A SCHEME FOR THE IDENTIFICATION OF CLINICAL ISOLATES OF GRAM-NEGATIVE ANAEROBIC BACILLI BY CONVENTIONAL BACTERIOLOGICAL TESTS

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SEVERAL schemes have been developed for the identification of gram-negative anaerobic bacilli. Those most widely used in the USA are given in the Anaerobe Laboratory Manual of the Virginia Polytechnic Institute (Holdeman, Cato and Moore, 1977), the Wadsworth Anaerobic Bacteriology Manual (Sutter, Vargo and Finegold, 1975) and the CDC Laboratory Manual (Dowell and Hawkins, 1974). API Laboratory Products Ltd (Invincible Road, Farnborough, Hants) have produced a commercial test strip for the identification of anaerobes (API-20 Anaerobes) which has many limitations (Dr B. Watt, personal communication; Duerden, unpublished results); the API-ZYM test strip (Tharagotnet *et al.*, 1977) awaits further evaluation. Simpler schemes have been used to separate strains of *Bacteroides* and related organisms into the major groups rather than distinct species; these include the antibiotic-resistance tests of Sutter and Finegold (1971), which are now incorporated in the commercial Mastring identification test (Mast Laboratories Ltd, 38 Queensland Street, Liverpool, L7 3JG), and dye-tolerance tests developed from those of Baird-Parker (1957) and Suzuki, Ushijima and Ichinose (1966).

Gas-liquid chromatographic (GLC) analysis of the short-chain fatty-acid products of metabolism has been of major importance in the classification of the Bacteroidaceae but it can be used only to allocate strains to one of the major genera or subgroups and does not provide identification to specific or subspecific level (Deacon, Duerden and Holbrook, 1978).

In diagnostic bacteriology, it is often difficult to distinguish the pathogenic members of the Bacteroidaceae from others that are merely part of the normal flora colonising devitalised tissue. However, evidence has accumulated that certain species and subspecies have greater pathogenic potential than others and that the isolation and recognition of these may be of particular significance (Werner, 1974; Smith, 1975; Duerden, 1979). A simple and reliable method for

the identification of isolates is therefore needed for use in the diagnostic bacteriological laboratory.

In 1976 we presented a provisional scheme for the identification of gram-negative anaerobic bacilli by means of conventional bacteriological tests (Duerden *et al.*, 1976) based upon studies with 165 strains, mostly of the *B. fragilis* group. Since then, understanding of the classification and relationships of gram-negative anaerobic bacilli has improved and we have studied many more strains drawn from a wider variety of species. Detailed results of some of these studies have already been published (Holbrook, Duerden and Deacon, 1977; Deacon *et al.*, 1978), and we now present a more comprehensive identification scheme.

MATERIALS AND METHODS

Organisms

The results were assembled and the identification scheme was derived from the examination of 1017 strains of gram-negative anaerobic bacilli. These organisms and their sources are listed in table I. The following reference strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT: *Bacteroides fragilis* [*B. fragilis* subspecies (ss.) *fragilis*] NCTC nos. 9343, 9344, 8560, 10584 and 10581; *B. vulgatus* NCTC nos. 10583 and 11154; *B. thetaiotaomicron* NCTC10582; *B. eggerthii* NCTC11155; *B. splanchnicus* NCTC nos. 10825 and 10826; *B. melaninogenicus* ss. *intermedius* NCTC nos. 9336 and 9338; *B. asaccharolyticus* NCTC9337; *B. praeacutus* NCTC11158; *B. corrodens* NCTC10939; *Fusobacterium necrophorum* NCTC nos. 10575, 10576 and 10577; *F. polymorphum* NCTC10562; *F. necrogenes* NCTC10723; *F. varium* NCTC10560; *B. multiaacidus* NCTC nos. 10934 and 10935; and *Leptotrichia buccalis* NCTC10249.

B. melaninogenicus ss. *melaninogenicus* ATCC15930 [see Holbrook and Duerden, 1974; International Committee on Systematic Bacteriology (ICSB), 1977] was from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md 20852, USA. *B. ovatus* ATCC8483, *B. uniformis* (previously designated *B. thetaiotaomicron*) ATCC8492, and *B. distasonis* ATCC8503 were from Dr Ella M. Barnes, Agricultural Research Council Food Research Institute, Colney Lane, Norwich, NR4 7UA.

The clinical isolates were from routine specimens submitted to the diagnostic bacteriological laboratories of the Edinburgh Royal Infirmary, Sheffield Royal Infirmary, Sheffield Royal Hospital, Sheffield Children's Hospital, and the Central Microbiological Laboratories, Western General Hospital, Edinburgh. The faecal, vaginal and oral strains were isolated in our research laboratories from normal healthy subjects as part of investigations of the *Bacteroides* spp. found in the normal human flora (Holbrook, 1976; Holbrook, Ogston and Ross, 1978; Duerden, 1979). Most of the strains described as "obtained from colleagues" were sent to us in connexion with collaborative studies initiated by the ICSB, Taxonomic Sub-committee on Gram-negative Anaerobic Rods (see Holbrook *et al.*, 1977; Deacon *et al.*, 1978).

Characterisation of strains

The culture media used have been described by Duerden *et al.* (1976). All strains were tested for the ability to grow in air, air + CO₂, and under anaerobic conditions; sensitivity to metronidazole in a disk diffusion test confirmed that test strains were obligate anaerobes (Prince *et al.*, 1969; Watt and Jack, 1977).

In the initial studies (Duerden *et al.*, 1976; Holbrook *et al.*, 1977) strains were subjected to the following set of morphological, biochemical, tolerance and antibiotic-disk resistance tests.

TABLE I
The identity and source of 1017 strains of gram-negative anaerobic bacilli

Species or subspecies (ss.)	Number of strains of the stated species obtained from						Total number of strains
	clinical specimens	faeces	mouth	vagina	reference centres	colleagues	
<i>B. fragilis</i>	236	15	0	0	5	0	256
<i>B. vulgatus</i>	11	45	0	3	2	0	61
<i>B. distasonis</i>	6	41	0	1	1	0	49
<i>B. ovatus</i>	1	0	0	0	1	0	2
<i>B. thetaiotaomicron</i>	36	37	0	3	1	0	77
<i>B. eggerthii</i>	2	30	0	0	1	0	33
<i>B. variabilis</i>	1	0	0	0	1	0	2
<i>B. uniformis</i>	1	8	0	0	2	0	11
<i>B. splanchnicus</i>	1	16	0	1	2	0	20
<i>B. melaninogenicus</i>							
ss. <i>melaninogenicus</i>	10	5	35	19	2	9	80
ss. <i>intermedius</i>	25	7	59	24	2	6	123
ss. <i>levii</i>	0	0	0	0	0	1	1
<i>B. bivius</i>	7	0	0	16(21)*	0	7	30(21)*
<i>B. disiens</i>	0	0	0	0	0	2	2
<i>B. oralis</i>	4	0	6	10	0	9	29
<i>B. ruminicola</i>	4	1	1	7	0	5	18
<i>B. oralis</i> / <i>ruminicola</i> group	1	3	3	1	0	0	8
<i>B. asaccharolyticus</i>	53	17	4	31	1	1	107
<i>B. praeacutus</i>	0	0	0	0	1	0	1
Non-pigmented non-saccharolytic strains	3	12	0	7	0	0	22
<i>B. corrodens</i>	8	0	0	2	1	7	18
<i>Bacteroides</i> spp.	2	4	1	3	0	1	11
<i>F. necrophorum</i>	1	0	0	0	3	0	4
<i>F. necrogenes</i>	1	0	0	0	1	0	2
<i>F. varium</i>	0	0	0	0	1	0	1
<i>F. polymorphum</i>	1	0	2	0	1	0	4
<i>Fusobacterium</i> spp.	8	0	6	0	0	0	14
<i>L. buccalis</i>	0	0	1	0	1	0	2
<i>B. multiacidus</i>	0	0	0	0	2	0	2
<i>B. ochraceus</i>	0	0	0	0	0	6	6

* Twenty-one strains were either *B. bivius* or *B. disiens* but were not fully identified.

Morphological and biochemical tests. Microscopic and colonial morphology; haemolytic effect on blood agar; pigment production; motility; lipase activity; oxidase test; catalase test; hydrogen-sulphide production; indole production; gelatinase test; aesculin hydrolysis; dextranase production; nitrate reduction; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol. Fermentation tests with arabinose and xylose were added subsequently. The methods used are described by Duerden *et al.* (1976).

Tolerance tests. Growth in the presence of (1) the bile salts sodium taurocholate and sodium deoxycholate, separately and in combination, and (2) the dyes brilliant green, Victoria blue 4R, gentian violet and ethyl violet (separately), as described by Duerden *et al.* (1976).

Antibiotic-disk resistance tests. Resistance to disks containing neomycin 1000 µg and 10 µg, kanamycin 1000 µg and 30 µg, penicillin 1.5 units, methicillin 10 µg, erythromycin 60 µg, colistin 10 µg, rifampicin 15 µg, lincomycin 2 µg, clindamycin 2 µg, bacitracin 0.1 unit, vancomycin 15 µg, chloramphenicol 10 µg, tetracycline 10 µg and metronidazole 5 µg (see Duerden *et al.*, 1976).

GLC analysis. The short-chain fatty acid products of metabolism of 203 strains, including all the reference strains and the strains from the ICSB collaborative studies, were analysed as

detailed by Deacon *et al.* (1978). Volatile acids were considered to be formed as major products of metabolism when $> 10 \mu\text{mol/ml}$, and non-volatile acids when $20 \mu\text{mol/ml}$, were detected (see Deacon *et al.*, 1978).

Selected discriminant tests

From the results of the early studies, the following short set of tests was selected for their particular discriminatory value: *tolerance tests* with sodium taurocholate, Victoria blue 4R and gentian violet (separately); *antibiotic-disk resistance tests* with metronidazole $5 \mu\text{g}$, neomycin $1000 \mu\text{g}$, kanamycin $1000 \mu\text{g}$, penicillin 2 units and rifampicin $15 \mu\text{g}$ per disk; *tests* for pigment production, indole production, digestion of gelatin, hydrolysis of aesculin, and fermentation of glucose, rhamnose, trehalose, mannitol and xylose, with tests for the fermentation of lactose and sucrose added when necessary.

The methods used for these tests were those of Duerden *et al.* (1976) with the following modifications. (1) The basic liquid medium for the fermentation tests and tests for gelatin digestion, indole production and aesculin hydrolysis in the sets of tests carried out in one laboratory (Sheffield) was a modification of the BM medium of Nash (see Deacon *et al.*, 1978). The results were comparable with those obtained previously and this medium supported a better growth of some fastidious strains. (2) In the preparation of tolerance-test media, the stock solutions of bile salts and dyes were added to the (cooled) autoclaved basal medium. (3) Tests for nitrate reduction were done with Trypticase Nitrate Broth (BBL).

RESULTS

Six strains of *Bacteroides ochraceus* were studied but are excluded from this report. They were able to grow in air + CO_2 and were resistant to metronidazole, an antimicrobial agent to which only anaerobic bacteria are susceptible (Prince *et al.*, 1969). On this evidence they should be removed from the Bacteroidaceae.

The following results given for the different species and subspecies of the Bacteroidaceae are typical patterns derived as a composite from our studies with the rest of the 1011 strains tested. They were originally based upon studies with reference strains and have been modified as a result of our experience with fresh isolates from clinical sources and from the normal flora. Where results were found to be variable within a species or subspecies, this is indicated in the tables (see footnotes to tables II, III and IV) and discussed in the text.

Gram-negative anaerobic bacilli can be separated into four broad groups: (1) the fragilis group, (2) the melaninogenicus-oralis group, (3) the asaccharolytic group and (4) the fusobacteria. Strains can usually be allocated to one of these groups according to the results of tolerance and antibiotic-disk resistance tests (table II) although an additional test for glucose fermentation or GLC analysis is needed to separate some members of the asaccharolytic group from the melaninogenicus-oralis group.

The fragilis group

Most strains in this group give the same pattern of results in antibiotic-disk

TABLE II

Typical patterns of results obtained in antibiotic-disk resistance and tolerance tests with *Bacteroides* spp.

Test	Pattern of results* obtained with strains of			
	fragilis group	melaninogenicus oralis group	asaccharolytic group	fusobacterium group
<i>Antibiotic susceptibility</i>				
Neomycin 1000 µg	R	S	S/R	S
Kanamycin 1000 µg	R	R	R	S
Penicillin 2 units	R	S/R	S	S
Rifampicin 15 µg	S	S	S	S/R
<i>Tolerance</i>				
Taurocholate	+	I	I	I or +
Victoria blue 4R	+	+/I	I	+
Gentian violet	I	I	I	+

* In antibiotic-susceptibility tests: R=resistant; S=sensitive; S/R=30–70% of strains gave each result; in *tolerance tests*: + = growth; I=inhibitor; +/I=30–70% of strains gave each result; I or + = different species give results as indicated in table VI.

resistance tests and tolerance tests; they are resistant to the neomycin, kanamycin and penicillin disks but sensitive to the rifampicin disk, and they are tolerant of taurocholate and Victoria blue 4R but inhibited by gentian violet (table II). There are a few exceptions to this pattern: some reference strains of *B. uniformis*, *B. variabilis* and *B. splanchnicus* are inhibited by sodium taurocholate but grow in bile-stimulation tests with bile broth as done at the VPI (Holdeman *et al.*, 1977); moreover, many fresh isolates that otherwise conform with the typical patterns of results of these species are tolerant of taurocholate.

GLC analysis shows that, for strains of the fragilis group, succinic acid, and generally acetic acid, are major products of metabolism after incubation for 2 days. Propionic, *iso*-butyric, *iso*-valeric and lactic acids are minor products of some strains. *B. splanchnicus*, however, produces significant quantities of *n*-butyric acid and a variety of other acids including *iso*-valeric, *iso*-butyric and propionic acids, but not lactic acid.

Strains allocated to the fragilis group can be divided into nine species by the results of tests for indole production, aesculin hydrolysis and the fermentation of glucose, lactose, sucrose, rhamnose, trehalose, mannitol and xylose. The results obtained with the nine species are shown in table III. *B. fragilis* strains generally give the typical pattern of results except that a few strains do not ferment xylose. *B. vulgatus* strains give variable results in the test for aesculin hydrolysis; c. 50% do not hydrolyse aesculin and some others do so only slowly. All *B. distasonis* strains ferment trehalose and xylose, and most strains also ferment rhamnose. Six species hydrolyse aesculin and produce indole; they are distinguished by the results of fermentation tests. *B. ovatus* strains give positive results in all the tests but few strains of this species were found in the present studies. *B. thetaiotaomicron* strains ferment all the test

TABLE III
Typical patterns of results obtained with species of the fragilis group in biochemical tests

Test	Patterns of results* obtained with strains of									
	<i>B. fragilis</i>	<i>B. vulgatus</i>	<i>B. distasonis</i>	<i>B. thetaiotaomicron</i>	<i>B. ovatus</i>	<i>B. uniformis</i>	<i>B. variabilis</i>	<i>B. eggerthii</i>	<i>B. splanchnicus</i>	
Indole production	-	-	-	+/-	+	+	+	+	+	+
Gelatin digestion	+/-	+	+(-)	+/-	+	+	+	+	+	+
Aesculin hydrolysis	+	+/-	+	+	+	+	+	+	+	+
Fermentation of										
glucose	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+
rhamnose	-	+	+(-)	+	+	-	+	+	-	-
trehalose	-	-	+	+	+	+	-	-	-	-
mannitol	-	-	+	-	+	+	-	-	-	-
xylose	+(-)	+	+	+	+	+	+	+	+	+/-

* + = > 95% of strains gave a positive result; - = > 95% of strains gave a negative result; +/- = 30-70% of strains gave each result; +(-) = 70-95% of strains gave a positive result.

carbohydrates except mannitol. *B. uniformis* strains do not ferment rhamnose or mannitol; *B. variabilis* strains are similar except that they ferment rhamnose and not trehalose. *B. eggerthii* strains ferment rhamnose but not sucrose or trehalose and *B. splanchnicus* strains ferment only glucose, lactose and in some cases xylose.

In our original tests (Duerden *et al.*, 1976) it appeared that a negative result in the gelatinase test was useful in the identification of *B. fragilis*, *B. distasonis* and *B. thetaiotaomicron*. However, subsequent experience showed that many strains of these species digested the gelatin disk, though slowly. The test was more commonly positive when performed in BM broth and we now regard the gelatinase test as having little discriminatory value.

The melaninogenicus-oralis group

This group comprises the pigmented strains that ferment glucose and the non-pigmented strains that share many similar properties. In general they are sensitive to the neomycin and rifampicin disks but resistant to kanamycin. In the tolerance tests they are inhibited by taurocholate and by gentian violet. Many strains are sensitive to penicillin but an increasing number of strains have been found to be resistant, principally in the non-pigmented species and *B. melaninogenicus* ss. *melaninogenicus*. Similarly, many strains of *B. ruminicola* and *B. oralis*, some strains of *B. bivius* and a few strains of *B. melaninogenicus* ss. *melaninogenicus* are tolerant of Victoria blue 4R.

Strains of the melaninogenicus-oralis group typically form succinic and acetic acids as major products. The production of minor or trace amounts of other short-chain fatty acids is variable and none may be produced. Lactic acid is produced variably and usually in small quantities. The fatty-acid profiles of the new species *B. bivius* and *B. disiens* are similar to those of other members of the group, but the one strain of the new weakly-fermentative subspecies of *B. melaninogenicus* ss. *levii* that we tested gave a different profile: *n*-butyric acid was a major product, with significant quantities of acetic, propionic, *iso*-butyric and *iso*-valeric acids; lactic and succinic acids were not produced.

Strains allocated to this group can be divided into seven species or subspecies according to the test results shown in table IV.

B. melaninogenicus ss. *intermedius* strains differ from the other members of this group in producing indole; they also digest gelatin quite rapidly but do not hydrolyse aesculin and few strains ferment lactose. The colonies on blood agar are usually black or dark brown and shiny after incubation for 3–4 days. *B. melaninogenicus* ss. *melaninogenicus* strains often produce brown rather than black colonies and many of them produce a distinctive colony with a dark brown centre surrounded by a pale annulus. They ferment lactose and sucrose and a minority of strains hydrolyse aesculin. *B. melaninogenicus* ss. *levii* is represented in our series by only one strain which produces dark-brown colonies slowly on blood agar and more promptly on lysed blood agar. It

TABLE IV

Typical patterns of results obtained with species and subspecies (ss.) of the melaninogenic-oralis group in biochemical and cultural tests

Test	Patterns of results* obtained with strains of						
	<i>B. melaninogenicus</i>				<i>B. bivius</i>	<i>B. disiens</i>	<i>B. oralis</i>
	<i>ss. intermedius</i>	<i>ss. levii</i>	<i>ss. melaninogenicus</i>				
Pigment production	+	+	+	—	—	—	—
Indole production	+	—	—	—	—	—	—
Gelatin digestion	+	+	+	+	+	+	+(—)
Aesculin hydrolysis	—	—	+/-	—	—	+(-)	+
Fermentation of							
glucose	+	+	+	+	+	+	+
lactose	-(+)	+	+	+	—	+	+
sucrose	+	—	+	—	—	+	+
rhamnose	—	—	-(+)	—	—	+/-	+(—)
trehalose	—	—	—	—	—	—	—
mannitol	—	—	—	—	—	—	—
xylose	—	—	—	—	—	—	+

*See footnote to table III; -(+) = 70–95% of strains gave a negative result.

appears to be asaccharolytic after incubation of fermentation tests for 48 h, but if these tests are continued for 4 days it ferments glucose and lactose.

B. bivius strains do not produce pigment although their colonies are often pale brown after prolonged incubation on lysed blood agar. They also differ from *B. melaninogenicus* ss. *melaninogenicus* in not fermenting sucrose. *B. disiens* strains differ from *B. bivius* only in not fermenting lactose. However, the two typical strains sent to us from the VPI were also moderately resistant to the neomycin disk. *B. oralis* strains ferment lactose and sucrose and some strains also ferment rhamnose, but none of them ferment xylose. All *B. ruminicola* strains, however, ferment xylose and most of them also ferment rhamnose.

The asaccharolytic group

The organisms listed in table V do not ferment glucose or other carbohydrates. They include the pigmented *B. asaccharolyticus* (formerly *B. melaninogenicus* ss. *asaccharolyticus*; Finegold and Barnes, 1977) which produces black or very dark-brown and often moist colonies on blood agar, *B. corrodens*, which produces characteristic "pitting" or "corroding" of the agar surface around colonies, *B. praeacutus*, and several other non-pigmented asaccharolytic organisms.

B. asaccharolyticus strains are inhibited in the three tolerance tests, resistant to kanamycin and sensitive to penicillin and rifampicin; most are also sensitive to the neomycin disk but a sizeable minority (c.30%) are resistant. They produce indole and digest gelatin rapidly but do not hydrolyse aesculin. GLC analysis shows that they produce a variety of acids including *n*-butyric

TABLE V

Typical patterns of results obtained with species of the asaccharolytic group in a combined set of tests

Test	Patterns of results *obtained with strains of			
	<i>B. asaccharolyticus</i>	<i>B. corrodens</i>	<i>B. praeacutus</i>	other non-pigmented asaccharolytic strains
<i>Tolerance</i>				
Taurocholate	I	I	I	I
Victoria blue 4R	I	+	+	I
Gentian violet	I	I	+	I
<i>Antibiotic susceptibility</i>				
Neomycin	S/R	S	S	S
Kanamycin	R	S	S	R
Penicillin	S	S	S	S(R)
Rifampicin	S	S/R	S	S
Pitting growth on primary culture	—	+	—	—
Pigment production	+	—	—	—
Indole production	+(—)	—	—	+(—)
Gelatin digestion	+	+	+	+
Aesculin hydrolysis	—	—	—	—

* See footnotes to tables II and III; S/(R)=70–95% of strains were sensitive. None of the strains fermented glucose.

acid; some strains produce succinic acid but others do not. Studies with *B. asaccharolyticus* have indicated that lactic-acid production may be mimicked or apparently supplemented by the occurrence of a product with a retention time that is very close to that of lactic acid with some column packings; this seems to merit further study.

B. corrodens strains are included here, but they share some characteristic results with the fusobacteria: they are tolerant of Victoria blue 4R but inhibited in the other tolerance tests, and sensitive to penicillin, neomycin and kanamycin; some strains are sensitive to rifampicin but others are resistant. However, the GLC profiles distinguish *B. corrodens* strains from the fusobacteria. They give few positive results in our basic series of tests except that they all digest gelatin; but the identification of strains as *B. corrodens* can be confirmed by positive results in the oxidase test and tests for the reduction of nitrate and the production of urease (Jackson and Goodman, 1978).

B. praeacutus strains are inhibited by taurocholate but tolerant of both dyes and are sensitive to the four antibiotic disks. They give negative results in the remainder of our basic series of tests except that they digest gelatin. The reference strain NCTC11158 is motile and reduces nitrate.

The other non-pigmented asaccharolytic strains are a somewhat heterogeneous collection that are insufficiently characterised at present to assign specific status to them. Some of them share many characteristics with *B. asaccharolyticus* except for pigment production; they give the same results in tolerance and antibiotic-disk resistance-tests, digest gelatin and produce indole. These strains can probably be assigned to the species *B. putredinis*

(Holdeman *et al.*, 1977). Other strains, however, give different patterns of results and are at present not identified further.

The fusobacteria

The results given in table VI were obtained mostly with reference strains; our experience with fresh isolates of fusobacteria is small. Microscopically, most of our test strains have the typical "fusiform" appearance but some do not and are indistinguishable from other small gram-negative bacilli. There are variations between different species in the group in the results of tolerance tests and antibiotic-disk resistance tests but they all share a common characteristic in the GLC analysis of their fatty-acid metabolic products—*n*-butyric acid is a major product.

Leptotrichia buccalis (Vincent's organism) and *B. multiacidus* are included with the fusobacteria in table VI, but they are distinct from the genus *Fusobacterium*. *L. buccalis* shares many phenotypic characteristics with fusobacteria but forms lactic acid as a major metabolic product and does not form *n*-butyric acid.

The results obtained with *B. multiacidus* in tolerance and antibiotic-disk resistance tests were similar to those obtained with fusobacteria, but *B. multiacidus* is strongly saccharolytic, does not produce *n*-butyric acid and is not fusiform in morphology. Its relationship with other *Bacteroides* spp. remains to be determined.

TABLE VI

Patterns of results obtained with reference strains of Fusobacterium spp., Leptotrichia buccalis and B. multiacidus

Test	Patterns of results *obtained with reference strains of					
	<i>F. necrophorum</i>	<i>F. necrogenes</i>	<i>F. varium</i>	<i>F. polymorphum</i>	<i>L. buccalis</i>	<i>B. multiacidus</i>
<i>Antibiotic susceptibility</i>						
Neomycin	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S
Penicillin	S	S	S	S	S	S
Rifampicin	R	R	R	S	S	R
<i>Tolerance</i>						
Taurocholate	I	+	+	I	I	I
Victoria blue 4R	+	+	+	+	+	+
Gentian violet	+	+	+	+	+	+
Indole production	+	—	+	+	—	—
Gelatin digestion	—	—	—	—	—	—
Aesculin hydrolysis	—	+	—	—	+	+
Fermentation of						
glucose	+	+	+	—	+	+
lactose	—	—	—	—	+	+
sucrose	—	—	—	—	+	+
rhamnose	—	—	—	—	—	+/-
trehalose	—	+	—	—	+	—
mannitol	—	—	—	—	—	+/-
xylose	—	—	—	—	—	+

* See footnotes to tables II and III.

DISCUSSION

Gram-negative non-sporing anaerobic bacilli of the *Bacteroides-Fusobacterium* group are important members of the normal flora of the lower gastrointestinal tract, mouth and vagina (Gibbons *et al.*, 1963; Drasar, Shiner and McLeod, 1969; Gorbach *et al.*, 1973; Drasar and Hill, 1974) and are also significant causes of clinical infections, particularly after surgical or accidental injury related to these sites and in debilitated patients (Phillips and Sussman, 1974; Finegold, 1977). Improvements in anaerobic techniques (Collee, Rutter and Watt, 1971; Holdeman and Moore, 1973; Watt, 1973; Watt, Hoare and Collee, 1973; Watt, Collee and Brown, 1974) have provided routine diagnostic bacteriological laboratories with reliable methods for the isolation of bacteroides organisms from a wide variety of clinical conditions, but few attempts are generally made to identify the isolates; they are usually reported as "*Bacteroides* spp.", or at most the non-pigmented penicillin-resistant strains are reported as *B. fragilis*, the pigmented ones as *B. melaninogenicus* and the others as *Bacteroides* spp.

Studies in specialised laboratories around the world have clarified some of the problems in the classification of the Bacteroidaceae (Finegold and Barnes, 1977; ICSB, 1977, 1980). The fragilis group are commensals of the lower gastro-intestinal tract and pathogens in wound infections, abscesses and peritonitis. Holdeman and Moore (1974) included all members of the group in a single species, *B. fragilis*, with five subspecies: ss. *fragilis*, ss. *vulgatus*, ss. *distasonis*, ss. *ovatus* and ss. *thetaiotaomicron*. They believed that the species represented a continuum of variants with clusters of strains that were designated subspecies. However, Cato and Johnson (1976) found major differences between the subspecies in DNA homology studies and proposed that they should be reinstated to species rank; we have adopted this view in the present studies. Nevertheless, the species in the fragilis group share many properties. The results obtained in our tests form a continuous spectrum with clusters of strains that represent the named species. Most isolates can be allocated to a species but there remain some intermediate organisms that clearly belong with the fragilis group but cannot be allocated to a recognised species.

International collaboration has been particularly useful in developing the classification of the black-pigmented *Bacteroides* spp. and related organisms. *B. asaccharolyticus* has been segregated from the saccharolytic subspecies of *B. melaninogenicus* and studies have shown that *B. melaninogenicus* ss. *melaninogenicus*, *B. oralis*, *B. bivius*, *B. disiens* and *B. ruminicola* form a closely related group that share many characteristics (ICSB, 1977, 1980).

The term saccharolytic is used to describe strains that produce acid from carbohydrates by fermentation; *B. asaccharolyticus* utilises glucose by non-fermentative pathways. *B. oralis*, *B. bivius* and *B. disiens* are separated only on the basis of individual fermentation tests. Their classification as separate species requires confirmation by additional tests, such as DNA-base-ratio and homology studies, cell-wall analysis and antigenic analysis. Moreover, a type

strain of *B. oralis* has not been established, and the relationship between *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis* is the subject of an unresolved taxonomic debate; these labels may denote pigmented and non-pigmented variants of a single species. The production of pigmented colonies on media that contain blood has less taxonomic significance than was previously thought and may not be a valid criterion for separating these otherwise similar strains into different species (Sundqvist, 1976; Holbrook *et al.*, 1977). This supports a growing feeling that the *melaninogenicus* label should be re-defined or replaced. The *melaninogenicus*-*oralis* group are commensals of the mouth and vagina and are implicated in infections related to these sites.

The asaccharolytic group contains some of the most exacting strains encountered in these studies and their classification is unsatisfactory. Differentiation of slow-growing and unreactive strains on the basis of conventional tests is difficult. The pigmented *B. asaccharolyticus* is well recognised; it is a commensal in the large intestine and is commonly isolated from infections. Similarly, the corroding bacilli that are obligate anaerobes are designated *B. corrodens*. We have confirmed Jackson and Goodman's (1978) finding that *B. corrodens* produces urease. These workers have suggested that this species should be called *B. ureolyticus* to avoid confusion with *Eikenella corrodens*, a species of carboxyphilic organisms that can grow in air + CO₂, but the epithet *corrodens* has taxonomic precedence and the generic names should prevent any confusion. Identification of other non-pigmented non-saccharolytic strains is difficult, and few type or reference strains are available. The schemes given in the Wadsworth Anaerobic Bacteriology Manual (Sutter *et al.*, 1975) and the CDC Laboratory Manual (Dowell and Hawkins, 1974) reflect our difficulties with the identification of these organisms. The VPI Anaerobe Laboratory Manual (Holdeman *et al.*, 1977) gives the results obtained with more species, but the identity of our isolates was not resolved by reference to these patterns, except that some were probably *B. putredinis*. Some strains could be distinguished from *B. asaccharolyticus* only by their failure to produce pigment.

There are major doubts about the classification of the fusobacteria. Some of the strains isolated from clinical material and from the normal flora of the gingival crevice have exacting growth requirements and many tests are difficult to perform on them. Most strains that belong to the recognised species shown in table VI are readily identified, but many isolates do not give the recognised patterns of results and cannot be further identified at present.

The identification of *Bacteroides* isolates to species level is considered unnecessary in many diagnostic bacteriological laboratories. The normal flora contains many species and subspecies of *Bacteroides* and different species are found in different sites. Moreover there is considerable evidence that certain species have a specific pathogenic potential. The frequency of occurrence of the species in clearly pathogenic roles does not reflect their prevalence in the normal flora. In particular, *B. fragilis* forms only a small part (c. 9%) of fragilis-group isolates from normal faeces but 75% of isolates from infections related to the large intestine (Duerden, 1979). This suggests that *B. fragilis* has either an effective mechanism to evade the host defences or special aggressive

potential that may be related to cell-surface properties (Kasper, 1976) or the formation of diffusible products (Gesner and Jenkin, 1961; Müller and Werner, 1970). The identification of *B. asaccharolyticus* and *B. melaninogenicus* strains may also have particular significance (Duerden, 1979, 1980). The identification of *Bacteroides* isolates may, therefore, help in assessing the significance of laboratory findings and in determining the source of an infection when this is not immediately apparent.

The scheme described in this paper uses conventional bacteriological tests designed for work with Bacteroidaceae. It allows prompt and accurate identification of the *Bacteroides* spp. commonly encountered in specimens received by clinical laboratories and in the normal human flora. The series of tests does not form a sequential key. The tests were selected for use as a set to take account of small variations in the results of individual tests within several species. We do not suggest that this is the only approach to the identification of *Bacteroides* spp. in the diagnostic bacteriological laboratory. Other methods such as serological tests may afford a more prompt identification of certain groups (Lambe, 1974; Lambe and Jerris, 1976; Stauffer *et al.*, 1975). GLC analysis of the short-chain fatty acid products of metabolism has been given particular prominence in current systems of classification of Bacteroidaceae (Holdeman and Moore, 1974). We have included the results of GLC analysis in our descriptions of the groups but this is not essential for the identification of unknown isolates. GLC enables the rapid identification of clinical isolates to the generic level, but additional conventional tests remain necessary for species or subspecies identification (Deacon *et al.*, 1978). Our experience has shown that satisfactory results are obtained by the careful use of conventional procedures without the need for expensive and complicated equipment.

SUMMARY

More than 1000 strains of gram-negative anaerobic bacilli, including reference strains, clinical isolates, and members of the normal flora of the mouth, lower gastro-intestinal tract and vagina of healthy human subjects, were studied by conventional bacteriological methods and by gas-liquid chromatographic analysis of metabolic products in a series of investigations. A short combined set of tests with particular discriminant value was selected, and a scheme for the identification of the species and subspecies encountered in the diagnostic bacteriological laboratory was based upon our composite results. The tests are: antibiotic-disk resistance tests with neomycin 1000 µg, kanamycin 1000 µg, penicillin 2 units and rifampicin 15 µg per disk; tolerance tests with sodium taurocholate, Victoria blue 4R and gentian violet; and tests for pigment production, indole production, aesculin hydrolysis and the fermentation of glucose, lactose, sucrose, rhamnose, trehalose, mannitol and xylose. Gram-negative anaerobic bacilli are divided into four groups: (1) the fragilis group with nine species, which include the five subgroups previously classified as subspecies of *B. fragilis*; (2) the melaninogenicus-oralis group, which includes

the three saccharolytic subspecies (ss.) of *B. melaninogenicus*—ss. *melaninogenicus*, ss. *intermedius* and ss. *levii*—and four non-pigmented species; (3) the asaccharolytic group, which comprises *B. asaccharolyticus* (formerly *B. melaninogenicus* ss. *asaccharolyticus*), *B. corrodens* and other non-pigmented non-saccharolytic strains, and (4) the fusobacteria.

We are grateful to all the colleagues who have sent strains to us; in particular, we acknowledge several valuable discussions with Dr Ella M. Barnes who also co-ordinated the ICSB studies.

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