid were formed.

The enzymes involved in the decomposition of D-tartrate were shown to be adaptive in character. Attempts to adapt other cultures of the common saccharolytic species of *Clostridium* to the utilization of D-tartrate were unsuccessful. The ability to ferment D-tartrate is not general among the representatives of the genus *Clostridium*.

With the exception of L-malic acid, four carbon dicarboxylic acids other than D-tartaric were not readily attacked by the bacteria under the conditions of the experiments.

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