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GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF METABOLIC PRODUCTS IN THE IDENTIFICATION OF BACTEROIDACEAE OF CLINICAL INTEREST

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GRAM-NEGATIVE non-sporing anaerobic bacilli of the *Bacteroides-Fusobacterium* group are commonly isolated from clinical specimens (Moore, Cato and Holdeman, 1969; Finegold, 1974). Accurate and prompt generic identification is essential for effective treatment; complete identification may provide information on the pathogenic potential of certain biotypes for man. Significant advances in methods for the identification of clinical isolates have resulted from recent improvements in anaerobic culture procedures (Collee *et al.*, 1972; Holdeman and Moore, 1972; Watt, 1973), culture media (Gibbons and McDonald, 1960; Finegold, Sugihara and Sutter, 1971), and characterisation schemes (Duerden *et al.*, 1976). Conventional bacteriological procedures have been supplemented by a wide variety of more complex and often time-consuming approaches (see Aalbaek, 1973).

The short-chain fatty acids formed as end products of protein or carbohydrate metabolism are of particular importance in current systems of classification (Holdeman and Moore, 1974). The family Bacteroidaceae has been assigned two principal genera, *Bacteroides* and *Fusobacterium*, largely on the basis that *Fusobacterium* spp. produce major amounts of *n*-butyric acid. A third genus, *Leptotrichia*, comprises oral fusiform bacteria that do not produce major amounts of any fatty acid other than lactic. Classification of members of the Bacteroidaceae on the basis of their fatty-acid end products was first attempted by Guillaume, Beerens and Osteux (1956) by means of distillation and paper chromatographic separation; the use of gas-liquid chromatography (GLC) was developed by Werner (1969) and Cato *et al.* (1970).

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The aims of the present study were (1) to determine the fermentation products of members of the *Bacteroides-Fusobacterium* group in various media, (2) to evaluate the use of these media for semi-quantitative GLC analysis of the products, and (3) to assess the value of GLC in identifying a wide range of strains from various clinical sources by comparing the results with those obtained by morphological, biochemical, tolerance and antibiotic-resistance tests.

MATERIALS AND METHODS

Organisms

The following strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT: *Bacteroides fragilis* (*B. fragilis* ss. *fragilis*) nos. NCTC9344 and 8560; *B. thetaiotaomicron* (*B. fragilis* ss. *thetaitaomicron*) no. NCTC10582; *B. vulgatus* (*B. fragilis* ss. *vulgatus*) no. NCTC10583; *B. melaninogenicus* nos. NCTC9336 and 9337; *Fusobacterium polymorphum* no. NCTC10562; *F. necrogenes* no. NCTC10723; *F. (Bacteroides) Sphaerophorus necrophorum* no. NCTC10575; *Leptotrichia buccalis* no. NCTC10249.

B. oralis no. ATCC15930 (*B. melaninogenicus*, see Holbrook and Duerden, 1974) was from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md 20852, USA. *B. fragilis* ss. *vulgatus* no. ATCC8482, *B. fragilis* ss. *ovatus* no. ATCC8483, *B. fragilis* ss. *thetaitaomicron* no. ATCC8492 and *B. fragilis* ss. *distasonis* no. ATCC8503 were from Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich NOR 7OF.

The following strains were referred by the International Committee on Systematic Bacteriology (ICSB) Taxonomic Sub-Committee for Gram-negative anaerobic rods: *B. melaninogenicus* ss. *melaninogenicus* nos. WAL2721 and WAL2724 from Dr S. M. Finegold, UCLA School of Medicine, Los Angeles, Ca 90024, USA; nos. GUI1011 and GUI1034 from Dr K. Ueno, Department of Bacteriology, Gifu University School of Medicine, Tsukasa-Machi, Gifu-shi, Gifu-ken, Japan; no. VPI4196 from Dr Elizabeth Cato, Virginia Polytechnic Institute and State University (VPI), Blacksburg Va. 24060, USA; *B. oralis* nos. VPI7570A and VPI5832 from VPI; nos. J1, 7CM and 30 from Dr B. A. Phillips, National Institute for Research in Dairying, University of Reading, England; no. NP333 from Mr G. H. Bowden, London Hospital Medical College, Dental School, Turner Street, London; *B. ochraceus* (*Ristella ochraceus*) nos. 1956C and 2467B from Dr M. Sebald, Anaerobic Laboratory, Institut Pasteur, 25 Rue du Docteur Roux, Paris XV*, France; no. VPI2845 from VPI; and nos. 10, 79B and 73 from Dr W. H. van Palenstein-Helderman, Department of Preventive Dentistry, University of Utrecht, Netherlands.

Additional strains of Gram-negative anaerobic bacilli were isolated in our laboratory; they consisted of 113 from subgingival dental plaque and 40 from clinical specimens (19 from faeces, 19 from high vaginal swabs, 1 from a rectal abscess and 1 from an abdominal wound swab). In all, 185 isolates were examined.

Bacteriological procedures

Isolation of strains from clinical specimens was as detailed by Holbrook (1976) and Holbrook, Duerden and Deacon (1977). All strains were characterised by the procedures of Duerden *et al.* (1976), and all were independently identified by GLC analysis. Cultures were regularly checked for purity by examination of Gram-stained smears and by aerobic and anaerobic subculture on blood agar (BA) containing 5% human blood in Columbia Agar Base (Oxoid). The standard inocula and anaerobic procedures of Collee *et al.* (1972) were used.

Turbidity readings were performed on a Pye Unicam (Cambridge, England) SP600 spectrophotometer and the absorbance was read at 600 nm in 1-cm cuvettes.

GLC media

Basal media were sterilised by autoclaving at 103.5 kilo-pascals (15 lb per in²) for 15 min. Stored media were steamed for 30 min. before use to drive off dissolved oxygen. Filter-sterilised stock solutions of the following growth factors were prepared: haemin (haematin hydrochloride; BDH Chemicals Ltd, Poole, England) 500 µg per ml in 0.01N NaOH; menadione (vitamin K₃; Sigma Chemicals, London) 100 µg per ml in distilled water (see Barnes and Impey, 1971); cysteine hydrochloride (BDH) 3.75% (w/v) in distilled water; and Na₂CO₃ 4% (w/v) in distilled water. These were added aseptically to all steamed (cooled) basal media to give final concentrations of haemin 5 µg per ml, menadione 1 µg per ml, cysteine hydrochloride 0.075% (w/v), and Na₂CO₃ 0.04% (w/v). As required, filter-sterilised aqueous stock solutions of glucose (20% w/v) and vitamin B₁₂ (cyanocobalamin; BDH) 10 µg per ml were added to give final concentrations of glucose 1% (w/v) and vitamin B₁₂ 0.1 µg per ml. The vitamin B₁₂ solution, protected from light, was stored at 4°C.

BM medium was a modification of the medium described by Williams *et al.* (1975); it contained 1% Trypticase (Baltimore Biological Laboratories, BBL), 1% Proteose Peptone (Oxoid), 0.5% Yeast Extract (Difco) and 0.5% sodium chloride. The pH was adjusted to 7.4 and the medium was autoclaved. Filter-sterilised horse serum (2% v/v) (Wellcome Reagents Ltd, Beckenham, England) and growth factors were added to the steamed (cooled) medium immediately before inoculation.

The other media were modifications of BM medium without trypticase. Full strength PPY medium contained 2% Proteose Peptone (Oxoid), 1% Yeast Extract (Difco), 0.5% sodium chloride and growth factors; variations of PPY medium were supplemented with glucose (PPYG), with glucose and 2% horse serum (PPYSG), and with serum alone (PPYS). Half-strength PPY (½PPY) contained 1% proteose peptone, 0.5% yeast extract, 0.5% sodium chloride and growth factors; variations of this medium were supplemented with vitamin B₁₂ (½PPY12) and vitamin B₁₂ and serum (½PPY12S).

Gas liquid chromatography

Apparatus. A dual flame-ionisation gas chromatograph, Series 104 (Pye Unicam, Cambridge, England), fitted with heated injection ports, was used with a Servoscribe 1S recorder, model 541.20 (Belmont Instruments, 6 Belmont Drive, Giffnock, Glasgow G46 7PA), set at the 10-mV range with a chart speed of 120 mm per h. The oxygen-free nitrogen carrier gas, hydrogen and air were supplied by British Oxygen Co. Ltd, London.

Two identical coiled-glass columns (Pye Unicam), length 1.5 m and internal diameter 4 mm, were packed in the laboratory with Chromosorb 101, 80/100 mesh (Johns-Manville, Denver, Colo., available from Gas Chromatography Services Ltd, 23 Old Chester Road, Lower Bebington, Wirral, Merseyside L63 7LA). The columns were conditioned for 24 h at 250°C before gentle repacking; fresh polymer was added to leave a 2.5-cm gap at the top of the column. After further overnight conditioning at 250°C, the columns were purged at the operating temperature of 190°C for 16 h before use.

Operating conditions. The chromatograph was operated isothermally at 190°C for both volatile and non-volatile (methylated) acid analyses. The injection ports and column oven were at 190°C and the detector oven temperature was 250°C. The carrier-gas flow rate in the analysing column was c. 35 ml per min. The carrier gas and hydrogen flow rates of the non-analysing column were adjusted for optimal acetic-acid response. The columns were kept in condition by maintaining the flow of carrier gas overnight at the operating temperature. There were no problems of column bleed with the porous polymer. Flow rates were checked each day before analyses commenced.

Sample preparation and analysis. The procedures were derived from those of Carlsson (1973). In general, GLC analyses were performed on 2-day cultures; 4- or 7-day cultures were used for a few slow-growing strains. Cultures were acidified with four drops of 50% H₂SO₄ to approximately pH 2 and centrifuged at 800 g for 1 h; the cell-free supernates were stored at 4°C in screw-capped 7-ml bijou bottles until analysed. For analysis of volatile acids, a 0.6 µl sample of supernate was injected directly on to the analysing column with a

5- μ l syringe (SGE, Melbourne, Australia, available from Phase Separations Ltd, Deeside Industrial Estate, Clywd, North Wales CH5 2LR). No pretreatment or extraction procedures were used. Contamination of the top 2–3 cm of the column occurred during volatile acid analyses and necessitated periodic replacement with fresh polymer. Overnight conditioning followed by 8-h purging restored normal operation.

For the non-volatile lactic and succinic acids the acidified culture supernates were methylated according to the method of Holdeman and Moore (1972) and a 0.6- μ l sample of the chloroform extract was injected on to the column operated under the same conditions as those used for volatile analyses. Electrometer attenuation was 2×10^{-2} for volatile analyses and 5×10^{-2} for non-volatile analyses.

The peaks were routinely identified by direct measurement of the relative retention times with the acetic-acid peak as a reference. Accurate identification was made by comparing retention times of test samples with those obtained with a series of chromatographically-pure aqueous standards.

Standards. Aqueous 0.01M standards of acetic, propionic, *iso*-butyric, *n*-butyric, *iso*-valeric and *n*-valeric acids (BDH Analar) were used in the volatile analyses to establish absolute retention times and quantitation values. A combined acid standard containing a 0.01M concentration of each acid was used to establish relative retention times; it was used each day to monitor column performance and sensitivity settings of the instrument. Aqueous 0.02M lactic and succinic acid standards were included with each batch of non-volatile (methylated) acid analyses. Samples of uninoculated sterile medium were included as controls in every batch of each type of analysis.

The approximate concentration values of acids (μ .moles per ml) in the test samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards.

RESULTS

Comparison of media for GLC analysis

Preliminary trials showed that PYG medium (Holdeman and Moore, 1972) was unsatisfactory for the culture of some strains of *Bacteroides* spp. whereas good growth of a wide range of species within the genus was obtained in media based on proteose peptone (BM, $\frac{1}{2}$ PPY12, PPYG, PPYSG, see *Methods*). There were no growth failures, and even asaccharolytic strains grew well from small inocula, although some required 3–4 days to do so. Some cultures of asaccharolytic strains produced little turbidity and good growth was confirmed by microscopy.

Initial studies with wild strains. BM medium was used for the GLC analyses on 30 of 72 subgingival isolates, and the remaining 42 strains were tested in $\frac{1}{2}$ PPY12 medium; 32 of the 72 strains were independently identified as *B. melaninogenicus* ss. *melaninogenicus*, and 40 strains were ss. *intermedius*. There were no differences in the ranges of relative amounts of fatty acids produced by the two subspecies in the two media. All 72 strains produced succinic and acetic acids in significant quantities ($> 10 \mu$.moles per ml) and all produced minor amounts of *iso*-valeric acid with trace amounts of *iso*-butyric acid. Most strains produced traces of propionic acid but none produced *n*-butyric acid. The two subspecies were generally distinguished by the amount of lactic acid produced: 38 of 40 ss. *intermedius* strains produced 60–150 (median 95) μ .moles lactic acid per ml, whereas 28 of 32 ss. *melaninogenicus* strains produced 4–60 (median 38) μ .moles per ml in single analyses on 3-day cultures. The

large amount of acetic acid in sterile BM medium led us to exclude it from our further quantitative GLC studies.

Studies with reference strains grown in a range of derived media. The changes in fatty acid production by five reference strains of the *B. melaninogenicus*-*B. oralis* group in the different media are shown in table I. No valid type strain of *B. oralis* is currently available, but strain NP333 shares many of the characteristics originally ascribed to this species (Loesche, Socransky and Gibbons, 1964). The three pigmented saccharolytic strains produced characteristic fatty-acid profiles that were consistent in all the media; succinic and acetic acids were the major products. *n*-Butyric acid was not produced by any saccharolytic strain, but it was a significant product of the asaccharolytic strain NCTC9337. This strain also differed from the four saccharolytic strains by not producing succinic acid in any medium.

The incorporation of glucose in the culture medium induced only minor changes in the range of acids produced. Strain NP333 produced *iso*-valeric and lactic acids only in glucose-free media. Glucose stimulated acetic acid production by the strains labelled VPI4196, ATCC15930 and NP333. Strain NCTC9336 produced less acetic, lactic, *iso*-butyric and *iso*-valeric acids in

TABLE I
Production of volatile fatty acids by five reference strains of *Bacteroides* spp. in six different peptone media*

Test strain	Test medium*	Approximate concentration of the stated fatty acid produced†						
		Acetic	Propionic	<i>iso</i> -Butyric	Butyric	<i>iso</i> -Valeric	Lactic	Succinic
<i>B. melaninogenicus</i> ss. <i>intermedius</i> NCTC9336	PPY	21	<1	2	0	3	110	26
	PPYS	25	<1	2	0	5	171	46
	‡PPY12	15	<1	1	0	3	90	25
	‡PPY12S	15	<1	2	0	3	131	26
	PPYG	16	<1	<1	0	1	62	35
	PPYSG	22	<1	<1	0	2	64	37
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i> VPI4196	PPY	11	<1	1	0	2	13	35
	PPYS	11	<1	1	0	2	14	37
	‡PPY12	6	<1	1	0	1	14	22
	‡PPY12S	6	<1	<1	0	1	13	25
	PPYG	20	<1	1	0	1	8	4
	PPYSG	20	1	1	0	1	6	39
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i> ATCC15930	PPY	10	<1	0	0	0	0	21
	PPYS	9	1	0	0	0	0	17
	‡PPY12	5	0	0	0	0	0	14
	‡PPY12S	10	<1	0	0	0	11	11
	PPYG	18	1	0	0	0	0	26
	PPYSG	14	0	0	0	0	0	27
<i>B. oralis</i> NP333	PPY	6	<1	0	0	1	32	17
	PPYS	4	<1	0	0	<1	11	17
	‡PPY12	4	<1	0	0	<1	9	8
	‡PPY12S	3	<1	0	0	<1	9	10
	PPYG	6	1	0	0	0	0	18
	PPYSG	10	1	0	0	0	0	22
<i>B. melaninogenicus</i> ss. <i>asaccharolyticus</i> NCTC9337	PPY	21	5	3	12	6	144	0
	PPYS	25	4	2	11	5	121	0
	‡PPY12	12	9	1	2	2	64	0
	‡PPY12S	19	12	2	4	3	145	0
	PPYG	25	6	3	13	6	148	0
	PPYSG	17	1	1	6	3	148	0

* See *Methods* section for details of the media.

† Fatty acid concentration expressed in μ moles per ml estimated by measurement of peak height with reference to aqueous standards corrected for values obtained with medium only. Results are those of single analyses for each sample.

glucose-enriched media; this was not attributable to less growth. Glucose did not affect acid production by strain NCTC9337.

The addition of serum had little or no effect on growth or fatty acid production by the four saccharolytic strains. The effect of serum on strain NCTC9337 was inconsistent: production of acetic acid was slightly enhanced by serum in the absence of glucose; growth and production of propionic acid were enhanced when serum and vitamin B₁₂ were added; production of other acids was not affected.

The effect of vitamin B₁₂ supplement. Strain NCTC9337 was grown for 23 h in $\frac{1}{2}$ PPY, $\frac{1}{2}$ PPY12, $\frac{1}{2}$ PPY12S, PPY and PPYS medium. The figure shows the volatile fatty-acid profiles obtained from the combined acid standard and the cultures, and table II shows the concentrations of the individual volatile fatty acids achieved in the various media. The production of acetic, *iso*-butyric and *iso*-valeric acids was directly proportional to turbidity. The production of propionic acid was significantly higher in the $\frac{1}{2}$ PPY12 and $\frac{1}{2}$ PPY12S media.

FIGURE.—The volatile fatty-acid profiles of *B. melaninogenicus* ss. *asaccharolyticus* strain NCTC9337, grown for 23 h in five different media, compared with the plot of a combined acid standard (VFA) to show the effect of vitamin B₁₂ (cyanocobalamin) and horse serum on growth and acid production. The various media are indicated at the bottom of the chart and the turbidity (T) of each test culture is given at the top of the chart. Each ml of the combined acid standard contained 10 μ .moles of the following acids: acetic (A), propionic (P), *iso*-butyric (iB), *n*-butyric (nB), *iso*-valeric (iV) and *n*-valeric (nV). W = "Water" peak. Details of the media used and the chromatographic conditions are given in the *Methods* section. Chart speed = 120 mm per h.

TABLE II

The volatile fatty acids produced by strain NCTC9337 in 23-h cultures of proteose peptone media

Medium	Turbidity at 600 nm	Approximate concentration (μ .moles per ml) of the stated acid				
		Acetic	Propionic	iso-Butyric	Butyric	iso-Valeric
1/2 PPY	0.365	0.87	<0.2	0.44	1.52	1.44
1/2 PPY12	0.63	4.2	3.04	0.68	1.44	1.8
PPY	0.79	6.1	<0.2	0.56	2.88	2.24
1/2 PPY12S	0.96	7.3	6.0	1.06	2.48	2.6
PPYS	1.15	9.6	0.7	1.06	5.76	3.37

Footnotes as in table I.

Serum and vitamin B₁₂ independently stimulated growth; together they produced an additive effect. The production of *n*-butyric acid was slightly inhibited by vitamin B₁₂.

Definitive studies of fatty acid production

Fatty acid profiles of reference strains grown in PPYSG. Table III shows the fatty acids produced by 12 reference strains of Bacteroidaceae in single analyses on 2-day cultures in PPYSG medium. The *Bacteroides* strains all produced similar fatty-acid profiles that were clearly distinct from the profiles of *Fusobacterium* and *Leptotrichia* strains. All *Bacteroides* strains produced succinic, acetic, lactic and propionic acids. The *Fusobacterium* strains produced moderate amounts of *n*-butyric acid and small quantities of lactic and propionic acids but not iso-butyric or iso-valeric acids; one strain produced a trace of succinic acid. The *Leptotrichia* isolate produced traces of acetic and succinic acids and a moderate amount of lactic acid only.

TABLE III

Approximate concentrations of fatty acids produced by 12 reference strains of the genera *Bacteroides*, *Fusobacterium* and *Leptotrichia* in 2-day cultures in PPYSG medium

Strain identity	Strain no.	Approximate concentration (μ .moles per ml) of the stated acid*						
		Acetic	Propionic	iso-Butyric	Butyric	iso-Valeric	Lactic	Succinic
<i>Bacteroides fragilis</i>	NCTC9344	22	1	0	0	<1	4	35
<i>Bacteroides fragilis</i>	NCTC8560	21	1	0	0	<1	4	33
<i>Bacteroides thetaiotaomicron</i>	NCTC10582	21	<1	0	0	0	3	26
<i>Bacteroides thetaiotaomicron</i>	ATCC8492	12	<1	0	0	0	2	19
<i>Bacteroides ovatus</i>	ATCC8483	21	1	0	0	<1	3	33
<i>Bacteroides distasonis</i>	ATCC8503	4	4	<1	0	<1	7	22
<i>Bacteroides vulgatus</i>	ATCC8482	5	5	<1	0	<1	17	18
<i>Bacteroides vulgatus</i>	NCTC10583	20	1	0	0	0	0	35
<i>Fusobacterium necrogenes</i>	NCTC10723	6	2	0	13	0	2	0
<i>Fusobacterium polymorphum</i>	NCTC10562	18	2	0	14	0	3	2
<i>Fusobacterium necrophorum</i>	NCTC10575	7	5	0	12	0	3	0
<i>Leptotrichia buccalis</i>	NCTC10249	1	0	0	0	0	22	2

* See footnote to table I.

Studies with isolates grown in glucose-enriched and glucose-free media. GLC was performed on glucose-enriched and glucose-free cultures of 55 saccharolytic subgingival-crevice isolates that included representative strains of *B. melaninogenicus*, *B. oralis*, *Fusobacterium* spp. and *L. buccalis*. Table IV shows the median values and the range of results obtained with 49 isolates tested in glucose-enriched media (PPYG; PPYSG) and 38 isolates tested in glucose-free media (PPY; $\frac{1}{2}$ PPY12). Thirty-two isolates were tested in both glucose-enriched and glucose-free media. The volatile fatty-acid profiles of the 49 saccharolytic strains in the *B. melaninogenicus*-*B. oralis* group were similar to the profiles of the four saccharolytic reference strains. *B. melaninogenicus* ss. *intermedius* strains tended to produce slightly more acetic acid than the ss. *melaninogenicus* strains, but there was considerable overlapping in the ranges of results. The ranges for the other acids produced by these two subspecies also overlapped; the different median values may reflect population differences, but individual strains could not be reliably distinguished. Similarly, *B. oralis* strains could not be distinguished from strains of *B. melaninogenicus* ss. *melaninogenicus* on the basis of the volatile fatty acids produced in the media used. Propionic acid was a minor product of most strains. The reduction in *iso*-valeric and *iso*-butyric acid production in glucose-enriched media correlated with increased growth in these media. *n*-Butyric acid was not produced by any of these strains. The two fusobacterium and four leptotrichia isolates gave volatile fatty-acid profiles that were distinct from each other and consistent with the profiles of the respective reference strains. The leptotrichia strains produced more acetic and succinic acids than the reference strain; two of the isolates produced traces of propionic acid in PPYG medium.

TABLE IV

Concentrations of fatty acids produced by 55 subgingival isolates in glucose-free and glucose-enriched media

Test species or subspecies	Number of strains	Medium*	Number of analyses	Median concentration (and range) of the stated acid† (μ .moles per ml)				
				Acetic	Propionic	<i>iso</i> -Butyric	Butyric	<i>iso</i> -Valeric
<i>B. melaninogenicus</i> ss. <i>intermedius</i>	20	Glu ⁻	17	18 (5-28)	<1 (0-1)	<1 (0-2)	0	3 (1-9)
		Glu ⁺	16	20 (5-25)	1 (0-5)	<1 (0-<1)	0	<1 (0-3)
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i>	22	Glu ⁻	22	9 (5-15)	<1 (0-2)	<1 (0-1)	0	2 (0-4)
		Glu ⁺	25	15 (5-27)	<1 (0-4)	0 (0-<1)	0	<1 (0-2)
<i>B. oralis</i>	7	Glu ⁻	3	14 (9-23)	<1 (0-1)	0 (0-<1)	0	1 (0-4)
		Glu ⁺	12	14 (8-26)	0 (0-2)	0	0	0 (0-<1)
<i>Fusobacterium</i> sp.	2	Glu ⁻	2	14	2	0	14	0
		Glu ⁺	2	11	2	0	14	0
<i>Leptotrichia buccalis</i>	4	Glu ⁻	1	9	0	0	0	0
		Glu ⁺	4	10 (4-20)	0 (0-<1)	0	0	0

* Glu⁻ = Glucose-free medium; Glu⁺ = glucose-enriched medium.

† See footnote in table I.

Quantitative lactic- and succinic-acid estimations were performed for 29 of the *B. melaninogenicus* and *B. oralis* strains (table V). The lactic acid concentrations were consistently reduced four-fold in glucose-enriched media with 21 strains tested in both glucose-enriched and glucose-free media. *B. melaninogenicus* ss. *intermedius* isolates produced less lactic acid (≤ 25 μ .moles per ml) than was produced by the reference strain NCTC9336. This finding was clarified by later studies reported below. *B. oralis* strains produced less lactic acid than *B. melaninogenicus* strains, but the ranges obtained in both glucose-enriched and glucose-free media overlapped. Five *B. oralis* strains failed to produce lactic acid in glucose-enriched media, but four strains produced 10 μ .moles or more per ml.

Clinical isolates grown in fully supplemented medium. As a result of the initial studies, PPYSG medium was selected for GLC analysis of the fatty acids produced by 42 clinical isolates of *Bacteroides* spp. Twelve asaccharolytic strains were tested after incubation for 7 days; all other isolates were tested after 2 days. The only *B. fragilis* isolate tested produced major amounts of succinic and acetic acids and a trace of propionic acid; two *B. ovatus* isolates produced similar profiles but one isolate also produced lactic acid 5 μ .moles per ml. One strain identified as *B. corrodens* produced a range of acids similar to that of asaccharolytic *B. melaninogenicus* strains; acetic and lactic acids were the major products with propionic, *iso*-butyric, *n*-butyric and *iso*-valeric acids as minor products; succinic acid was not produced. Seventeen strains of *B. melaninogenicus* ss. *asaccharolyticus* produced typical profiles; lactic (27–150 μ .moles per ml) and acetic (5–27 μ .moles per ml) acids were the major products and *n*-butyric acid was produced in moderate amounts (5–13

TABLE V

Production of lactic and succinic acids by 29 subgingival isolates of B. melaninogenicus and B. oralis in glucose-enriched and glucose-free media

Test species or subspecies	Medium*	Number of analyses	Median concentration (and range) of the stated acid (μ .moles per ml)	
			Lactic	Succinic
<i>B. melaninogenicus</i> ss. <i>intermedius</i>	Glu ⁻	10	75 (30-100)	26 (6-52)
	Glu ⁺	5	12 (5-25)	19 (10-38)
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i>	Glu ⁻	13	57 (23-91)	38 (10-74)
	Glu ⁺	15	8 (2-33)	21 (12-40)
<i>B. oralis</i>	Glu ⁻	2	20 & 33	16 & 17
	Glu ⁺	11	5 (0-15)	24 (10-40)

* Glu⁻ = Glucose-free medium; Glu⁺ = glucose-enriched medium.

μ .moles per ml); seven strains did not produce succinic acid but five strains produced >10 μ .moles per ml (10–19 μ .moles per ml); propionic and *iso*-valeric acids were minor products.

The saccharolytic strains of *B. melaninogenicus* studied in this section included eight faecal, two subgingival and eight high-vaginal-swab (HVS) isolates. All 18 strains (10 ss. *intermedius*; 8 ss. *melaninogenicus*) produced similar volatile fatty-acid profiles. The median values and the ranges of concentrations of the acids produced were virtually identical to those reported for the same subspecies in glucose-enriched media in table IV. Lactic and succinic acid results differed between the two subspecies on this occasion and also differed from the results reported in table V.

The ranges of values and the median and mean values obtained from faecal, subgingival and HVS isolates are compared in table VI. The values reported for the subgingival isolates include the results for strains included in table V and for nine strains of *B. melaninogenicus* ss. *melaninogenicus* from the collaborative survey (see below).

Subgingival strains of *B. melaninogenicus* ss. *intermedius* produced lactic and succinic acids in quantities similar to those produced by faecal strains of the same subspecies. Eight of 11 subgingival and faecal strains consistently produced lactic acid <30 μ .moles per ml and seven of 11 strains produced succinic acid <40 μ .moles per ml. Two faecal and one subgingival strain produced lactic acid >45 μ .moles per ml. HVS strains of *B. melaninogenicus* ss. *intermedius* produced more lactic and succinic acids than the subgingival or faecal strains. Three of four HVS strains consistently produced lactic acid >60 μ .moles per ml and the other strain produced lactic acid >60 μ .moles

TABLE VI

*Comparison of lactic and succinic acid production by saccharolytic strains of B. melaninogenicus from different clinical sites**

Test subspecies	Source	Number of strains	Number of analyses	Range of concentrations of the stated acid (μ .moles per ml)	Median values	Mean values
<i>B. melaninogenicus</i> ss. <i>intermedius</i>	Subgingival	6	10	Lactic 6–46	20	20.5
				Succinic 10–81	38	39.5
	Faecal	5	7	Lactic 10–52	25	28
				Succinic 9–71	40	37
	HVS†	4	7	Lactic 34–110	65	74
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i>				Succinic 24–85	68	65
	Subgingival	17‡	17	Lactic 2–33	8	9
				Succinic 12–40	19	23
	Faecal	3	3	Lactic 4–38	20	20.6
				Succinic 11–69	46	42
	HVS	4	4	Lactic 10–55	33	33.5
				Succinic 35–150	83	95

* All analyses on 2-day cultures in glucose-enriched medium.

† HVS = High vaginal swab.

‡ Includes our strains analysed in collaborative survey.

per ml in one of two cultures tested. All four HVS strains produced succinic acid $>66 \mu\text{moles}$ per ml although two of four strains did so in only one of two cultures tested. All analyses were performed on 2-day cultures in PPYSG medium and reasons for the culture-to-culture variation shown by a few strains were not elucidated.

Subgingival and faecal strains of *B. melaninogenicus* ss. *melaninogenicus* produced similar quantities of lactic and succinic acids. Seventeen of 20 strains produced lactic acid $<13 \mu\text{moles}$ per ml and 18 of 20 strains produced succinic acid $<40 \mu\text{moles}$ per ml. The high median value of $46 \mu\text{moles}$ per ml for succinic acid production by faecal strains in table VI is not significant where only three strains were studied. Three of four HVS strains of *B. melaninogenicus* ss. *melaninogenicus* produced lactic acid $>30 \mu\text{moles}$ per ml and succinic acid $>80 \mu\text{moles}$ per ml.

Three additional strains were provisionally identified as *B. melaninogenicus* ss. *intermedius* on the basis of indole production, gelatin digestion and the slow, weak fermentation of glucose. These isolates, however, produced *n*-butyric acid on two occasions in PPYSG medium; two also failed to produce succinic acid and were therefore more typical of asaccharolytic strains, but the third isolate produced succinic acid $18 \mu\text{moles}$ per ml.

Collaborative studies with B. melaninogenicus ss. *melaninogenicus*, *B. oralis* and *B. ochraceus*. Forty strains were tested as part of a collaborative investigation instigated by the ICSB Taxonomic Sub-Committee on Gram-negative anaerobic rods. Twelve strains were referred to us by the sub-committee, six strains were referred by other colleagues and 22 strains were isolated from subgingival plaque. Table VII shows the range of fatty acids produced by the strains. Loesche's strain 7CM designated *B. oralis* has been excluded from the *B. oralis* group in the table as it is more typical of a *B. fragilis* strain (see Holbrook, Duerden and Deacon, 1977); similarly, the referred strains ATCC15930, VPI7570A and no. 30 were found to produce black-pigmented colonies on lysed-blood agar and were included in the *B. melaninogenicus* ss. *melaninogenicus* group.

Table VIII shows population differences between the groups represented in the collaborative study. The individual results were assigned to four grades

TABLE VII
Fatty acids produced in PPYSG medium by strains included in the collaborative study

Test species or subspecies	Number of strains	Median concentration (and range) of the stated acid† (μmoles per ml)						
		Acetic	Propionic	iso-Butyric	Butyric	iso-Valeric	Lactic	Succinic
<i>Bacteroides melaninogenicus</i> ss. <i>melaninogenicus</i>	23*	20 (10-46)	1 (0-4)	0 (0-<1)	0	<1 (0-2)	7 (0-33)	22 (14-40)
<i>Bacteroides oralis</i>	10	15 (4-26)	<1 (0-2)	0	0	0 (0-<1)	0 (0-10)	21 (11-36)
<i>Bacteroides ochraceus</i>	6	10 (3-14)	0 (0-2)	0	0	0 (0-<1)	0 (0-5)	19 (11-31)
Loesche's strain 7CM	1‡	22	<1	0	0	0	0	45

* Includes ATCC15930, VPI7570A and 30 (see text and Holbrook and Duerden, 1974).

† See footnote to table I.

‡ Not included in *B. oralis* (see text).

TABLE VIII

The production of fatty acids by the 40 strains examined in the collaborative study

Fatty acid	Concentration of acid produced*	Percentage of strains producing the stated concentration of acid		
		<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i> (23 strains)	<i>B. oralis</i> (11 strains)	<i>B. ochraceus</i> (6 strains)
Acetic	+	0	18.2	66.7
	++	100	81.8	33.3
Propionic	—	13	36.4	50
	tr	34.8	36.4	16.7
<i>iso</i> -Butyric	+	52.2	27.2	33.3
	—	82.6	100	100
<i>n</i> -Butyric	tr	17.4	0	0
	—	100	100	100
<i>iso</i> -Valeric	—	21.7	63.6	83.3
	tr	69.5	36.4	16.7
<i>n</i> -Valeric	+	8.7	0	0
	—	100	100	100
Lactic	—	8.7	63.6	83.3
	tr	69.6	18.2	16.7
	+	17.4	18.2	0
	++	4.3	0	0
Succinic	+	39.1	45.5	50
	++	60.9	54.5	50

* The system used to grade the concentration values (μ .moles per ml) was as follows. (a) Volatile acids: ++ = >10; + = 1.1–10; tr = 0.2–1.0; — = <0.2. (b) Lactic and succinic acids: ++ = >20; + = 10–20; tr = 2–10; — = <2.0.

that appear to represent significant differences between strains. Individual strains of *B. melaninogenicus* ss. *melaninogenicus* could not be differentiated from strains of *B. oralis* on the basis of GLC results alone. With one exception, the median values and ranges of values in respect of the acids produced by *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis* strains were similar to the values obtained previously (see table IV) and overlapped those obtained with the *B. ochraceus* strains. However, *B. ochraceus* strains generally produced smaller quantities of acetic and lactic acids. It is of interest to note that three referred strains of *B. melaninogenicus* ss. *melaninogenicus* (WAL2724, WAL2721 and VPI7570A) produced acetic acid >28 μ .moles per ml.

A study of culture-to-culture variation. Despite careful attention to detail, particularly with regard to size and condition of inocula, incubation time, sample size at injection and the daily use of appropriate standards and controls, variation occurred in the quantitative results obtained from different cultures of the same strains. The results of analyses on two separate 2-day cultures of each of three reference strains in the same medium are shown in table IX. In all analyses the major products of each strain showed small variations but were always present in significant amounts. Minor products varied in concentration but were consistently detected; trace products were occasionally absent.

TABLE IX

The quantitative variation in fatty acid production by three reference strains of Bacteroides species or subspecies, analysed in PPYSG medium on two separate occasions

Organism	Study*	Approximate concentration of the stated acid†						
		Acetic	Propionic	iso-Butyric	Butyric	iso-Valeric	Lactic	Succinic
<i>B. oralis</i> strain NP333	I	19	2	0	0	1	3	36
	II	10	1	0	0	0	0	22
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i> strain ATCC15930	I	20	1	0	0	0	0	25
	II	14	0	0	0	0	0	27
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i> strain VPI4196	I	26	2	1	0	1	12	38
	II	20	1	1	0	1	6	39

* I = Collaborative study; II = media comparison study.

† See footnote to table I.

All analyses were performed on 2-day cultures.

DISCUSSION

All of the 185 Gram-negative, non-sporing, obligate anaerobes studied were identified to species or subspecies level by simple morphological, cultural, biochemical, tolerance and antibiotic-resistance tests (see Duerden *et al.*, 1976). All isolates were correctly identified to genus level by independently-performed GLC analyses of the short-chain fatty acids produced in glucose-free or glucose-enriched broth cultures. This study confirms that presumptive identification of genus can be made on the basis of Gram stain, colonial morphology and fermentation products (see Holdeman and Moore, 1974), and that GLC data must be supplemented with other test data in order to sub-classify the *Bacteroides* and *Fusobacterium* genera.

Essentially similar fatty-acid profiles were obtained with test strains of *B. ovatus* and *B. fragilis* and with reference strains of *B. fragilis* representing all of the *B. fragilis* subspecies now accorded species rank (see Cato and Johnson, 1976). The major products were succinic and acetic acids with traces of lactic and propionic acids; occasional strains produced traces of iso-valeric and iso-butyric acids. Moreover, all the *B. fragilis*-like strains tested produced fatty acids similar to those produced by the saccharolytic *B. melaninogenicus* strains; quantitative differences and minor qualitative differences existed, but none was of clear discriminatory value at the species or subspecies level. Furthermore, it was impossible to differentiate *B. oralis* from *B. melaninogenicus* ss. *melaninogenicus* and *B. corrodens* from *B. melaninogenicus* ss. *asaccharolyticus*. The status of *B. oralis* (Loesche *et al.*, 1964) as a separate species is currently debated (ICSB Minute, in press); the strains may be non-pigmented variants of *B. melaninogenicus* ss. *melaninogenicus* (Harding *et al.*, 1976; Holbrook *et al.*, 1977). The similarity of *B. oralis* strains to *B. ruminicola* has also been noted (Sundqvist, 1976; Terada, Uchida and Mitsuoka, 1976). It appears that the strains may only be differentiated by arabinose, xylose and salicin fermentation. The six *B. ochraceus* strains were not distinguished with certainty from the *B. melaninogenicus*-*B. oralis* group. Strains of the two

saccharolytic subspecies of *B. melaninogenicus* produced succinic and acetic acids as major products in all of the test media; production of lactic acid was variable, and there were frequently minor amounts of propionic, *iso*-valeric and *iso*-butyric acids, but *n*-butyric acid was not produced. The strains were clearly distinct from the asaccharolytic subspecies which, in glucose-enriched or glucose-free media, typically produced moderate to large quantities of acetic, lactic and *n*-butyric acids and minor amounts of propionic, *iso*-butyric and *iso*-valeric acids; some produced succinic acid. Isolates belonging to the genus *Fusobacterium* typically produced acetic and *n*-butyric acids with traces of lactic and propionic acids, but did not produce *iso*-butyric or *iso*-valeric acid. The only significant products of isolates belonging to the genus *Leptotrichia* were moderate amounts of lactic and acetic acids. These conclusions are summarised in table X.

The use of aqueous, semi-quantitative GLC analyses of the fermentation products of a wide range of reference and clinical strains of bacteroides organisms in this study revealed the considerable biological variation as reflected in the end products of fermentation that exists within recognised groups of related organisms. In the *B. melaninogenicus*-*B. oralis* group, strain-to-strain and culture-to-culture variation occurred despite careful standardisation of methods; this obliged us to follow the example of Holdeman and Moore (1972) and we developed a scoring system that took account of these variations but allowed recognition of meaningful differences between strains that might be useful in identification.

Shah *et al.* (1976) recently compared the biochemical properties of 45 *B. melaninogenicus* strains and reported differences in the fatty acid profiles of the two saccharolytic subspecies. Lactic and propionic acids were not detected in BM broth cultures of either subspecies. No *B. melaninogenicus* ss. *melaninogenicus* strain was found to produce *iso*-butyric acid but most ss. *intermedius* strains produced traces of it. Our results differ from those of Shah *et al.* (1976) who also noted discrepancies between their findings and those of Holdeman and Moore (1972) and Lambe (1974). In our experience the detection of lactic or succinic acid in concentrations of $<5 \mu\text{moles per ml}$ in the aqueous volatile analyses was unreliable and methylation was necessary. Other small differences in the cultural or chromatographic procedures may account for other variations between our results and those of Shah *et al.* (1976). We consider that the occurrence of such discrepancies in the determination of minor products precludes the use of such small differences for subspecies identification or taxonomic differentiation of strains without support from other tests. We agree with Moore (1970) who stated that the *major* fatty acid products are stable characteristics and it is apparent that these products are consistently and reliably detected by different groups of workers.

The observation that vaginal isolates of saccharolytic *B. melaninogenicus* strains produce more lactic and succinic acids than oral or faecal strains of the same subspecies is interesting. Insufficient numbers were examined to draw definite conclusions; a population difference may exist between the intestinal and vaginal strains and biotyping might be possible on this basis.

TABLE X
Typical fermentation patterns in the family Bacteroidaceae

Species and subspecies	Concentration* of the stated acid produced					
	Acetic	Propionic	iso-Butyric	Butyric	iso-Valeric	Lactic
<i>B. fragilis</i> -like strains	++†	tr	(tr)	0	(tr)	tr
<i>B. melaninogenicus</i>	++	0-tr(++)	0+	0	0+	++
ss. <i>intermedius</i>	++	0-tr(++)	0-tr	0	0+	++
ss. <i>melaninogenicus</i>	++	0-tr(++)	0-tr	0	0-tr	++
<i>B. oralis</i>	++	0+	0	0	0-tr	++
<i>B. ochraceus</i>	++	0+	0	0	0-(tr)	++
<i>B. melaninogenicus</i>	++	+	(tr)-	++	+	0+
ss. <i>asaccharolyticus</i>	++	+	0	++	0	0-tr
<i>Fusobacterium</i> spp.	tr-++	0-(tr)	0	0	0	tr
<i>Leptotrichia buccalis</i>						

* See footnote to table VIII for the system used to grade the concentration values. The fatty acid concentrations were estimated as described in the footnote to table I.

† One reference strain each of *B. distasonis* and *B. vulgatus* produced + value only.

() = Occasional strains only.

The symbols in bold type indicate results likely to be of discriminatory value.

The BM medium of Williams *et al.* (1975), similar to the isolation medium of Schaedler, Dubos and Costello (1965), proved to be an excellent culture medium and, with meat particles added, it is a useful maintenance medium for a wide range of anaerobic bacteria. We found that the high acetic-acid content of BM medium, contributed by trypticase, limited its value for our GLC studies and it was used only briefly in our preliminary series with freshly-isolated oral strains.

The effect of restricted changes in the growth medium on fatty acid production was evaluated with five reference strains of the *B. melaninogenicus*-*B. oralis* group in six proteose peptone media that did not contain trypticase (BBL). Each strain produced a characteristic range of acids that was consistent in all the media except for the putative *B. oralis* strain NP333 that produced traces of *iso*-valeric and lactic acids in glucose-free media only. Glucose in general affected the amounts but not the range of acids produced by saccharolytic strains. It did not affect acid production by the single asaccharolytic strain NCTC9337. Similar observations have been reported previously (Werner, Pulverer and Reichertz, 1971; Werner, 1974; Mayhew, Onderdonk and Gorbach, 1975) and support the general conclusion of Holdeman and Moore (1975) that chromatographic analyses could be run on any medium.

The choice of fully-supplemented PPYSG medium, containing proteose peptone, 2% serum and 1% glucose, for definitive GLC studies was based on the following considerations. (1) The full-strength PPY media generally gave better growth rates of a wide range of strains and the addition of serum enhanced the growth of some strains without affecting the fatty-acid profiles. We were concerned to achieve rapid growth wherever possible, to minimise incubation times and to facilitate rapid identification of our isolates. (2) Many clinical isolates are likely to be saccharolytic strains that utilise glucose preferentially and grow more rapidly on this substrate. Asaccharolytic strains may show a slightly-increased lag phase in glucose-enriched media (Lev and Milford, 1975) but in our experience their fatty-acid profiles were not affected. Where glucose is the preferred substrate for energy production, its metabolism will generally cause catabolite repression of amino-acid utilisation, but Loesche and Gibbons (1968) and Wahren and Gibbons (1970) have shown that fermentation of amino acids by some strains of *F. nucleatum* and *B. melaninogenicus* is not repressed by glucose. It therefore seemed reasonable to include glucose in the medium.

The growth and fatty-acid production of the *B. melaninogenicus* ss. *asaccharolyticus* strain NCTC9337 was significantly affected by vitamin B₁₂. Growth and propionic-acid production were stimulated by cyanocobalamin 0.1 µg per ml, but production of *n*-butyric acid was partially inhibited. The initial observation of growth stimulation was made in the course of other studies. The concentration used in the present study was previously found to provide maximum growth stimulation (Deacon, unpublished data). Varel and Bryant (1974) reported that *B. fragilis* strain NCTC9343 required vitamin B₁₂ 0.1 ng per ml for good growth in a defined medium. To our knowledge, the minimal vitamin-B₁₂ growth requirements of *B. melaninogenicus* ss.

asaccharolyticus strains have not been determined and we are not aware of any previous reports of the pronounced effects that high concentrations of the vitamin can have on the growth and metabolism of these strains. Further studies are in progress.

The GLC approach used in this study provided quantitative data useful in identification of our isolates. There is a high degree of correlation with the other independently-performed tests. GLC may expedite the identification of clinical isolates to generic level without reliance on many other observations and tests, but it is clear that some basic data are necessary, even at this level, and additional information is required for species or subspecies identification. The appeal of GLC techniques for clinical laboratories is likely to be increased with recent reports suggesting that it is possible to detect the presence of anaerobes in clinical specimens by direct analyses on pus or other material (Bricknell, Sugihara and Brook, 1976; Gorbach *et al.*, 1976; Phillips, Tearle and Willis, 1976). However, this calls for skilled and experienced staff, able to monitor and interpret daily analyses. There must also be a sufficient number of specimens yielding anaerobes to justify the capital outlay for the equipment. At present, we consider that if clinical microbiologists are encouraged to use existing GLC facilities available in reference laboratories, they will contribute to the further refinement and development of the procedures and to clarification of the remaining areas of confusion in the taxonomy of the anaerobic bacteria.

SUMMARY

The acid end-products of 185 isolates from the family Bacteroidaceae were separated and analysed by gas-liquid chromatography on broth cultures. Different media were evaluated and definitive studies were performed in a fully supplemented complex medium. The limitations of this approach to the identification of a wide range of strains from various clinical sources were determined and the results were compared with those of a series of morphological, biochemical, tolerance and antibiotic-resistance tests.

All test strains were identified to generic level by simple microscopic and colonial observations and GLC analysis; additional tests were required to allow species or subspecies identification of most strains. Population differences were detected between some species or subspecies isolated from different clinical sites by quantitative analyses of fatty acids, but individual strains could not always be separated because of overlapping ranges of distribution of acids that were common products of more than one species or subspecies. Small differences in minor products between different species or subspecies were variable and are not considered adequate for discrimination at these taxonomic levels without support from other observations.

The potential application of the GLC technique to the rapid and accurate identification of these organisms in hospital laboratories is considered.

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REFERENCES

- AALBAEK, B. 1973. Bacteroidaceae, status of classification and identification of anaerobic non-sporing, gram-negative rods. *Nord. VetMed.*, **25**, 409.
- BARNES, E. M. AND IMPEY, C. S. 1971. The isolation of anaerobic bacteria from chicken caeca with particular reference to members of the Family Bacteroidaceae. In *Isolation of Anaerobes*, Society for Applied Bacteriology Technical Series No. 5, edited by D. A. Shapton and R. G. Board, London and New York, p. 115.
- BRICKNELL, K. S., SUGIHARA, P. T. AND BROOK, I. 1976. Optimizing methylation conditions for gas-liquid chromatography. Assay of lactic acid in biological samples. Annual Meeting American Society for Microbiology, abstract C 108.
- CARLSSON, J. 1973. Simplified gas chromatographic procedure for identification of bacterial metabolic products. *Appl. Microbiol.*, **25**, 287.
- CATO, E. P., CUMMINS, C. S., HOLDEMAN, L. V., JOHNSON, J. L., MOORE, W. E. C., SMIBERT, R. M. AND SMITH, L. D. S. 1970. Outline of clinical methods in anaerobic bacteriology, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg.
- CATO, E. P. AND JOHNSON, J. L. 1976. Reinstatement of species rank for *Bacteroides fragilis*, *B. ovatus*, *B. distasonis*, *B. thetaiotaomicron* and *B. vulgatus*: designation of neotype strains for *B. fragilis* (Veillon and Züber) Castellani and Chalmers and *B. thetaiotaomicron* (Distaso) Castellani and Chalmers. *Int. J. syst. Bact.*, **26**, 230.
- COLLEE, J. G., WATT, B., FOWLER, E. B. AND BROWN, R. 1972. An evaluation of the Gaspak System in the culture of anaerobic bacteria. *J. appl. Bact.*, **35**, 71.
- DUERDEN, B. I., HOLBROOK, W. P., COLLEE, J. G. AND WATT, B. 1976. The characterization of clinically important Gram-negative anaerobic bacilli by conventional bacteriological tests. *J. appl. Bact.*, **40**, 163.
- FINEGOLD, S. M. 1974. Intra-abdominal, genito-urinary, skin and soft tissue infections due to non-sporing anaerobic bacteria. In *Infection with non-sporing anaerobic bacteria*, edited by I. Phillips and M. Sussman, Edinburgh, p. 160.
- FINEGOLD, S. M., SUGIHARA, P. T. AND SUTTER, V. L. 1971. Use of selective media for isolation of anaerobes from humans. In *Isolation of anaerobes*, Society for Applied Bacteriology. Technical Series No. 5, edited by D. A. Shapton and R. G. Board, London and New York, p. 99.
- GIBBONS, R. J. AND MACDONALD, J. B. 1960. Hemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. *J. Bact.*, **80**, 164.
- GORBACH, S. L., MAYHEW, J. W., BARTLETT, J. G., THADEPALLI, H. AND ONDERDONK, A. B. 1976. Rapid diagnosis of anaerobic infections by direct gas-liquid chromatography of clinical specimens. *J. clin. Invest.*, **57**, 478.
- GUILLAUME, J., BEERENS, H. AND OSTEUX, R. 1956. La chromatographie sur papier des acides aliphatiques volatils de C₁ à C₆. Son application à la détermination des bactéries anaérobies. *Annls Inst. Pasteur Lille*, **8**, 13.
- HARDING, G. K. M., SUTTER, V. L., FINEGOLD, S. M. AND BRICKNELL, K. S. 1976. Characterization of *Bacteroides melaninogenicus*. *J. clin. Microbiol.*, **4**, 354.
- HOLBROOK, W. P. 1976. The isolation of *Bacteroides melaninogenicus* from the human mouth. *J. dent. Res.*, **55**, Special issue D, Abstract no. 81, D126.
- HOLBROOK, W. P. AND DUERDEN, B. I. 1974. A comparison of some characteristics of reference strains of *Bacteroides oralis* with *Bacteroides melaninogenicus*. *Archs oral Biol.*, **19**, 1231.
- HOLBROOK, W. P., DUERDEN, B. I. AND DEACON, A. G. 1977. The classification of *Bacteroides melaninogenicus* and related species. *J. appl. Bact.*, **42**, 259.
- HOLDEMAN, L. V. AND MOORE, W. E. C. 1972. (Editors). *Anaerobe Laboratory Manual*, Virginia Polytechnic Institute and State University, Blacksburg.

- HOLDEMAN, L. V. AND MOORE, W. E. C. 1974. Gram-negative anaerobic bacteria. In Bergey's manual of determinative bacteriology, 8th ed., edited by R. E. Buchanan and N. E. Gibbons, Baltimore, p. 384.
- HOLDEMAN, L. V. AND MOORE, W. E. C. 1975. Identification of anaerobes in the clinical laboratory. *Am. J. med. Technol.*, **41**, 49.
- LAMBE, D. W. JR 1974. Determination of *Bacteroides melaninogenicus* serogroups by fluorescent antibody staining. *Appl. Microbiol.*, **28**, 561.
- LEV, M. AND MILFORD, A. F. 1975. Sensitivity of a *Bacteroides melaninogenicus* strain to monosaccharides: effect on enzyme induction. *J. Bact.*, **121**, 152.
- LOESCHE, W. J. AND GIBBONS, R. J. 1968. Amino acid fermentation by *Fusobacterium nucleatum*. *Archs oral Biol.*, **13**, 191.
- LOESCHE, W. J., SOCRANSKY, S. S. AND GIBBONS, R. J. 1964. *Bacteroides oralis*, proposed new species isolated from the oral cavity of man. *J. Bact.*, **88**, 1329.
- MAYHEW, J. W., ONDERDONK, A. B. AND GORBACH, S. L. 1975. Effects of time and growth media on short-chain fatty acid production by *Bacteroides fragilis*. *Appl. Microbiol.*, **29**, 472.
- MOORE, W. E. C. 1970. Relationships of metabolic products to taxonomy of anaerobic bacteria. *Int. J. syst. Bact.*, **20**, 535.
- MOORE, W. E. C., CATO, E. P. AND HOLDEMAN, L. V. 1969. Anaerobic bacteria of the gastrointestinal flora and their occurrence in clinical infections. *J. infect. Dis.*, **119**, 641.
- PHILLIPS, K. D., TEARLE, P. V. AND WILLIS, A. T. 1976. Rapid diagnosis of anaerobic infections by gas-liquid chromatography of clinical material. *J. clin. Path.*, **29**, 428.
- SCHAEGLER, R. W., DUBOS, R. AND COSTELLO, R. 1965. The development of the bacterial flora in the gastrointestinal tract of mice. *J. exp. Med.*, **122**, 59.
- SHAH, H., WILLIAMS, R. A. D., BOWDEN, G. H. AND HARDIE, J. M. 1967. Comparison of the biochemical properties of *Bacteroides melaninogenicus* from human dental plaque and other sites. *J. appl. Bact.*, **41**, 473.
- SUNDQVIST, G. K. O. 1976. Bacteriological studies of necrotic pulps. *Odontological dissertations*, No. 7, University of Umeå, Sweden.
- TERADA, A., UCHIDA, K. AND MITSUOKA, T. 1976. Die Bacteroidaceenflora in dem Faeces von Schweinen. *Zentbl. Bakt. ParasitKde*, I. Abt. Orig. A, **234**, 362.
- VAREL, V. H. AND BRYANT, M. P. 1974. Nutritional features of *Bacteroides fragilis* subsp. *fragilis*. *Appl. Microbiol.*, **28**, 251.
- WAHREN, A. AND GIBBONS, R. J. 1970. Amino acid fermentation by *Bacteroides melaninogenicus*. *Antonie van Leeuwenhoek*, **36**, 149.
- WATT, B. 1973. The influence of carbon dioxide on the growth of obligate and facultative anaerobes on solid media. *J. med. Microbiol.*, **6**, 307.
- WERNER, H. 1969. *Bacteroides convexus* Eggerth und Gagnon 1933 identisch mit *Bacteroides fragilis* (Veillon und Züber 1898) Castellani und Chalmers 1919. *Zentbl. Bakt. ParasitKde*, Abt. Orig., **211**, 344.
- WERNER, H. 1974. Differentiation and medical importance of saccharolytic intestinal Bacteroides. *Arzneimittel-Forsch.*, **24**, 340.
- WERNER, H., PULVERER, G. AND REICHERTZ, C. 1971. The biochemical properties and antibiotic susceptibility of *Bacteroides melaninogenicus*. *Med. Microbiol. Immunol.*, **157**, 3.
- WILLIAMS, R. A. D., BOWDEN, G. H., HARDIE, J. M. AND SHAH, H. 1975. Biochemical properties of *Bacteroides melaninogenicus* subspecies. *Int. J. syst. Bact.*, **25**, 298.