STRAIN VARIATIONS IN C. BIFERMENTANS[1]

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Clostridium bifermentans was first isolated and described by Tissier and Martelly (1902), and was generally accepted as a distinct and definite species until Hall (1922) observed that some bifermentans strains were motile, therein differing from the original description. Correlated with this difference in motility, he found certain other differences—in agglutination and in deep colony morphology. He proposed dividing this species into two species, retaining the name *C. bifermentans* for the non-motile strains, and designating the motile strains as *C. centrosporogenes.*

Kahn (1924), after an extensive comparative study of the anaerobes, recognized this designation, but suggested that perhaps it should be regarded as "a strain variant of *C. sporogenes* and that the cultural features exhibited by Hall's type are not sufficient to warrant a new specific name." Similarly Weinberg and Ginsbourg (1927), in a review of proposed new species, state that *C. centrosporogenes* is merely a particular strain (souche particulière) of *C. sporogenes.*

Sturges and Drake (1928), however, examining Hall's strains of *C. bifermentans* and *C. centrosporogenes* in sealed capillary tubes, found motility in all strains examined, and suggested that *C. centrosporogenes* was probably much more closely related to *C. bifermentans* than to *C. sporogenes.*

The proteolytic ability of *C. bifermentans* is another point on which the results of different investigators vary. Tissier and Martelly (1902), Hall (1922), Reddish and Rettger (1924) and

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Weinberg and Ginsbourg (1927) considered this species as strongly proteolytic. Kendall, Day, and Walker (1922) found moderate proteolysis, and Kahn (1924) reported that proteolysis was "definitely mild in type."

This reported variance in the proteolysis of *C. bifermentans* (which name in some cases undoubtedly included *C. centrosporogenes* since some of the results were reported before Hall's proposed division of the species) was readily accounted for when a preliminary investigation was made of the proteolytic ability of one of Hall's bifermentans and one of his centrosporogenes strains. The comparatively slow action of the bifermentans strain suggested that Hall's division of the group might be substantiated by another and a more definite criterion than aerobic motility tests.

It was further planned to study fermentation reactions, morphology, colony form, and other possible indications of a logical grouping. To show the similarities and dissimilarities of *C. sporogenes* to the *C. bifermentans* group, the former organism was included in all tests.

EXPERIMENTAL

Strains used

The strains used in this study were:

1. *C. bifermentans**.....Lister Institute
2. *C. bifermentans*.....Hall 50
3. *C. bifermentans*.....Hall 70
4. *C. bifermentans*.....Hall 258
5. *C. bifermentans*.....Hall 408 C
6. *C. centrosporogenes*.....Hall 76
7. *C. centrosporogenes*.....Hall 262
8. *C. centrosporogenes*.....Hall 472
9. *C. centrosporogenes*.....Hall 516

10 to 21 inclusive. Strains isolated in this laboratory:

C. sporogenes.....Hall 52
C. sporogenes.....Hall 54

* It should be noted that this strain was named before a division into bifermentans and centrosporogenes had been suggested.

Purity of these strains was insured by plating and by repeated examinations. In the experimental work, every culture was examined for contamination.

Morphology

The most consistent and convenient observations on morphology were made on a minced pork medium, buffered to pH 8.0 with sodium phosphate. Cultures were grown at 37°C., and Gram stains made. Measurements are given for a typical strain (no. 1) in table 1. The reason for a departure from the usual

TABLE 1

Measurements of strain 1

	WIDTH			LENGTH		
	Minimum	Majority	Maximum	Minimum	Majority	Maximum
	μ	μ	μ	μ	μ	μ
Vegetative rods:						
16 hours	0.5	0.7	1.1	1.3	2.5	7.0
40 hours	0.5	0.9	1.6	1.0	4.0	6.5
3 days	1.2	1.4	1.6	2.2	4.5	6.0
7 days	0.9	1.0	1.1	4.0	8.0	260.0
Sporangia:						
All ages	0.7	0.8	1.1	1.8	2.5	3.5
Free spores:						
All ages	0.6	0.8	0.9	1.0	1.4	2.2

custom of reporting measurements in a few figures is obvious. Almost all rods and sporangia are Gram-positive, regardless of age. Spores swell the rods slightly, but definitely, in most of the sporangia. Apparently about 95 per cent of the cells form spores.

While most strains rarely show more than three or four rods in a chain, strains 9, 12, 13 and 14 often have as many as twenty in a chain. In strains 9 and 14, these chains break up as the sporangia develop, but in strains 12 and 13 long chains of mature sporangia are seen. Strains 2, 3, 5, and 7 show little, if any, growth before twenty hours. Vegetative rods measure about the same as strain 1, but sporangia are larger, measuring 0.9 to 1.8μ by 2.5 to 5.0μ. Sporangia of strains 12 and 13 are inter-

mediate in width, this measurement varying from 1.2 to 1.4μ. Strain 8 is exceptional in that the spores rarely swell the rod.

C. sporogenes, under the same conditions, measures 0.7 to 0.9μ by 3 to 5μ at sixteen hours, and 0.5 to 0.7μ by 1 to 4μ at forty hours. After forty hours most of the cells become Gram-negative. Less than 25 per cent of the cells form spores. The spores swell the rods markedly.

Motility was demonstrated for all these 21 strains by examination in sealed capillary tubes. Examined under cover slips, young cultures of strains 1, 5, 6, and 8 to 21 usually, but not always, showed a few motile cells. Motility was but rarely demonstrated by this method for strains 2, 3, 4, and 7. *C. sporogenes* cultures usually contain a large number of motile cells, regardless of the method of examination or age of culture.

Colony form

Thompson (1926) claimed to be able to differentiate between *C. bifermentans* and *C. centrosporogenes* by the types of colonies formed on blood agar. Accordingly, dilution plates were poured, using a veal infusion agar (pH 7.5). To each plate, 0.5 cc. of fresh sterile defibrinated beef blood was added before the agar was poured. These plates were incubated in an atmosphere of hydrogen at 37°C. for forty-eight hours.

Under these conditions, deep colonies develop to a diameter of from 0.1 to 1.0 mm. Several types of colonies may be observed; ameboid masses, containing irregular granules of varying size and density; colonies of simple lens shape; dense lobate colonies appearing to be aggregates of 5 to 20 lens-shaped lobes; and irregular masses with small bud-like protuberances. Crowded plates show only the first type of colony. Edges of colonies may be either entire or irregular to filamentous.

A zone of hemolysis surrounds each colony. Strains 2, 3, 4 and 7 produce a narrow zone of hemolysis, while the other strains produce a comparatively wide zone—as wide or wider than the diameter of the colony. The hemolytic zone of *C. sporogenes* is several times as wide as the diameter of its colony.

Viscosity

Kahn (1924) mentioned a "mucoid-like deposit" in cultures of *C. centrosporogenes*. We have made similar observations in bouillon, gelatin, and meat media. Strain 1, alone, consistently shows this property. In general 2, 3, 4, 7, 12 and 13 usually show a minimum of ropiness or none at all, while the other strains usually show a considerable amount. *C. sporogenes* never produces any viscosity.

Correlated with this property of viscosity is the occurrence of a clear zone in the upper part of gelatin and broth cultures.

TABLE 2

Acid production from carbohydrates

	GLUCOSE	LEVULOSE	GALACTOSE	MANNOSE	MALTOSE	GLYCEROL	SORBITOL
Strains 1, 5, 8 to 12 inclusive..	+	+	0	+	+	+	+
Strains 2, 3, 4, 6 and 7.....	+	+	0	+	+	+	0
C. sporogenes 52 and 54.....	+	+	Slight	0	+	Slight	+

Fermentations

A basic medium containing 1 per cent peptone and 0.3 per cent beef extract was used. To this was added 1 per cent of the carbohydrate. The cultures were incubated three days in hydrogen at 37°C. The pH of the medium before inoculation was between 7.0 and 7.5. Inoculations were made from cultures grown for twenty-four hours in the basic medium. The carbohydrates fermented are shown in table 2. Gas production is variable in the bifermentans group except that it is almost always produced from glucose, maltose and mannose.

Protein metabolism

Observations of the digestive action of these strains on gelatin, coagulated egg white and coagulated blood serum media showed slow liquefaction of these substrates by strains 2, 3, 4 and 7. Digestion by the other strains was more rapid and more extensive. *C. sporogenes* liquefied these media rapidly after a short lag period.

Gelatin was selected as the most suitable medium for obtaining quantitative data for possible differentiation. Progress of proteolysis was measured in terms of electrical conductivity change in the medium. The relation between the production of ammonia, which is an end product of protein metabolism, and conductivity change has been discussed and a technique for the purpose described by Parsons and Sturges (1926).

TABLE 3

Conductivity change in gelatin

Expressed as reciprocal ohms $\times 10^8$

STRAIN	20 HOURS	44 HOURS	4 DAYS	11 DAYS	51 DAYS
1	3.2	7.0	11.7	14.9	18.3
2	1.1	2.1	4.1	9.2	14.9
3	0.4	1.1	3.1	7.3	13.3
4	0.5	1.5	3.7	7.5	11.0
5	3.4	7.9	11.5	15.2	19.4
6	2.5	5.3	10.6	16.8	20.1
7	1.2	3.1	5.4	9.8	12.2
8	2.3	6.3	10.6	14.0	18.8
9	2.2	5.1	8.8	13.9	21.4
10	3.3	5.5	7.4	12.0	18.4
11	2.6	6.6	11.1	15.1	19.4
12	1.9	3.6	5.5	8.9	18.5
13	1.9	3.5	5.3	8.6	20.0
14	2.2	4.4	7.9	13.1	22.3
15	2.5	4.5	8.2	15.1	19.6
16	2.0	4.4	8.1	12.9	18.7
17	1.8	3.5	6.4	12.0	20.4
18	1.8	3.6	6.6	10.6	20.8
19	2.0	5.2	10.1	13.7	20.4
20	3.8	8.9	14.4	16.8	21.8
21	2.9	5.6	10.2	13.4	17.3
C. sporogenes 52	0.2	13.4	20.0	24.4	28.0
C. sporogenes 54	0.5	12.2	18.6	21.9	26.2

Nutrient gelatin was inoculated from twenty-four-hour cultures of each strain, and incubated in an atmosphere of hydrogen at 37°C. Resistances of cultures were read at twenty hours, forty-four hours, four days, eleven days, and fifty-one days.

Ten tubes were inoculated with each strain, and duplicate tubes were used at each interval. Readings were made at 30°C.

From the resistance, conductivity was computed, and the similarly determined conductivity of the uninoculated controls was subtracted. Conductivity change is expressed in reciprocal ohms $\times 10^3$. For the sake of brevity and clarity, and since the readings on duplicate tubes showed but few deviations of more than 2 per cent from the average, only the averages are reported in table 3.

DISCUSSION

The data on morphology show several points of difference between *C. sporogenes* and all the other strains. The former is more persistent in its motility; its spores swell the rods more; vegetative rods of *C. sporogenes* cultures become smaller as the cultures mature, while rods in cultures of the bifermentans group become larger; the majority of the rods in *C. sporogenes* cultures lose their property of retaining the Gram stain, decrease in size and die without sporulating, while most of the organisms in cultures of the bifermentans group produce spores.

Several possible groupings of the 21 strains are suggested by the data. Number 8 differs in the fact that its spore does not swell the rod. Strains 9, 12, 13 and 14 show more chain formation, 12 and 13 being more persistent in this respect. Strains 2, 3, 4 and 7 differ in the slowness of their growth, the sensitiveness of their motility, and the width of their sporangia. Strains 12 and 13 are intermediate with regard to sporangium width.

As to colony formation, no consistent differences are observed, except in the ratio of the diameter of the colony to the width of the zone of hemolysis. *C. sporogenes* is distinctly more hemolytic. Strains 2, 3, 4 and 7 are feebly hemolytic. The other strains are intermediate.

The production of viscosity again distinguishes *C. sporogenes* from the bifermentans group. It also gives an indication, although an undependable one, of a differentiation between strains 2, 3, 4, 7, 12 and 13, and the remainder of the group.

The fermentation of mannose furnishes a definite and precise

differentiation of *C. sporogenes* from the bifermentans group. The reactions in galactose and glycerol, although less definite, appear to be constant. The fermentation of sorbitol demarcates strains 2, 3, 4, 6 and 7 from the remainder of this group.

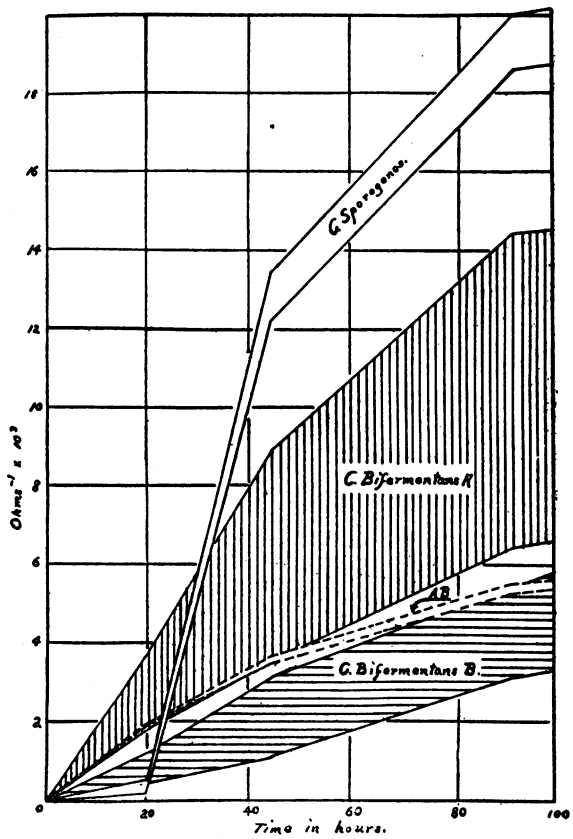

FIG. 1. RANGE OF CONDUCTIVITY CHANGE IN GELATIN, ALL STRAINS—EARLY AGES

The data on digestion of gelatin in table 3 suggest no basis for a division of the bifermentans strains. However, in figures 1 and 2 we have divided these strains into groups on the basis of previously described differences in morphology, hemolysis, viscosity and macroscopic observations of liquefaction of proteins.

The highest and the lowest values (conductivity change) for each day in each group are plotted as points. The areas between the lines connecting these points will be referred to as zones. The zoning arrived at in this manner gives some support to these other differentiations as shown by the separation of the zone designated *A* from that designated *B*.

Strains 12 and 13, however, exhibit an intermediate type of activity as was the case with their other properties. The course

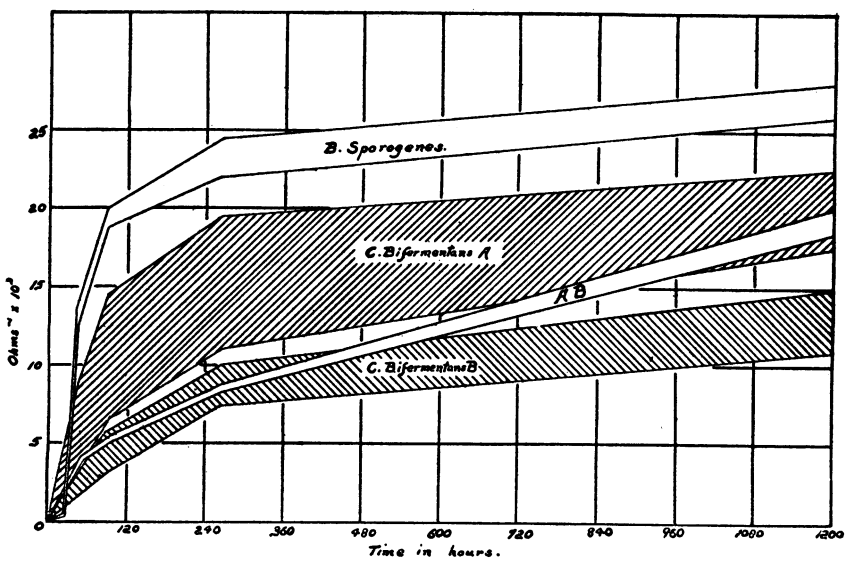

FIG. 2. RANGE OF CONDUCTIVITY CHANGE—OLD AGES

of their gelatin digestion is represented by the narrow zone, *AB*, which for the first forty-five hours lies within zone *A*, then deflects to enter zone *B* at about ninety hours, emerging again (see fig. 2) and reëntering zone *A*. If, therefore, these two cultures should be included either in Group A or in Group B, the reconstructed zones would show a considerable overlapping and any such differentiation would be impossible. Similarly, a grouping made according to the fermentation of sorbitol would give widely overlapping zones.

The difference between the bifermentans group and *C. sporogenes*, in gelatinolytic activity, is so clearly shown in the charts as scarcely to need comment. The latter shows a lag period extending for twenty hours, followed by a period of maximum activity, during which proteolysis, as measured by ammonia accumulation, proceeds to a point never attained by members of the bifermentans group.

TABLE 4

Grouping of bifermentans strains according to type of reaction

DIFFERENTIAL CHARACTERS	STRAINS, SOURCES AND PREVIOUS DESIGNATION			
	1 (Lister) "Bifermentans" 5 (Hall) "Bifermentans" 8 (Hall) "Centrosporogenes" 9 (Hall) "Centrosporogenes" 10–21 Authors' Strains	6 (Hall) "Centrosporogenes"	12 Authors' Strains 13 Authors' Strains	2 (Hall) "Bifermentans" 3 (Hall) "Bifermentans" 4 (Hall) "Bifermentans" 7 (Hall) "Centrosporogenes"
Rapidity of growth.....	A	A	A	B
Sensitiveness of motility....	A	A	A	B
Sporangium width.....	A	A	AB	B
Extent of hemolysis.....	A	A	A	B
Viscosity.....	A	A	B	B
Clear zone in gelatin.....	A	A	B	B
Fermentation of sorbitol..	A	B	A	B
Rate of digestion.....	A	A	AB	B

Consideration of these charts will readily show the possibilities of erroneous conclusions when observations of one or two strains are used as a basis for differentiation of anaerobic species. For instance, there are strains of *C. bifermentans* whose position lies nearer to the *C. sporogenes* zone than to the *B* zone.

Another important point illustrated by these charts is the possibility of fallacies arising from observations limited to one or two ages. Thus the conductivity readings for *C. sporogenes* indicate consecutively: first, coincidence with the less proteolytic strains of *C. bifermentans*; then, coincidence with the more pro-

teolytic strains; and finally, a distinct disparity from both. The disagreement as to the extent of proteolysis caused by *C. bifermentans* may possibly be explained in this way.

Each of the characters studied indicates a greater difference between *C. sporogenes* and the bifermentans group than between the individual members of the group. Correlation of the differentiations within the group indicates two types differing in morphology, hemolysis, viscosity, sorbitol fermentation and proteolysis. The reaction common to strains 1, 5, 8, 9, 10, 11, and 14 to 21 inclusive are designated in table 4 as Type A reactions, and the reactions common to strains 2, 3, 4 and 7 as Type B reactions. Strain 6 has Type A reactions except on sorbitol. Strains 12 and 13 have some Type A, some Type B, and some intermediate (AB) reactions.

While the reactions designated as Type A and Type B represent real differences, none of these differences, except sorbitol fermentation, are both sufficiently great and sufficiently constant to be recommended for general classification. Furthermore, the fact that there are cultures giving, in some characters, Type A reactions, and in other characters, Type B or intermediate reactions, militates against using these reactions to divide the species.

Finally, it is to be noted that Hall's differential designation of his eight strains could not be substantiated by differences in any one of the characters studied.

SUMMARY AND CONCLUSIONS

With the assumption that *C. bifermentans* and *C. centrosporogenes* constituted a somewhat unified group, a study was made of all available strains (twenty-one) belonging to this group.

Since no consistent differences were found between the strains labelled *C. bifermentans* and the strains labelled *C. centrosporogenes*, the use of the latter name would seem unjustifiable.

The variations observed suggest another and a more logical grouping, but the nature of the variations and the existence of intermediate strains necessitate all of the strains being considered as a single species.

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