THE FUSOBACTERIUM GENUS

I. Biochemical and Serological Classification

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Fusiform bacilli are normally present in human mouths and throats, and on the external genitalia; also in the oral cavities of various species of animals. They have been observed, furthermore, in a variety of human pathological conditions. In spite of their frequent occurrence in nature, there has been little progress in the classification of these organisms since Leiner (1907) first observed that there was more than one cultural type. This situation may be ascribed largely to the difficulty of obtaining satisfactory growth upon solid media. Most of the attempts at classification have been limited to a very large extent to morphological observations. Furthermore, it has been held generally that all spindle-shaped organsism are members of the fusiform genus. The resulting confusion is evidenced by the fact that the relevant literature contains many contradictory reports concerning staining properties, motility, oxygen requirements and biochemical reactions.

The first systematic study was that of Krumwiede and Pratt (1913), who divided fifteen strains into two groups, upon their ability to ferment sucrose. Knorr (1922) established three morphological types among his strains, as did Smith (1932), by the use of the dark-field illumination technique. In 1927 Varney published a detailed description of eighteen cultures from normal and abnormal mouths. Four types were estab-

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lished, two by serological methods, and two by morphological studies. Biochemical observations were not reported.

Slanetz and Rettger (1933) made a significant advance in the study of the fusobacteria by devising a potato medium upon which abundant growth could be obtained. A morphological and biochemical study of fifty-three strains revealed four types. Types I and II were indistinguishable biochemically, both fermenting glucose and producing indol, but were separated by colony appearance and cell size. Type III was larger than I and II, and fermented sucrose as well as glucose. Indol formation was irregular. Type IV was a large bacillus which fermented lactose as well as glucose and sucrose, but did not form indol. The agglutination test was considered as unsuitable for the purpose of classification. Quite recently Bachmann and Gregor (1936) divided nine strains into two groups by means of agglutination and complement fixation tests. No biochemical reactions were reported.

Unfortunately, it is not possible to compare the types proposed by the various investigators since, in no instance, has one group of cultures been successfully studied by both biochemical and serological methods. The present report deals with a morphological, cultural, biochemical and serological study of eighty strains originating from a variety of sources. All of the cultures were Gram-negative, non-motile, non-sporulating and, when isolated, strictly anaerobic.

METHODS

Media employed. Slanetz and Rettger found gentian violet to be a valuable aid in primary isolation, on account of its favorable selective action. The medium which proved most satisfactory during the present investigation was the same as that of Slanetz and Rettger, except that it contained glucose and cysteine. It has the following composition:

Proteose peptone	1.0 per cent
Liebig's meat extract	0.3 per cent
Cysteine hydrochloride	0.05 per cent
Glucose	0.1 per cent

Potato extract (aqueous)	10.0	per cent
Agar	2.0	per cent
Distilled water		q.s.

Adjusted to pH 7.6

For making primary isolations gentian violet was added in a final concentration of 1:20,000.

Stock cultures were carried in a semi-solid agar (0.3 per cent) having the above composition, aside from the agar. For routine purposes the same medium without the agar was used. All media were sterilized at 15 pounds pressure for 15 minutes, cooled quickly, and inoculated immediately, when this was possible. When media were held overnight they were stored in an anaerobic jar.

Method of isolation. Swabs containing the original material were streaked on the gentian violet agar. When the inoculum came from human mouths, members of the *Fusobacterium* genus were usually obtained directly in pure culture. Material from animal sources, however, frequently contained other organisms to such an extent as to overgrow the more delicate fusiform bacilli; this made it necessary to dilute the original material. In making isolations from cases of pulmonary infection precaution was exercised to avoid contamination with mouth flora. Sputum as such was diluted with two volumes of sterile saline solution and centrifuged lightly. After removing the supernatant fluid, the washed residue was re-suspended in an equal volume of saline solution, shaken vigorously and whirled at high speed for ten minutes. The supernatant liquid yielded *Fusobacterium*, frequently accompanied by minute cocci.

The gentian violet agar plates were incubated anaerobically for two days at 37°C., at the end of which period the *Fusobacterium* colonies could be recognized by their deep violet color and characteristic ground-glass appearance when viewed with the hand lens. Subcultures from these colonies were usually pure.

Anaerobic technique. The method for securing strictly anaerobic conditions was that described by Weiss and Spaulding (1937). The procedure consists in evacuating a glass jar (Hempel desiccator) which is connected with a system containing a Cenco Hyvac pump, mercury manometer and hydrogen tank. A small

flat procelain dish containing a catalyst (shredded palladinized asbestos) is placed conveniently in each jar, the ground surfaces of which are smeared lightly with cello-seal. When the jar has been evacuated to the negative pressure desired, the pump is disconnected by means of a three-way stopcock. Hydrogen is then passed through the system and into the jar until atmospheric pressure is restored. If the addition of a small amount of carbon

TABLE 1

Sources of strains

	TYPES
Human:	
Normal mouths	H_1–H_{27}, and H_{9_a}, H_{12_a}
Carious teeth	H_{28}–H_{30}
Fuso-spirochetal infections (mouth and throat)	VA_1–VA_{10}
Lung abscess	LA_1–LA_4, LA_7, LA_{12}, LA_{14}–LA_{16}
Lung abscess with empyema	LA_5, LA_6, LA_8–LA_{10}
Bronchiectasis	LA_{11}, LA_{13}
Vagina:	
Normal	V_1, V_2, V_5
Cervical laceration	V_3
Purulent discharge	V_4
Animal:	
Chimpanzee:	
Normal mouth	Ch_1
Chronic lung infection	CH_2
Monkey mouths (normal)	M_1–M_4
Dog mouths (normal)	D_1–D_5
Rabbit mouths (normal)	R_1–R_3
Guinea pig mouths (normal)	GP_1–GP_3

dioxide is desired, this may be accomplished before introducing the hydrogen, by attaching the carbon dioxide tank to the system.

Sources of strains

Thirty-two isolations were obtained from so-called normal mouths of twenty-two persons, including two from carious teeth. Ten cultures originated in various cases of mouth infection; for two of these (VA_1 and VA_2) the writers are indebted to Dr. Ruth Tunnicliff. Sixteen "lung abscess" cultures were derived from

various pulmonary infections. Four of these strains (LA_1 through LA_4) were kindly supplied by Dr. Charles Weiss. Five vaginal isolations were made from both normal and pathological conditions. Seventeen cultures were obtained from the mouths and throats of animals (see table 1).

FREQUENCY AND DISTRIBUTION

Fusobacterium colonies appeared on all of the plates streaked with swabs from human mouths. They were less frequently obtained from the throats of animals. All of the swabs from pulmonary as well as mouth and throat infections yielded positive cultures. The material from three out of five normal human vaginae also contained fusobacteria; on the other hand, thirty swabs obtained during pregnancy were negative. With one exception, washings from the pregnancy cases were distinctly acid, ranging from pH 4.4 to 6.6. Repeated attempts to obtain isolations from human and animal feces were unsuccessful.

CLASSIFICATION OF STRAINS

Fermentation studies

The ability of some of the fusiform bacilli to ferment sucrose and lactose, while others lacked this property, suggested to us that a more comprehensive investigation of the fermentative properties might reveal further differential tests. Therefore, the eighty strains were studied for their ability to produce acid and gas from eighteen fermentable substances. In addition to the common mono- and disaccharides, this list included cellobiose, trehalose, raffinose, inulin, soluble starch, xylose, rhamnose, salicin, glycerol, mannitol, sorbitol and inositol.

Two methods were employed for determining the carbohydrolytic activity. In the first, the test materials were added aseptically to the basal potato broth medium from autoclaved aqueous solutions of the test substances; in the second, the test solutions were sterilized by filtration. The second procedure is preferable. In each instance the inoculum consisted of 0.1 cc. of a twenty-four-hour potato broth culture. Durham fermentation tubes may be used, since the anaerobic jar holding the tubes

need not be evacuated to the point where the liquid is displaced from the inverted tubes.

The basal medium for the fermentation tests consisted of 1.0 per cent proteose peptone, 0.3 per cent Liebig's meat extract and 0.1 per cent cysteine hydrochloride. Excellent growth occurred in this medium when the carbohydrate present was utilized. The medium must be freshly prepared, however, before use; indeed, satisfactory results cannot be obtained unless the inoculations are made immediately after sterilization.

Sulphonaphthalein indicators could not be added directly to the medium, since they were occasionally destroyed by the organisms. The pH determinations were made by a spot-plate procedure. After twenty-four hours, three days and seven days incubation at 37°C., a bi-convex loopful of the broth culture was added to approximately 0.5 cc. of distilled water containing one drop of a 0.16 per cent solution of the proper indicator. White porcelain spot-plates furnished an excellent background. Although tedious, this method possesses the important advantage of permitting determinations beyond the range of a single indicator. Since the primary division of the authors' cultures is based upon differences in the final pH, this method plays an important rôle in the classification scheme proposed in this report.

Two distinct types of fermentative reactions were observed. One set of cultures (group I) was mildly saccharolytic and produced a relatively weak acidity, the final pH averaging 6.2. The remaining cultures (group II) gave rise to a much greater final acidity (pH 4.6), and in addition were active toward a wider range of carbohydrates. The separation of the two groups on this basis was distinct.

Glucose and levulose were fermented by all of the strains. None of the cultures produced acid from xylose, rhamnose, glycerol, inulin, mannitol, sorbitol and inositol. Galactose was attacked by some members of both groups but with only slight acidity. Maltose and trehalose were acidified by group II, but not by group I. The ability to ferment these two substances constitutes in itself a valuable differential test for the two groups.

Group I is divided into two types. The strains fermenting only glucose and levulose are designated as belonging to type I A; the remaining cultures, all capable of attacking sucrose, constitute type I B. Group II, although it contains no less than nine fermentative patterns, is separated only into a lactose-negative type, II A, and a lactose-positive, type IIB.

TABLE 2

Summary of fermentative types

Final pH in 1.0 per cent carbohydrate cysteine broth

TYPE	NUMBER OF STRAINS	GLUCOSE, LEVULOSE	SUCROSE	MALTOSE	TREHALOSE	SALICIN	CELLOBIOSE	LACTOSE	RAFFINOSE	SOLUBLE STARCH
					Group I					
I A	29	ac	0	0	0	0	0	0	0	0
I B	19	ac	ac	0	0	0	0	0	0	0
					Intermediate					
	2	AC	AC	0	0	0	0	0	0	0
	3	AC	AC	AC	AC	0 or AC	0 or AC	0	0	0
					Group II					
II A	10	AC	AC	AC	AC	0 or AC	0 or AC	0	0	0
II B	1	AC	AC	AC	AC	0	0	AC	0	0
	16	AC	AC	AC	AC	AC	AC	AC	0 or AC	0 or AC

ac indicates final pH between 6.0 and 6.5; AC indicates final pH between 4.4 and 5.2; 0 indicates no detectable acidity.

Two cultures in group I (LA_8 and LA_{10}) and three in group II (H_6, H_9 and H_{14}) proved to be atypical in that they possessed some of the biochemical characteristics of the opposite groups. These five cultures were placed together as an intermediate group.

The amount of titratable acidity produced by group II in 1.0 per cent carbohydrate media was found to be approximately twice that of group I, suggesting that group II is able to utilize an intermediate product of carbohydrate metabolism not attacked

by group I. Using the Thunberg technique, as modified by Kendall and Ishikawa (1929), the ability of the two groups to activate pyruvic, acetic and succinic acids was determined. Unfortunately, none of these substances proved of differential value, since pyruvic acid was activated by both groups, and acetic and succinic acids by neither group.

Biochemical studies

Indol. With two exceptions, the group I cultures produced indol, as shown by the Böhme-Ehrlich technique. Group II was uniformly negative by this test substance. The potato extract broth medium, without glucose and cysteine, was the most suitable medium.

Hydrogen sulfide. Group I formed large amounts of hydrogen sulfide, in the absence of cysteine, while group II blackened lead acetate only in the presence of this agent. Glucose was omitted from the medium. In gelatin or semi-solid agar containing cysteine, the hydrogen-sulfide-positive strains produced a turbid zone at the surface of the medium after a few hours' exposure to the air. This opaque layer contained elemental sulfur.

Nitrate reduction. Group I cultures, in general, reduced nitrates, with the liberation of ammonia. Group II, on the other hand, was uniformly negative for nitrite after five days' incubation. The usual naphthylamine-sulphanilic acid test was used for the detection of nitrite, the Thomas test for ammonia production, and zinc dust (ZoBell, 1932) for residual nitrate. The results were most satisfactory in the absence of added glucose and cysteine.

Hemolysis. None of our strains was hemolytic for cow's blood, but group I colonies were sometimes surrounded by a greenish area after twenty-four hours' exposure to the air. This "viridans" effect was found to be associated with the production of hydrogen sulfide.

Liquefaction of gelatin. Gelatin was never liquefied.

Serological studies

Serological methods have been of comparatively little value in the classification of the fusobacteria. Both Pratt (1927) and

Slanetz and Rettger (1933) emphasized the antigenic individuality of their strains. On the other hand, Varney (1927) established two of his four types by means of the agglutination test, and recently (1936) Bachmann and Gregor distinguished two groups by serological methods.

Agglutination tests. Relatively large doses of living organisms were employed for the production of antisera. Twenty-hour agar plate cultures were suspended in physiological salt solution and adjusted to an approximate turbidity of 10, McFarland scale. Five injections, increasing from 1.0 to 5.0 cc., were given at two-day intervals. Only cultures forming a homogeneous suspension were used. After five days following the last injection the titre was usually at the maximum (1:10,000 to 1:20,000). Ten antisera were prepared; however, one serum showed a high prozone reaction with every antigen tested and was discarded. Three of the strains were lethal for rabbits, necessitating the use of formalized antigens.

Performance of successful agglutination tests with most of the *Fusobacterium* strains was accomplished with considerable difficulty, since there was a strong tendency toward spontaneous agglutination. Cells obtained from agar cultures exhibited less non-specific agglutination than did broth cultures. Relatively stable antigens were finally obtained, however, by using twenty-hour meat infusion potato agar cultures suspended in alkaline (pH 8.0) formalized M/20 solution of $Na_2HPO_4 \cdot 12H_2O$. Readings were made after three hours' incubation at 45°C., and after twenty-four hours' at 37°C. Extreme care was necessary in interpreting the results. Upon standing, many of the larger morphological types (group II) settled out in a manner entirely distinct from auto-agglutination. Viewed casually, this sedimentation bore a striking resemblance to flagellar agglutination, but when the tubes were shaken again a homogeneous suspension resulted. True agglutination of the large group II cells resulted in loose flocculation, while group I underwent typical granular clumping. The discovery that a few of the strains were serum-sensitive necessitated the use of a normal serum control. This phenomenon did not appear to be associated with the presence

of rough variants. Stock antigens stored for several months in the ice box were still satisfactory.

The eighty antigens were tested against the nine antisera; representative reactions are presented in table 3. The strain specificity reported by previous investigators is evident from the sample results shown. It becomes apparent that a classification of the genus based solely upon the simple agglutination test is open to criticism, certainly unless an appreciable number of antisera of each type is employed.

Viewing the agglutination results in a general way, group I is definitely separated from group II, thus confirming the biochemi-

TABLE 3

Agglutination reactions

ANTIGEN		ANTISERUM									
		Group I					Group II				Control
		Type A				Type B	Type A		Type B		
Strain	Type	H_1	Ch_2	Ch_1	LA_4	H_{12}	H_4	H_{21}	LA_9	GP_1	
H_{30}	I A	640	1,280	1,280	320	160	40	0	0	0	0
VA_6	I A	160	10,240	10,240	20	2,560	40	40	0	0	0
H_{22}	I B	160	160	0	160	5,120	0	0	0	0	0
LA_1	I B	320	160	80	0	320	0	0	0	0	0
H_{11}	II A	1,280	40	320	0	40	10,240	0	0	0	0
VA_3	II A	160	160	20	0	0	2,560	0	0	0	0
H_{29}	II B	0	80	40	20	0	640	20	10,240	160	0
LA_{13}	II B	0	20	0	0	0	40	0	2,560	0	0

cal differentiation. The intermediate group is divided, four strains showing antigenic relationship to group I, and one to group II. There is some evidence that group II is composed of two distinct serological types corresponding to types II A and II B. Many of the type II B strains, however, possessed agglutinogens for the entire group. The antigenic composition of both groups was investigated further by means of agglutinin absorption and precipitation tests (see table 4).

Agglutinin-absorption tests. Reciprocal absorption tests confirmed the presence of group specific antigens. The high degree of strain specificity, however, made interpretation of the results

difficult. Attempts to recognize the presence of definite antigenic types in group I were unsuccessful; in fact, sufficient data were obtained to justify the conclusion that this group possesses a complex antigenic structure. Group II, on the other hand, was divided, as suggested by the simple agglutination tests, into two sub-groups corresponding to types II A and II B. There was a further indication that type II B strains contain agglutinogens for both types II A and II B.

Precipitin tests. The successful use of the precipitation technique by Lancefield (1933, 1934) for typing the streptococci, and by Julianelle and Wieghard (1935) for the classification of the

TABLE 4

Summary of classification scheme

	BIOCHEMICAL								SEROLOGICAL			
	Fermentation of			Final pH in carbohydrate broth	Production of		Reduction of		Agglutination by antisera against			
									Group I		Group II	
	Sucrose	Maltose, Trehalose	Lactose		Indol	H_2S	NO_3	NO_2	A	B	A	B
Group I, type A.....	−	−	−	6.2	+	+	+	+	+	+	−	−
Group I, type B.....	+	−	−	6.3	+	+	+	+	+	+	−	−
Intermediate.....	+	±	−	4.8–5.1	+	+	±	±	±	±	±	−
Group II, type A.....	+	+	−	4.7	−	−	−	−	−	−	+	−
Group II, type B.....	+	+	+	4.6	−	−	−	−	−	−	±	+

± indicates both positive and negative reactions within the group.

staphylococci, suggested the desirability of applying this technique to the fusobacteria. The method of Lancefield in which crude extracts are employed was at first closely followed. When most of these antigens failed to produce any precipitation whatsoever, larger volumes of cells were used, but still with negative results. Believing that much of the desired antigen might have been lost in separating the cells from the medium in which they were grown, cell-free broth antigens were prepared, but again with unsatisfactory results. Finally, the bacterial growths on solid media were washed off and boiled for one hour in M/20 HCl. A study of twenty extracts prepared in this manner re-

sulted in some confusion. The group II antigens reacted only with homologous antisera, but the group I extracts failed to react with any of the antisera. Whereas group II had been divided previously into the two types by the agglutination tests, it appeared by this method to be homogeneous. Five of the antisera were entirely unsatisfactory as precipitating agents. Similar (negative) results were obtained in attempts to isolate a precipitable substance.

The biochemical and serological bases of classification receive some support from the morphological and cultural studies conducted by us. However, since the last two must be regarded as having somewhat limited taxonomic value in themselves, and because they constitute a major part of the work on growth requirements and variation, they are presented in the second of the two papers which constitute this series.

RELATION BETWEEN SOURCE AND TYPE

Most investigators have failed to note a correlation between types and sources of strains. On the other hand, Slanetz and Rettger isolated their types II and III from pathological conditions only. In the present study it is noteworthy that all but three of the animal strains belonged to group I.

DISCUSSION

If it were not for the presence of the intermediate group, the question might well be raised as to whether groups I and II actually belong to the same genus. Fortunately, the five intermediate strains possess properties characteristic of both groups. As a result, it is possible to trace a gradual transition from the weakly saccharolytic and highly nitrogen-active strains of group I (type A) to the highly fermentative members of group II (type B), which are relatively indifferent to the presence of nitrogen compounds.

The occurrence of essentially different fermentation processes was substantiated further by potentiometric oxidation-reduction determinations made in this laboratory by Dr. R. W. H. Gil-

lespie.[2] The potential time curves clearly demonstrated that the two groups may be distinguished by this method.

The primary separation into two groups was made, not upon the ability of different strains to attack different carbohydrates, but rather upon the widely different final acidities which they produced. The fact that the other biochemical tests, the serological reactions and the oxidation-reduction studies, support this division appears to us to be sufficient ground for proposing that groups I and II be designated as separate species of the genus, *Fusobacterium*.

The division of group I into two types by the fermentation of sucrose is not confirmed by the agglutination tests. Instead, the group appears to possess a complex antigenic structure. Although no evidence could be obtained that these serological subgroups are significant, a receptor-analysis study would in all probability yield more definite information.

The separation of group II into two types upon the basis of lactose fermentation was confirmed to some extent by the agglutination reactions. If the agglutinin-absorption results have any value, it is in the suggestion that types II A and II B are probably distinct entities. On the other hand, the precipitin tests failed to distinguish these two types.

Morphological and cultural characteristics may also serve as differential criteria for the groups, but since their taxonomic value is somewhat limited in the system employed here, they will be discussed separately in the second report of this series.

A comparison of the proposed classification with that of Slanetz and Rettger may be summarized as follows: Group I, type A, comprises Slanetz and Rettger's types I and II; group I, type B, contains a portion of their type III; group II, type A, contains the remainder of their type III; group II, type B, corresponds to their type IV.

CONCLUSIONS

1. Eighty strains of *Fusobacterium* were divided into two main groups by their biochemical and serological behavior.

[2] We are indebted to Dr. Gillespie for permission to make use here of these unpublished data.

2. Group II is distinguished from group I by (*a*) its ability to produce approximately twice as great an acidity from carbohydrates as the latter, (*b*) its property of fermenting maltose and trehalose, and (*c*) its inability to form indol, produce hydrogen sulfide and reduce nitrates.

3. Each of the two groups is made up of two important fermentative sub-groups, or types.

4. A method for preparing satisfactory agglutination antigens is described.

5. The precipitin technique failed to yield reliable results.

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