STUDIES ON ANAEROBIC BACTERIA

IX. ANTIGENIC RELATIONS OF CLOSTRIDIUM BIFERMENTANS AND CLOSTRIDIUM CENTROSPOROGENES[1]

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The systematic position, or right to specific rank, of the anaerobic bacillus, *Clostridium centrosporogenes*, has been somewhat insecure, since its conception as *Bacillus centrosporogenes* by Hall in 1922. Originally created to cover 4 strains resembling *Clostridium bifermentans* in many respects, the new species was recorded as actively motile at 8 hours, though non-motile at 24 hours. Deep colonies of *C. centrosporogenes* in meat infusion agar were described as more fluffy and diffuse, but this character was recognized as affected by consistency of the agar. Failure of *C. centrosporogenes* to react in an agglutinative serum for one strain of *C. bifermentans* was also cited. These last points of difference were considered minor and only motility was used in separation of *C. bifermentans* and *C. centrosporogenes* in Hall's key to anaerobes. Robertson (1929) questioned the specific rank of *C. centrosporogenes*, if separation from *C. bifermentans* were to rest upon motility alone.

In considering the position of the new species Hall recognized its physiological likeness to *Clostridium sporogenes* but decided against close affinity because of the central position of its spores and its positive glycerol fermentation. Nevertheless, Kahn (1924) and Weinberg and Ginsbourg (1927) suggested that the new culture was merely a variant of *C. sporogenes*. This possibility was considered by Drake and Sturges (1929) in a study of

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21 strains of the *bifermentans* group (including certain strains labelled *centrosporogenes*), but they were able to distinguish the entire group from *C. sporogenes* by dimensions of the cells, activity in motility, Gram reaction, hemolysis, fermentation of mannose, conductivity in gelatin media, etc. Differentiation within the *bifermentans*:*centrosporogenes* group by these characters was not justified in their opinion, nor were they able to confirm the separation on the appearance of colonies on blood agar, suggested by Thompson (1926). Various other publications, Kahn (1925); Hall and Whitehead (1927); Quick and Kahn (1929); and Valley (1929), have listed both organisms but, since they were not primarily concerned with species problems, need not be considered here.

Bergey's Manual (1923, 1925, 1930, and 1934) has recognized the two species with separation based mainly upon motility. The same was true of the tentative key of Spray presented in 1933; this, however, was changed in the 1934 key and the two names listed as synonyms, the species being considered as *Clostridium bifermentans* and motile. The Medical Research Committee (1919) and Lehmann-Neumann-Breed (1931) recognized only *Bacillus bifermentans* and that as non-motile. However, Zeissler and Rassfeld (1929) listed *Bacillus putrificus-tenuis* (considered synonymous with *B. bifermentans*) as possessing peritrichiate flagella.

It appears then that the point of most contention is whether *C. bifermentans* is motile or non-motile. Tissier and Martelly (1902) first described it as non-motile but gave no conditions of the test. Hall's acceptance in 1922 and redescription of it as a non-motile organism was apparently based on the fact that his 3 strains had never, to his knowledge, shown motility and were, in fact, non-motile under conditions allowing motility of certain other strains, which as we have seen, he designated *B. centrosporogenes*. It is significant, however, that one of his motile strains had previously been accepted by many as *B. bifermentans*, being the strain No. 506 of the Lister Institute, originally contributed by M. Robertson. The possibility of motility in *C. bifermentans* seems not to have been questioned

again until Sturges and Drake (1928) attempted to sort out their collection of strains of that general group, in comparison with certain type strains of each species furnished by Hall. They noted in some cases a rapid loss of motility during examination and therefore used the capillary tube technique for the protection of delicate anaerobes against aeration during the test of motility. In this way they were able to show motility in Hall's *B. bifermentans* strains as well as his *B. centrosporogenes.* Flagella stains made from young cultures also revealed peritrichiate flagella on cells of both "species", very convincing evidence of which is their excellent plate showing flagella on Hall's "non-motile" Strain 50 of *B. bifermentans.* Zeissler (1929) also recorded flagellation and motility of certain strains of *B. bifermentans* in the Lister and Pasteur Institutes, although in English and French literature the same strains are reported as non-motile. Glathe and Cunningham (1933) agreed that the Lister strains No. 506 and No. 2914 (the latter originally Hall's strain 50) are motile, as are also 8 strains which they isolated from farmyard manure. They are inclined to emend the Tissier-Martelly description of *B. bifermentans* to admit motile strains.

The only point at issue then is the reported inagglutinability of *C. centrosporogenes* in a *C. bifermentans* serum and of *C. bifermentans* in one of *C. centrosporogenes* (Hall, 1922). No reaction was found at 1:20 dilution, though no detail of the test is given. Titers of the sera were low, 1:160 and 1:2000, and only 3 strains each were tested. With the more complete analysis of agglutinogens now possible by the technique of flagellar and somatic agglutination, there is reason to retest the cross agglutination and so to put to still another test the identity of the species.

CULTURES AND METHODS USED

Nine cultures were available, several of which are, in fact, strains cited in the papers mentioned above. (See table 1.) The numbers and grouping of the last 7 cultures are those of Drake and Sturges (1929). After the routine testing of the cultures as to purity, observations were made upon their motility by the capillary tube method of Sturges and Drake. In 12-

hour cultures in liver-infusion broth motility was observed in all cases, Sturges' Nos. 2 and 3 and Lister 506 being least active. On the basis therefore of these observations and with regard for the key cultures, historically speaking, the following were chosen for production of sera: Lister *bifermentans* 506, Hall's *centrosporogenes* 76, and Sturges' *bifermentans* 2 (= Hall's 50).

Antigens for injection and later tests were produced as surface growth on liver extract agar of pH 7.6, enriched with 0.1 per

TABLE 1

Cultures used

NAME AS RECEIVED	SOURCE	INITIAL INFORMATION
B. bifermentans Strain 506	Lister Institute	Motile at 8 hours; not at 24 hours. Hall, 1922
B. centrosporogenes Hall strain 76	American Type Culture	One of the original strains of the species; motile. Hall, 1922
C. bifermentans Strain 2 = Hall's Strain 50 Strain 3 = Hall's Strain 70	W. S. Sturges	Hall considered non-motile. Flagella and motility demonstrated by Sturges and Drake, 1928
Strain 5 = Hall's Centro—408 C Strain 8 = Hall's Centro—472	W. S. Sturges	Motility easily demonstrated by Hall, and by Sturges and Drake
Strain 12 Sturges Strain 13 Sturges	W. S. Sturges	Motility easily demonstrated but "other characters intermediate between above groups"
Strain 6 = Hall's Centro 76	W. S. Sturges	Motility easily demonstrated. Sorbitol fermented

cent glucose and 0.5 per cent bacto tryptone. Three-day cultures yielded a moderate crop of cells, purely vegetative, which were collected with one washing by centrifugation.

As in previous work of this kind (McCoy and McClung, 1935) sera were produced by injection of living cells (so-called whole-cell or W condition) and by injection of cells heat-killed by 2 hours' steaming (so-called somatic or O condition). Dosage and intervals of injection were substantially as in the former paper. Collection of sera and their preservation by refrigeration alone

were also similar, but the details of setting up and incubating for the macroscopic tube tests with these organisms were changed somewhat from our former practice. Incubation at 54°C. for 6 to 8 hours, followed by refrigeration overnight was used after comparison of tests run under various other conditions, completeness of flocculation with sharp end point at titer being considered. Some advantage in stability of the test antigen through the long incubation was found in the use of 0.4 per cent instead of the usual 0.85 per cent saline. The technique for absorption of agglutinins was essentially that of our former publications.

EXPERIMENTAL RESULTS

Preliminary tests of the cross reactions of all 9 cultures showed definite reaction in both W and O sera (tables 2 and 3). Flocculi were delicate and in most cases one would hesitate to claim presence of an H (labilotropic) factor upon appearance of flocculation alone. As more convincing demonstration of the respective O (stabilotropic) and H elements in the antigen and their corresponding agglutinins in the sera, a series of absorption tests was undertaken. For simplicity of presentation, results will be given for only *bifermentans* 506, *centrosporogenes* 76, and *bifermentans* 2, for which complete reciprocal crosses can be given. It should be emphasized, however, that in every test, whether of direct agglutination, absorption, or test after absorption, the additional 6 organisms were used in one-way cross reactions. In no case was there reason to question their behavior, nor to suppose that among them was any which would not fall within the behavior of the 3 strains, whose reactions are completely known.

Distribution of the H factor among the three strains. In analysis of agglutinogens by the H-O technique, the proof of presence and identity of the H factor between strains of a species is first to be considered. Indeed in the case of *bifermentans*:*centrosporogenes* proof of possession of the same H factor by both organisms is of peculiar interest because of the supposed relation of H antigen to motility. Analyzed for H or so-called flagellar factor the agglutinative relations of the chosen strains are set

out in table 4. Our *bifermentans* 506 and *centrosporogenes* 76 undoubtedly possess the same H antigen, as shown by ability: to stimulate production of H agglutinins in the animal body, to absorb such agglutinins from either serum, and to react *in vitro* with delicate but typical H flocculation in a purified H antiserum

TABLE 2

Cross agglutination of 9 strains of C. centrosporogenes: C. bifermentans in W sera*

TEST ANTIGEN	W SERA FOR		
	Centr 76	Bi 506	Bi 2
	W antigen		
Centr 76	**2560**	1280	5120
Bi 506	1280	**2560**	10240
Bi 2	2560	640	**10240**
Bi 3	1280	1280	5120
Bi 5	1280	2560	5120
Bi 6	5120	5120	10240
Bi 8	1280	2560	10240
Bi 12	2560	320	5120
Bi 13	1280	640	10240
	O antigen		
Centr 76	5120	5120	2560
Bi 506	5120	5120	5120
Bi 2	2560	5120	5120
Bi 3	1280	5120	2560
Bi 5	2560	1280	5120
Bi 6	2560	5120	5120
Bi 8	1280	2560	2560
Bi 12	1280	2560	2560
Bi 13	1280	2560	2560

Homologous titers are set in boldfaced type.

* Whole-cell.

(*i.e.*, a W serum absorbed to virtually complete removal of O agglutinins). The *bifermentans* 2 of Sturges, which it will be remembered is the so-called non-motile Strain 50 of Hall, fulfills the first two requirements, thus demonstrating its H-agglutinogenic and H-absorptive capacity. Its ability to flocculate in purified H antiserum is variable: slight in sera of *centrosporogenes*

76 and *bifermentans* 506 to considerable in the homologous serum, all O-absorbed to −40 homologous titer. Perhaps this difference corresponds to chance variation in degree of motility of the particular cultures taken for antigen, though no records were kept on this point. Another factor may be the greater potency of the *bifermentans* 2 serum, in this particular case. At any rate what difference there may be between *bifermentans* 2 and *centrosporogenes* 76 and *bifermentans* 506 would seem quantitative rather than qualitative. Incidentally, other cases of such H variation were found: Strain 3 of Sturges (= Hall's 70) behaved

TABLE 3

Cross agglutination of 9 strains of C. centrosporogenes: C. bifermentans in O sera*

O antigen

TEST ANTIGEN	O SERA FOR		
	Centr 76	Bi 506	Bi 2
Centr 76	**20480**	10240	2560
Bi 506	10240	**10240**	5120
Bi 2	5120	10240	**2560**
Bi 3	5120	2560	5120
Bi 5	5120	5120	2560
Bi 6	5120	2560	2560
Bi 8	10240	5120	5120
Bi 12	5120	5120	2560
Bi 13	5120	5120	2560

Homologous titers are set in boldfaced type.

* Somatic.

as Strain 2 and Strains 12 and 13 slightly so, in the evidence of variable H development in different batches of antigen, as shown by flocculation.

Somatic agglutination. Possession of a common somatic antigen by the 3 strains is shown clearly in table 5, in which complete reciprocal absorption of somatic agglutinins is evident. And it is further indicated that only one somatic factor is involved; note the virtually complete removal in all cases, whether by homologous or heterologous absorptions. One may assume therefore complete identity of the somatic antigen, not simply the presence of a group somatic factor, as has been reported

TABLE 4

Tests of presence and identity of H factor in C. centrosporogenes 76, C. bifermentans 506 and C. bifermentans 2 by reciprocal absorption experiments

SERUM (W)	ABSORBING AGENT	TEST ANTIGEN (W)	TITER	
			W absorption (Before → after)	O absorption (Before → after)
Centr 76	Centr 76	Centr 76	2560 → −80*	2560 → 2560
		Bi 506	1280 → −80	1280 → 1280
		Bi 2	2560 → −80	2560 → −160
	Bi 506	Centr 76	2560 → −80	2560 → 2560
		Bi 506	1280 → −80	1280 → 1280
		Bi 2	2560 → −80	2560 → −160
	Bi 2	Centr 76	2560 → −80	2560 → 1280
		Bi 506	1280 → −80	1280 → 2560
		Bi 2	2560 → −80	2560 → −160
Bi 506	Centr 76	Centr 76	1280 → −80	1280 → 640
		Bi 506	2560 → −80	2560 → 5120
		Bi 2	640 → −80	640 → 160
	Bi 506	Centr 76	1280 → −80	1280 → 2560
		Bi 506	2560 → −80	2560 → 1280
		Bi 2	640 → −80	640 → 320
	Bi 2	Centr 76	1280 → −80	1280 → 1280
		Bi 506	2560 → −80	2560 → 640
		Bi 2	640 → −80	640 → 320
Bi 2	Centr 76	Centr 76	5120 → −80	5120 → 10240
		Bi 506	10240 → −80	10240 → 5120
		Bi 2	10240 → −80	10240 → 5120
	Bi 506	Centr 76	5120 → −80	5120 → 10240
		Bi 506	10240 → −80	10240 → 5120
		Bi 2	10240 → −80	10240 → 1280
	Bi 2	Centr 76	5120 → −80	5120 → 5120
		Bi 506	10240 → −80	10240 → 5120
		Bi 2	10240 → −80	10240 → 5120

* −80 or −160 indicates no agglutination at these dilutions, the lowest tested in the particular series.

TABLE 5

Proof of identity of somatic antigen by reciprocal absorptions between C. centrosporogenes 76, C. bifermentans 506, and C. bifermentans 2

SERUM (O)	ABSORBING AGENT	TEST ANTIGEN (O)	TITER	
			Before absorption	After absorption
Centr 76	Centr 76	Centr 76	20480	−40*
		Bi 506	10240	−40
		Bi 2	5120	−40
	Bi 506	Centr 76	20480	−40
		Bi 506	10240	−40
		Bi 2	5120	−40
	Bi 2	Centr 76	20480	−40
		Bi 506	10240	−40
		Bi 2	5120	−40
Bi 506	Centr 76	Centr 76	10240	−80
		Bi 506	10240	−80
		Bi 2	10240	−80
	Bi 506	Centr 76	10240	−80
		Bi 506	10240	−80
		Bi 2	10240	−80
	Bi 2	Centr 76	10240	−80
		Bi 506	10240	−80
		Bi 2	10240	−80
Bi 2	Centr 76	Centr 76	2560	−40
		Bi 506	5120	−40
		Bi 2	2560	−40
	Bi 506	Centr 76	2560	−40
		Bi 506	5120	−40
		Bi 2	2560	−40
	Bi 2	Centr 76	2560	−40
		Bi 506	5120	−40
		Bi 2	2560	−40

* −40 or −80 indicates no agglutination at these dilutions, the lowest tested in the particular series.

occasionally. Cross reaction to titer, and as often to one tube beyond homologous titer (*e.g.*, in the cross tests of *centrosporogenes* 76 and *bifermentans* 506 *before* absorption) would also suggest identity rather than group reaction involving a somatic factor.

It will be remembered that in tables 2 and 3 there was presented evidence of somatic reaction in the direct cross tests between all strains studied. In those tests also the reaction occurred in relatively high dilution, approaching or exceeding homologous titer in many cases. This point is worthy of emphasis, because it is sometimes found that in a mixed H-O serum produced for a motile organism, the O or somatic agglutinins are subordinate (Savage and White, 1925). It is suggested in such cases that the H or flagellar antigen is species-specific and the O often group-specific. In other cases, of completely non-motile organisms, such specificity as there may be in the agglutination reaction lies within somatic factors. Specificity of antigens, however, may be well developed; indeed in groups like the streptococci, extreme strain specificity may be found (Andrewes and Christie, 1932). The condition of *bifermentans*: *centrosporogenes* strains is in effect intermediate. H factors are present as we have seen, but they are not dominant and easily differentiated in direct cross tests. This condition presents an interesting transition of antigenic specificity, inasmuch as by their weak and variable motility the organisms also are indeterminate.

C. bifermentans versus C. centrosporogenes

As to the species relation of the 9 strains studied, there seems no question. The differences reported are quantitative rather than qualitative and are, in fact, no greater than have been met in previous studies of strains within a species. In the present case, the evidence of the presence of H factor, though in less amount, in certain of the *C. bifermentans* strains is particularly interesting, since it correlates well with their weaker motility. Thus, the serological data are in good accord with Drake and Sturges' conclusion that there is no consistent nor exclusive

difference between the strains labelled *C. bifermentans* and *C. centrosporogenes*, and that there is therefore no valid reason for recognizing *C. centrosporogenes*. Further, the evidence of H factor in *C. bifermentans* supports their observation that the species is motile, and with this emendation the original description is valid and sufficient.

SUMMARY

Identity of both H and O factors in the antigenic complex of *Clostridium centrosporogenes* 76, *Clostridium bifermentans* 506, and *Clostridium bifermentans* 2 has been shown by reciprocal agglutinin absorption experiments. Tests of 6 other strains in these sera show every probability of their having the same agglutinogens, although the evidence is incomplete.

Presence of the H factor in *Clostridium bifermentans* is consistent with its motility and presence of flagella, as was shown by Sturges and Drake in 1928.

This serological evidence, coupled with the failure of Drake and Sturges and others to find valid morphological and physiological differences between strains of so-called *Clostridium bifermentans* and *Clostridium centrosporogenes*, would indicate that the concept of a single species, *Clostridium bifermentans*, is adequate.

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