The Relationships of Bacteria Within the Family Bacteroidaceae as shown by Numerical Taxonomy

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SUMMARY

A comparison, by numerical taxonomy methods, was made of 72 named or freshly isolated strains of Gram-negative anaerobes which were considered to belong within the family Bacteroidaceae. In the first analysis 57 strains were compared. Representative strains were then selected and compared with additional named strains in a second analysis. Four phena were identified: (1) strains of *Sphaerophorus*, *Fusobacterium* and *Bacteroides melaninogenicus*, (2) *Bacteroides*, (3) unnamed poultry isolates, (4) a second group of unnamed poultry isolates. The most useful differential tests were found to be: (a) cell morphology, (b) the terminal pH value in glucose broth and the production of formic, acetic, propionic or butyric acids, (c) the production of propionic acid from threonine, (d) growth stimulation by bile, (e) the effect of various inhibitors.

INTRODUCTION

Difficulties in the identification and classification of the anaerobic non-sporing Gram-negative bacteria were first encountered in studies of the intestinal flora of chickens, when a group of organisms was isolated which bore no resemblance to any named strains (Barnes & Goldberg, 1962; Goldberg, Barnes & Charles, 1964). It was decided to use the methods of numerical taxonomy in an attempt to assign these organisms to a particular genus and at the same time to eliminate some of the confusion which exists within the family Bacteroidaceae. At present it is difficult even to attach particular characteristics to named organisms since several different classifications are in use (Prévot, 1938; *Bergey's Manual* 1957; Beerens, Castel & Fievez, 1962), and, owing to the difficulties of maintaining these organisms as freeze-dried cultures, there has been little interchange of isolates between workers of different countries.

The tests used in this investigation were based on the more important characters described in *Bergey's Manual* (7th ed. 1957) together with those tests which were considered important by Beerens, Schaffner, Guillaume & Castel (1963). It was hoped to obtain further information on the relationships of various named strains to each other, and also to select key tests for identification purposes, by using strains from as many sources as possible in a combination of tests.

METHODS

Sources of organisms. The origin of the strains used in the two computer analyses is given in Table 1.

Media and cultural conditions. Details of the media used and conditions of culture have already been described by Goldberg *et al.* (1964) and Barnes, Impey & Goldberg (1966). The incubation temperature was 37° unless otherwise stated.

Table 1. *The origin of the strains used in the two computer analyses*

Source	Computer analysis
From the National Collection of Type Cultures (NCTC)	
<i>Bacteroides necrophorus</i> 7155	1
<i>B. fragilis</i> 9343	1 and 2
<i>B. melaninogenicus</i> 9337	2
From the American Type Culture Collection (ATCC)	
<i>Bacteroides vulgatus</i> 8482, <i>B. ovatus</i> 8483, <i>B. thetaiotaomicron</i> 8492, <i>B. distasonis</i> 8503, <i>B. symbiosis</i> 12829, <i>Sphaerophorus varius</i> 8501, <i>S. necrophorus</i> 12290, <i>Fusobacterium polymorphum</i> 10953	1 and 2
From Dr H. Beerens (Institut Pasteur, Lille)	
<i>Fusiformis biacutus</i> 25, <i>Eggerthella clostridiformis</i> 22, 61, 353, 354, <i>Ristella variabilis</i> 285	1
<i>Ristella convexa</i> 265, <i>R. incommunis</i> 308, <i>R. variabilis</i> 264, 266, 267, <i>Sphaerophorus necrophorus</i> , <i>S. funduliformis</i> , <i>S. freundii</i>	1 and 2
<i>Sphaerophorus necrophorus</i> Fievez N117, N167, N252, <i>Fusiformis fusiformis</i> 389	2
From Dr T. Mitsuoka (Animal Physiology Laboratory, The Institute of Physical and Chemical Research, Tokyo, Japan): isolated from chicken caeca	
Strains AIII-45, AU23-33, CH32-17, CH32-20	1
CH36-9, N5-43, N209-12, N210-25, N212-47	
Strain AU21-27	
From Dr S. M. Finegold (Dept. of Medicine, Wadsworth Veterans Hospital, Los Angeles, California)	
<i>Bacteroides melaninogenicus</i> B477, B536, B537	2
From Dr R. J. Gibbons (Forsyth Dental Centre, Boston)	
<i>Fusobacterium polymorphum</i> , <i>Fusobacterium</i> JF5	2
Isolated by the authors	
From human faeces—strains 9/1 and 12/1	1
From chicken caeca—strains belonging to Groups 1, 2, 3 and 4 of Barnes & Goldberg (1965)	1 and 2
Group 1, strain EBF 59/96P	1 and 2
Group 2, strains EBF 59/91P, 95P	1
Group 3, strains EBF 61/31B, 66, 67, 68 and 69	1
Group 3, strain EBF 61/60B	1 and 2
Group 4, strains EBF 59/78, 85, 92P, 100, EBF 60/26, EBF 61/30, 63	1
Group 4, strains EBF 58/74, EBF 59/72, EBF 61/42, 56, 61	1 and 2
From duck caeca—strains EBD 1/1B, 3B and 4A	2
From turkey carcass—strains EBT 2/61 and 68	2

For *Bacteroides melaninogenicus* strains all the media were supplemented with menadione 0.5 µg./ml. and laked blood 5% (v/v) which was prepared by repeated freezing and thawing.

Morphology. Examinations for Gram reaction, size, shape and appearance, were carried out using 18–20 hr. cultures in Reinforced Clostridial Medium (RCM) of (Hirsch & Grinsted, 1954). Colonies on RCM agar (Hirsch & Grinsted, 1954) or blood agar (Beerens *et al.* 1963) were examined after 2 or 3 days incubation in a 90% hydrogen and 10% carbon dioxide atmosphere.

Lecithinase. Lecithinase production was determined using the method of Fievez (1963). The egg-yolk plates were examined daily up to 5 days.

Table 2. *Characters examined*

Test	Features scored
Morphology	
Gram reaction	Negative or variable
Shape of cells	Rods, beaded rods or filaments
Arrangement	Single rods, diplobacilli, or chains
Length	Short (0.2–0.59 μ), medium (0.6–3.0 μ), long (over 3 μ)
Diameter	Slender (0.2–0.9 μ), stout (1.0–2.0 μ)
Colonies on RCM agar	
Size	Small (2 mm or less), large (over 2 mm)
Edge	Entire or uneven
Surface	Raised or convex
Colour	White, grey or black
Appearance	Opaque or translucent
Colonies on blood agar	
Haemolysis	Negative or β haemolysis
Growth in RCM broth	
20 °C	Negative or positive
45 °C	Negative or positive
Turbidity	Flocculent or uniform
Gas production	Copious or negative
Glucose fermentation	
Terminal pH	pH 5.5 and below or above pH 5.5
Volatile fatty acids produced	
Formic, acetic, propionic or butyric acids	Positive when present at > 1 μ M/ml.
Fermentation of:	
Sucrose, cellobiose, galactose, arabinose, xylose, mannitol, inositol, salicin, and glycerol	Negative or acid produced
Cysteine milk	No change, acid, or acid and clot
Lecithinase production	Negative or positive
Gelatin liquefaction	Negative or positive
Indole production	Negative or positive
Hydrogen sulphide production	Negative, weakly positive, strongly positive
Propionic acid from threonine	Negative or positive
Growth stimulation by 10 % bile	Negative or positive
Growth in the presence of	
Brilliant green 1/100,000, penicillin 10 μ g./ml., neomycin 25 μ g./ml., polymyxin B 10 μ g./ml., and chlor-tetracycline 10 μ g./ml.	Negative or positive
Growth stimulation by menadione	Negative or positive

Biochemical tests. For carbohydrate fermentation, H₂S production, indole, etc., additions were made to the basal medium of Beerens (1953–54) as described by Goldberg *et al.* (1964).

Detection of volatile fatty acids. The volatile fatty acids produced from the fermentation of glucose were determined by the method of Guillaume, Beerens & Osteux (1956) as modified by Charles & Barrett (1963).

Bile Stimulation. Stimulation of growth was determined by adding 10 % bile to the Basal Glucose Phosphate (BGP) medium of Barnes & Goldberg (1962).

Effect of inhibitors. Growth in the presence of brilliant green 1/100,000 (v/v),

penicillin 10 µg./ml., neomycin 25 µg./ml., polymyxin B 10 µg./ml., or chlortetracycline 10 µg./ml. was tested by adding the required concentration of each inhibitor to RCM broth.

Theonine test. The production of propionic acid from threonine was detected following the method of Beerens (personal communication). This test was later modified by Beerens & Tahon-Castel (1965). The strains were examined using both techniques.

Characters. The organisms were examined for the characters given in Table 2.

Computer analysis. Both the computer analyses were carried out by Mr J. C. Gower (Rothamstead Experimental Station, Harpenden, Hertfordshire) using the similarity coefficient defined by Gower (1967) and a single linkage sorting programme (Sneath, 1957). The coefficient of similarity used allows for the inclusion of quantitative and multivalued qualitative characters as well as dichotomies for which negative matches do not contribute to the similarity coefficient. A detailed discussion of the choice of coding has been given by Thornley (1967).

In both analyses four tests were recorded as dichotomies, these were the production of formic, acetic, propionic or butyric acids, whilst hydrogen sulphide production was scored quantitatively (Table 2). All of the remaining tests were recorded as alternatives where equal weighting was given to the positive or negative answer. In the first analysis 83 and in the second 86 features were analysed.

RESULTS

Preliminary computer analysis. In the original computer analysis the main aim was to try and relate the chicken isolates to named strains. As they were intestinal in origin it was considered probable that they would relate more closely to *Bacteroides* than to the fusobacteria. It was also evident that morphologically they bore little resemblance to *Fusobacterium* species. Hence most of the named strains used for comparative purposes were either *Bacteroides* (*Ristella* in some classifications) or strains designated *Eggerthella* (Beerens *et al.* 1963). Only two strains of fusobacteria were included.

The 57 strains tested are listed in Table 1, whilst the characters analysed are given in Table 2. All the characters were used with the exception of haemolysis, lecithinase production and growth stimulation by menadione (as *Bacteroides melaninogenicus* strains were omitted from the first analysis). All of the strains were obligate anaerobes and inhibited by 10 µg./ml. chlortetracycline, none fermented glycerol or hydrolysed gelatin. These properties were therefore omitted from the analysis.

The similarity matrix is shown in Fig. 1. Five phenons were defined which contained 50 of the strains examined.

(1) *Eggerthella clostridiformis*. The four strains received from Beerens were identical with *Bacteroides necrophorus* strain NCTC 7155. Although at the time of analysis these strains were not considered to form spores, it was subsequently shown that all of these five strains produced spores after prolonged incubation on RCM agar (but not on the VL agar of Beerens). They were therefore excluded from further consideration.

(2) *Bacteroides*. This phenon included the ATCC strains, those of Beerens and *Bacteroides fragilis* strain NCTC 9343, together with 8 of the chicken isolates from Mitsuoka. *Sphaerophorus necrophorus* ATCC 12290 also had a high similarity to the *Bacteroides* strains.

(3) *Sphaerophorus*. The strain *S. varius* ATCC 8501 was closely related to three strains obtained from Beerens. One of the chicken isolates 59/96P also grouped with these strains although having a much lower similarity value.

(4) *Chicken isolates*. Group 4. Fourteen of the strains previously designated group 4 (Barnes & Goldberg, 1965) had a low similarity value to either *Bacteroides* or *Sphaerophorus*. A full description of these organisms was given by Goldberg, Barnes & Charles (1964) and the computer results justify their caution in not assigning the organisms to a particular genus.

(5) *Chicken isolates*. Group 3. A further group of 6 chicken isolates had the lowest similarity to all the other groups. They were originally designated group 3 by Barnes & Goldberg (1965). They have since been shown to be Gram-positive in very young cultures and their position within the family Bacteroidaceae is thus in doubt.

The only two fusobacteria, *Fusobacterium polymorphum* and *F. biacutus*, showed little similarity to each other. *Fusobacterium biacutus* has since been shown to produce spores (Dr H. Beerens, personal communication) and should therefore be eliminated from the family Bacteroidaceae.

Further analysis of representative strains

The results from the first analysis were sufficiently encouraging that the computer analysis was repeated to include additional named strains. In particular, three further strains of *Sphaerophorus necrophorus* were included as the strains originally obtained from Dr Beerens were atypical in certain characters. Two further strains of *Fusobacterium* were also tested but it was difficult to obtain representative strains of these organisms as they tend not to be maintained in stock culture collections. Four strains of *Bacteroides melaninogenicus* were added, the requirement for menadione being scored as a separate character. Representative chicken isolates were included, together with a few freshly isolated strains from ducks and turkeys.

Two further tests were used which had been considered important in the differentiation of the pathogenic strains of *Sphaerophorus* (Fievez, 1963). These were lecithinase production and haemolysis.

In all 40 strains were analysed; of these 25 strains had been used in the first analysis. The additional 15 strains are listed above. The similarity matrix is shown in Fig. 2. It is evident that the additional strains and the extra tests used widened the differences between the main groups of organisms shown in Fig. 1. Four phenons were now evident:

(1) Containing *Sphaerophorus*, *Fusobacterium* and *Bacteroides melaninogenicus* together with 3 poultry isolates.

(2) *Bacteroides*, together with the representative chicken isolate of Mitsuoka.

(3) The chicken isolates belonging to Group 4 (Barnes & Goldberg, 1965).

(4) The most representative chicken isolate belonging to Group 3 (Barnes & Goldberg, 1965) together with freshly isolated turkey and duck strains.

The family relationships are shown in the dendrogram Fig. 3, whilst the characteristics of the organisms are given in Tables 3 and 4.

In considering these results the discussion will be confined mainly to representative strains obtained from the Type Culture Collections and other workers. The poultry isolates will be discussed in detail elsewhere (Barnes & Impey, to be published).

Phenon 1: Sphaerophorus, Fusobacterium and Bacteroides melaninogenicus. The main characters which were common to *Sphaerophorus* and *Fusobacterium* and which

Fig. 2. Similarity matrix of 40 strains, 25 of which were also used in the first analysis.

differentiated these organisms from *Bacteroides* were the production of propionic acid from threonine, the high terminal pH in glucose broth, the production of butyric acid in glucose broth and the failure to produce acid from most of the other carbohydrates tested. All the strains tested grew in the presence of brilliant green 1/100,000.

Fig. 3. Taxonomic hierarchy of strains of *Sphaerophorus*, *Fusobacterium*, *Bacteroides* and unnamed poultry isolates.

The strains of *Sphaerophorus* differed morphologically from those of *Fusobacterium*. They also produced copious gas, and generally produced propionic acid as well as acetic and butyric acids from glucose. They were inhibited by polymyxin 10 µg./ml. They produced lecithinase and grew at 20°.

The *Fusobacterium* spp. were characterized by forming long rods or filaments with

Table 3. *Some of the more important differential characters within the family Bacteroidaceae*

Glucose fermentation												
	No. of strains*	Description of cells	Terminal pH		Volatile fatty acids produced				Propionic acid from threonine	Copious gas production	Stimulation by bile	Stimulation by menadione
			4.6 to 5.5	5.6 to 6.2	Formic	Acetic	Propionic	Butyric				
<i>Sphaerophorus</i> spp.	4	Medium, slender rods, round ends, single, or filaments	—	+	—	+	+	(3)†	+	+	—	—
<i>S. necrophorus</i> , Fievez strains	3	Long slender rods, round ends, and filaments	—	+	+	+	+	+	+	+	—	—
<i>Fusobacterium</i> spp.	4	Long slender rods and filaments with pointed ends	—	+	+	+	(3)	—	+	—	—	—
<i>B. melaninogenicus</i>	4	Medium slender rods with rounded ends; single	—	+	+	+	+	— (3)	—	—	—	+
<i>Bacteroides</i> spp.	12	Single, short, medium or long rods, generally slender, rounded ends	+	—	—	(7)	+	— (8)	—	—	+	(9)
Chicken isolates, group 4	5	Single, very large, stout rods, rounded ends	+	—	—	—	+	+	— (3)	—	—	—
Chicken isolates, group 3	4	Medium-to-long slender rods and beaded chains, pointed or rounded ends	+	(3)	—	(3)	—	—	+	(3)	—	—

* Strains EBD 1/1 B, 4 A, EBF 59/96 P and *B. symbiosis* ATCC 12829 have not been included in this Table.

† Parentheses denote number of strains with that particular reaction.

Table 4. Other characters of the anaerobic non-sporing Gram-negative rods

No. of strains*	β -haemolysis	Production of:			Growth at:		Behaviour in cysteine milk†	Growth in presence of:				Acid from:							
		Lecithinase	Indole	H ₂ S‡	20°	45°		Brilliant Green 1/100,000	Polymyxin 10 μ g./ml.	Neomycin 25 μ g./ml.	Penicillin 10 μ g./ml.	Sucrose	Cellobiose	Galactose	Arabinose	Xylose	Mannitol	Inositol	Salicin
<i>Sphaerophorus</i> spp. 4	-	+	+	+	+	+	NC	+	-	+	-	+	+	+	+	+	+	+	+
<i>S. necrophorus</i>	+	+	+	+	+	+	NC	+	+	-	+	+	+	+	+	+	+	+	+
Fievez strains	-	-	+	+	-	-	NC	+	-	+	+	+	+	+	+	+	+	+	+
<i>Fusobacterium</i> spp. 4	-	-	+	+	+	+	NC	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. melaninogenicus</i>	N.T.‖	N.T.	+	+	+	+	NC	-	+	+	+	+	+	+	+	+	+	+	+
<i>Bacteroides</i> spp. 12	+	-	-	+	+	+	NC	-	+	+	+	+	+	+	+	+	+	+	+
Chicken isolates	+	+	+	+	+	+	A + C(4)	-	+	+	+	+	+	+	+	+	+	+	+
Group 4							NC(3)												
Group 3	+	-	-	+	+	+	A + C(4)	+	+	+	+	+	+	+	+	+	+	+	+

* Strains EBD 1/1 B, 4 A, EBF 59/66p and *B. symbiosis* ATCC 12829 have not been included in this Table.† H₂S: + + +, strongly positive; +, positive; -, negative.

‡ NC, no change; A, acid; A + C, acid and clot.

§ Parentheses denote number of strains with that particular reaction.

|| N.T., not tested.

pointed ends; they were non-haemolytic; did not produce lecithinase; did not produce propionic acid from glucose.

The strains of *Bacteroides melaninogenicus* were related more closely to the *Sphaerophorus* strains than to *Bacteroides*. Their resemblance was based partly on their morphology, the production of formic, acetic and butyric acids in glucose broths and their failure to ferment sucrose, cellobiose, arabinose, xylose, mannitol, inositol and salicin. They differed from *Sphaerophorus* and *Fusobacterium* in their failure to produce propionic acid from threonine, inhibition by brilliant green 1/100,000, and growth in the presence of polymyxin 10 µg./ml. In these latter three characters they resembled *Bacteroides*.

Phenon 2: Bacteroides. Morphologically the *Bacteroides* strains could not be differentiated from *Sphaerophorus*. The group was differentiated by failure to produce propionic acid from threonine, fermentation of glucose with a low terminal pH (4.6–5.5), the failure to produce butyric acid. The organisms grew in the presence of polymyxin 10 µg./ml. and generally fermented a wider range of carbohydrates. Many but not all of the strains were stimulated by bile.

Phenon 3: Chicken isolates (group 4). These organisms were characterised by their large size and shape, the production of acetic and propionic acids in glucose broth and the fermentation of a wide range of carbohydrates, in particular mannitol. They showed a low similarity both to *Sphaerophorus* and *Bacteroides*.

Phenon 4: Chicken isolates (group 3). These organisms showed the least similarity to all other groups. In 6 hr broth cultures the organisms had a Gram-positive reaction but tended to be negative in 24 hr cultures. The organisms were very pleomorphic, their shapes ranging from coccoidal forms to long rods. Of the carbohydrates tested only glucose was fermented. Formic acid was produced by all the strains together with trace amounts of acetic and butyric acids with some of the strains.

DISCUSSION

There have been several detailed studies recently on individual genera within the family Bacteroidaceae, e.g. *Fusobacterium* (Baird-Parker, 1960), *Sphaerophorus* (Fievez, 1963), *Bacteroides* (Beerens *et al.* 1963), and the tests used above were based on those found to be important for each of these genera. The results obtained justify the division of the family into a number of groups but these groups can only be assigned to specific genera after agreement has been reached on the correct nomenclature.

The properties of the *Fusobacterium* strains agreed closely with those described by Baird-Parker (1960), whilst the *Sphaerophorus necrophorus* strains (Fievez N 117, N 167, N 252) conformed with the description of the species given by Fievez (1963), but differed in several characteristics from the other four *Sphaerophorus* strains tested (Table 4). The close relationship between *Fusobacterium* and *Sphaerophorus* which was shown in this analysis is supported by Sebald (1962), who found that the DNA base ratios were similar for the organisms within the two genera and suggested that they should be included together in one genus.

The separation of the *Bacteroides* strains into a separate group from *Fusobacterium* and *Sphaerophorus* is also supported by the DNA base ratio analyses of Sebald (1962) who showed that the G + C ratio was > 41 % as compared with 27–34 % for the other two genera. Beerens *et al.* (1963) suggested that many of the species described within the

genus *Bacteroides* (or *Ristella*) should be grouped together within one species *Eggerthella convexa*. This analysis confirmed the difficulty of differentiating between the various species but certain tests such as those used by Reinhold (1964) to differentiate *B. convexus* from *B. thetaiotaomicron* were not included.

It is evident that a number of type-culture collection strains have been incorrectly named—in particular the strain *Bacteroides necrophorus* NCTC 7155 which is identical with *Eggerthella clostridiformis* (Beerens) and does not resemble the other *Sphaerophorus* strains. The strain *S. necrophorus* ATCC 12290 is almost certainly a *Bacteroides* strain. *B. symbiosis* ATCC 12829 shows little resemblance to the other *Bacteroides* strains and needs further investigation.

There was no difficulty in recognizing the strains of *Bacteroides melaninogenicus* because of their typical black colonies and requirements for menadione; however, the taxonomic position of this organism within the family needs careful consideration. It was assigned by Beerens *et al.* (1962) to a separate genus, and support for this suggestion comes from its low similarity to the other *Bacteroides* strains.

One of the main purposes of the analysis was to determine which tests were most useful for identification purposes. Perhaps the most difficult problem was initially to determine which organisms might come within the family Bacteroidaceae, which is defined as containing Gram-negative anaerobic non-sporing rods. Strains were included in the first analysis which were subsequently shown to produce spores on particular media. Included amongst these was *Fusiformis biacutus* which has always been considered a non-sporing organism. Similarly, the *Eggerthella clostridiformis* strains of Beerens formed spores. The whole relationship of the Gram-negative sporing organisms to the Bacteroidaceae needs further investigation. The problem of obtaining a reliable Gram reaction has again been demonstrated. In these analyses the group 3 chicken isolates were included initially as Gram-negative bacteria but were subsequently found to be Gram-positive. However, they had a low similarity to all the other organisms tested, indicating that they also differed in many other properties.

As can be seen from Table 3, the morphological differentiation of the *Sphaerophorus* strains from *Bacteroides* was difficult, but *Fusobacterium* and the two groups of chicken isolates all had a characteristic morphology. Most of the traditional tests (Table 4) such as indole production, hydrogen sulphide production and the fermentation of various carbohydrates were too variable within the groups to be used to separate them. Amongst the differential tests considered to be most useful are those shown in Table 3. These were: (1) the terminal pH in glucose broth and the types of volatile fatty acids produced, i.e. formic, acetic, propionic or butyric acids, (2) the production of propionic acid from threonine, (3) growth stimulation by bile. Other useful tests shown in Table 4 included the effect of various inhibitors, in particular polymyxin 10 µg./ml.

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