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GAS CHROMATOGRAPHIC–MASS SPECTRAL STUDIES AFTER METHYLATION OF METABOLITES PRODUCED BY SOME ANAEROBIC BACTERIA IN SPENT MEDIA

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SUMMARY

The gas chromatographic analysis of metabolites such as volatile fatty acids (VFAs) and non-volatile fatty acids (NVFAs) for identification of anaerobic bacteria is now widely performed. Cultures of anaerobes tested for NVFAs as methyl esters were found to contain several unidentified compounds not previously detected and/or reported with methylation procedures. Gas chromatographic–mass spectrometric studies demonstrated that these compounds correspond to the methyl esters of both saturated and unsaturated short-chain fatty acids, and also of 2-hydroxy and 2-oxo acids. The distribution of these acids among different species of anaerobes was determined and their amounts were measured. The effects of supplementing the culture medium with either glucose or amino acids on the production of these acids are described. The use of very polar stationary phases is suggested for a better separation of all NVFAs.

INTRODUCTION

The analysis of volatile and non-volatile fatty acids (VFAs and NVFAs, respectively) by gas chromatography (GC) is a widely accepted method for the characterization of anaerobic bacteria at the genus or species level. Usually NVFAs are analysed as methyl or butyl esters [1–3], or after derivatization with heptafluorobutyric anhydride–pyridine–ethanol [4], and chromato-

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graphed on moderately polar stationary phases (e.g., SP-1000, OV-101, FFAP) with either packed or capillary columns.

Metabolic products detected after methylation include pyruvic, lactic, fumaric and succinic acids [1] and aromatic acids, e.g., phenylacetic acid [5] and 3-phenylpropionic acid [6]. They are identified by comparing their retention times with those of standards processed in the same way.

However, many anaerobes produce a variety of compounds which have not been identified, and which elute as sharp and relatively well resolved peaks. Using gas chromatography–mass spectrometry (GC–MS), we have recently identified two of these peaks as 2-hydroxybutanoic and 2-hydroxypentanoic acids as methyl esters (MeE) from spent media of *Fusobacterium* species and *Clostridium innocuum* [7]. Moreover, we observed that 2-hydroxybutanoic acid MeE eluted with 2,2-dimethoxypropanoic acid MeE, formed by methylation of pyruvic acid in addition to methyl pyruvate. This study was undertaken to investigate further the structures, taxonomic value and possibility of interference of these compounds with acids commonly found in anaerobic bacterial cultures.

EXPERIMENTAL

Bacteria and culture conditions

Reference strains and clinical isolates identified as members of *Clostridium* (12), *Peptostreptococcus* (3), *Propionibacterium* (3), *Bifidobacterium* (2), *Capnocytophaga* (2) and *Bacteroides* (6) genera were studied (Table I). They were identified according to the VPI Anaerobe Laboratory Manual [1]. Strains were grown in trypticase–glucose–yeast broth (TGY) at 37°C as described previously [8].

Analytical procedures

GC and GC–MS analyses were carried out as reported earlier [7]. Briefly, NVFAs were converted into methyl esters, which were extracted with chloroform. For GC analyses, the chloroform extract containing methyl esters was analysed as follows:

(1) with an Intersmat gas chromatograph (Model DFL121) equipped with a flame ionization detector and a glass column packed with 10% SP-1000 and 1% phosphoric acid (Supelco, Gland, Switzerland);

(2) by GC–MS determination with a Nermag R10-10C quadrupole GC–MS system using (i) a fused-silica capillary column coated with SP-1000 (Chrompack France, Les Ulis, France), with other details as described previously [7], and (ii) a 50 m×0.22 mm I.D. fused-silica capillary column coated with CP-Sil 88 (d_f=0.2 μm) (Chrompack France). In the latter instance the instrument was programmed from 80 to 120°C at 2°C/min. The GC–MS procedures were performed with either electron-impact ionization (EI) or positive ion

TABLE I

ANAEROBIC BACTERIAL STRAINS STUDIED

Species	Strain designation	Source[a]
Clostridium bifermentans	TM	1
Clostridium botulinum A	PP	1
Clostridium botulinum B	YB	1
Clostridium difficile	ATCC 9689^{Tb}	2
Clostridium difficile	538.87	3
Clostridium difficile	316.88	3
Clostridium paraputrificum	200.87	3
Clostridium perenne	189.87	3
Clostridium sordellii	82	1
Clostridium sporogenes	ATCC 3584^{T}	2
Clostridium sporogenes	144.87	3
Clostridium sporogenes	381.87	3
Peptostreptococcus anaerobius	ATCC 27337^{T}	2
Peptostreptococcus asaccharolyticus	360.88	3
Peptostreptococcus prevotii	AIP 413.88	3
Propionibacterium acnes	CIP 53117 = ATCC 6919^{T}	4
Propionibacterium avidum	AIP 1689B = ATCC 25577^{T}	1
Propionibacterium granulosum	135.87	3
Bifidobacterium adolescentis	194Ia = ATCC 15703^{T}	1
Bifidobacterium bifidum	Tissier^{T}	1
Capnocytophaga gingivalis	DSM 3290 = ATCC 33624^{T}	5
Capnocytophaga sputigena	DSM 3292 = ATCC 33612^{T}	5
Bacteroides bivius	VPI 6822 = ATCC 29303^{T}	6
Bacteroides distasonis	NCTC 11152 = ATCC 8503^{T}	7
Bacteroides fragilis	NCTC 9343 = ATCC 25285^{T}	7
Bacteroides vulgatus	DSM 1447 = ATCC 8482^{T}	5
Bacteroides endodontalis	ATCC 35406^{T}	2
Bacteroides gingivalis	ATCC 33277^{T}	2

[a]1, Collection des Anaérobies de l'Institut Pasteur (AIP), Paris, France; 2, American Type Culture Collection (ATCC), Rockville, MD, U.S.A.; 3, Clinical isolates from Unité des Anaérobies, Institut Pasteur, Paris, France; 4, Collection de l'Institut Pasteur, Paris, France; 5, Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G.; 6, Virginia Polytechnic Institute and State University (VPI), Blacksburg, VA, U.S.A.; 7, National Collection of Industrial Bacteria, Aberdeen, U.K.
[b]T = Type strain.

chemical ionization (PICI) with ammonia or trideuteroammonia (N^2H_3) as reactant gas.

Reagents

Most VFAs (acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic acids), most NVFAs (fumaric, succinic, phenylacetic, 3-phenylpropionic acids), methanol and chloroform were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). DL-Hydroxy acids, DL-oxo acids and L-amino

acids were obtained from Sigma (St. Louis, MO, U.S.A.) as either free acids or sodium salts. DL-5-Methylhexanoic acid was from ICN Biomedical, K&K Labs. Div. (Plainview, NY, U.S.A.), *trans*-4-methyl-2-pentenoic acid from Interchim (Montluçon, France), *trans*-2-hexenoic acid from Aldrich (Strasbourg, France) and *o*-toluic acid (internal standard) from Prolabo (Paris, France).

RESULTS

Gas chromatographic separation

Gas chromatograms obtained after methylation of spent culture media are shown in Fig. 1 for (A) *Clostridium difficile* ATCC 9689^T, (B) *Propionibacter-*

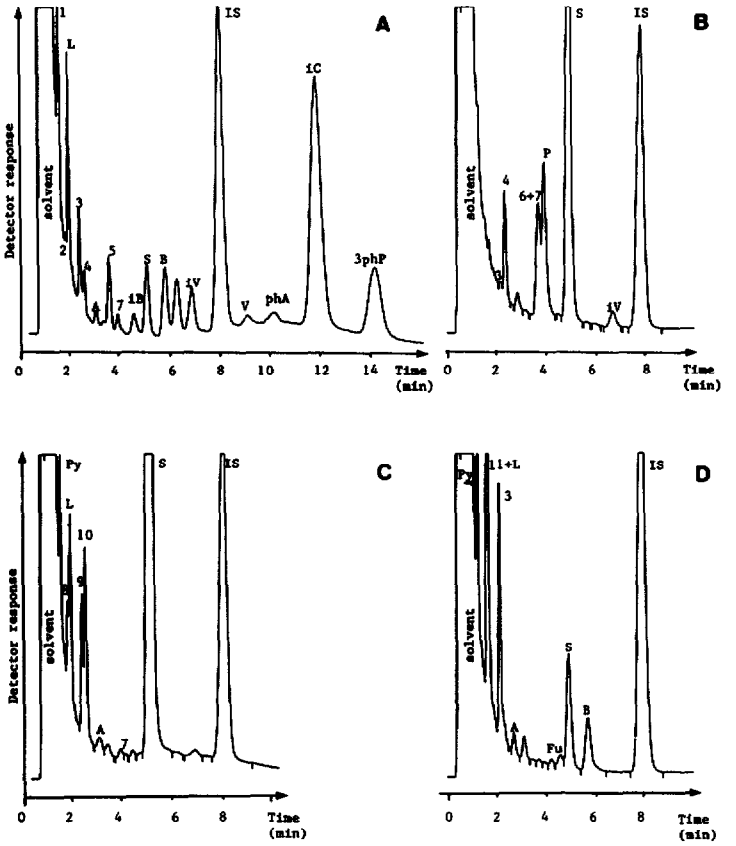


Fig. 1. Chromatograms of a chloroform extract of methylated TGY broth cultures of (A) *Clostridium difficile* ATCC 9689^T, (B) *Propionibacterium avidum* AIP 1689B^T, (C) *Capnocytophaga sputigena* DSM 3292^T and (D) *Peptostreptococcus prevotii* AIP 413.88 (see Table I for descriptions). GC conditions: oven temperature, 135 °C; injector and detector block temperature, 200 °C; nitrogen flow-rate, 30 ml min^{-1}; electrometer sensitivity, 10^{-10} A full-scale and attenuation 1; recording integrator sensitivity, 1 mV full-scale. Peaks: 1-11 = unknown compounds I-XI; IS = internal standard (*o*-toluic acid, final concentration 5 mmol l^{-1}); for other abbreviations, see

TABLE II

ORGANIC ACIDS DETECTED FROM SPENT MEDIA OF VARIOUS ANAEROBIC SPECIES AFTER METHYLATION

Systematic name	Common name	Abbreviation[a]	RRT[b]	Eluted as
4-Methylpentanoic	Isocaproic	4-Me $C_{5\ 0}$ (iC)	0.1225	Methyl ester
Hexanoic	Caproic	$C_{6\ 0}$ (C)	0.1380	Methyl ester
2-Oxopropanoic	Pyruvic	2-oxo $C_{3\ 0}$ (Py)	0.1587	Methyl ester
5-Methylhexanoic	Isoheptanoic	5-Me $C_{6\ 0}$	0.1638	Methyl ester
4-Methyl-2-pentenoic	-	4-Me $C_{5\ 1}$	0.1690	Methyl ester
3-Methyl-2-oxobutanoic	-	3-Me 2-oxo $C_{4\ 0}$	0.1961	Methyl ester
2-Hexenoic	-	$C_{6\ 1}$	0.2000	Methyl ester
2-Oxobutanoic	-	2-oxo $C_{4\ 0}$	0.2038	Methyl ester
2-Hydroxypropanoic	Lactic	2-OH $c_{3\ 0}$ (L)	0.2129	Methyl ester
2-Hydroxybutanoic	-	2-OH $C_{4\ 0}$	0.2645	Methyl ester
3-Methyl-2-oxopentanoic	-	3-Me 2-oxo $C_{5\ 0}$	0.2645	Methyl ester
2,2-Dimethoxypropanoic	-	–	0.2645	Methyl ester
4-Methyl-2-oxopentanoic	-	4-Me 2-oxo $C_{5\ 0}$	0.2851	Methyl ester
2-Hydroxy-3-methylbutanoic	-	2-OH 3-Me $C_{4\ 0}$	0.2916	Methyl ester
Ethanoic	Acetic	$C_{2\ 0}$ (A)	0.3600	Free acid
2-Hydroxypentanoic	-	2-OH $C_{5\ 0}$	0.3870	Methyl ester
Compound V[c]	-	-	0.4219	Methyl ester
2-Hydroxy-3-methylpentanoic	-	2-OH 3-Me $C_{5\ 0}$	0.4580	Methyl ester
2-Hydroxy-4-methylpentanoic	-	2-OH 4-Me $C_{5\ 0}$	0.4722	Methyl ester
Propanoic	Propionic	$C_{3\ 0}$ (P)	0.5032	Free acid
trans-Butenedioic	Fumaric	- (Fu)	0.5264	Dimethyl ester
2-Methylpropanoic	Isobutyric	2-Me $C_{3\ 0}$ (iB)	0.5600	Free acid
Butanedioic	Succinic	- (S)	0.6219	Dimethyl ester
Butanoic	Butyric	$C_{4\ 0}$ (B)	0.7251	Free acid
3-Methylbutanoic	Isovaleric	3-Me $C_{4\ 0}$ (iV)	0.8606	Free acid
Pentanoic	Valeric	$C_{5\ 0}$ (V)	1.1483	Free acid
Phenylethanoic	Phenylacetic	- (phA)	1.2709	Methyl ester
4-Methylpentanoic	Isocaproic	4-Me $C_{5\ 0}$ (iC)	1.5096	Free acid
3-Phenylpropanoic	3-Phenylpropionic	- (phP)	1.8064	Methyl ester
Hexanoic	Caproic	$C_{6\ 0}$ (C)	1.8064	Free acid

[a]The number to the left of the colon refers to the number of carbon atoms in the chain; the number to the right indicates the number of double bonds; 2-OH, 3-Me and 4-Me denote the position of the hydroxy or methyl group, respectively. Abbreviations in parentheses according to VPI Anaerobe Laboratory Manual [1], except phA and phP.

[b]Relative retention time with respect to *o*-toluic acid (retention time 7.75 min). GC conditions are described in the legend of Fig. 1.

[c]This compound has not yet been identified on the basis of GC–MS data (see text).

ium avidum AIP 1689B^T (ATCC 25577^T), (C) *Capnocytophaga sputigena* DSM 3292^T and (D) *Peptostreptococcus prevotii* AIP 413.88. These GC profiles exhibit most of the VFAs and NVFAs which may be detected from various anaerobes. Most of the peaks correspond to compounds described in the literature

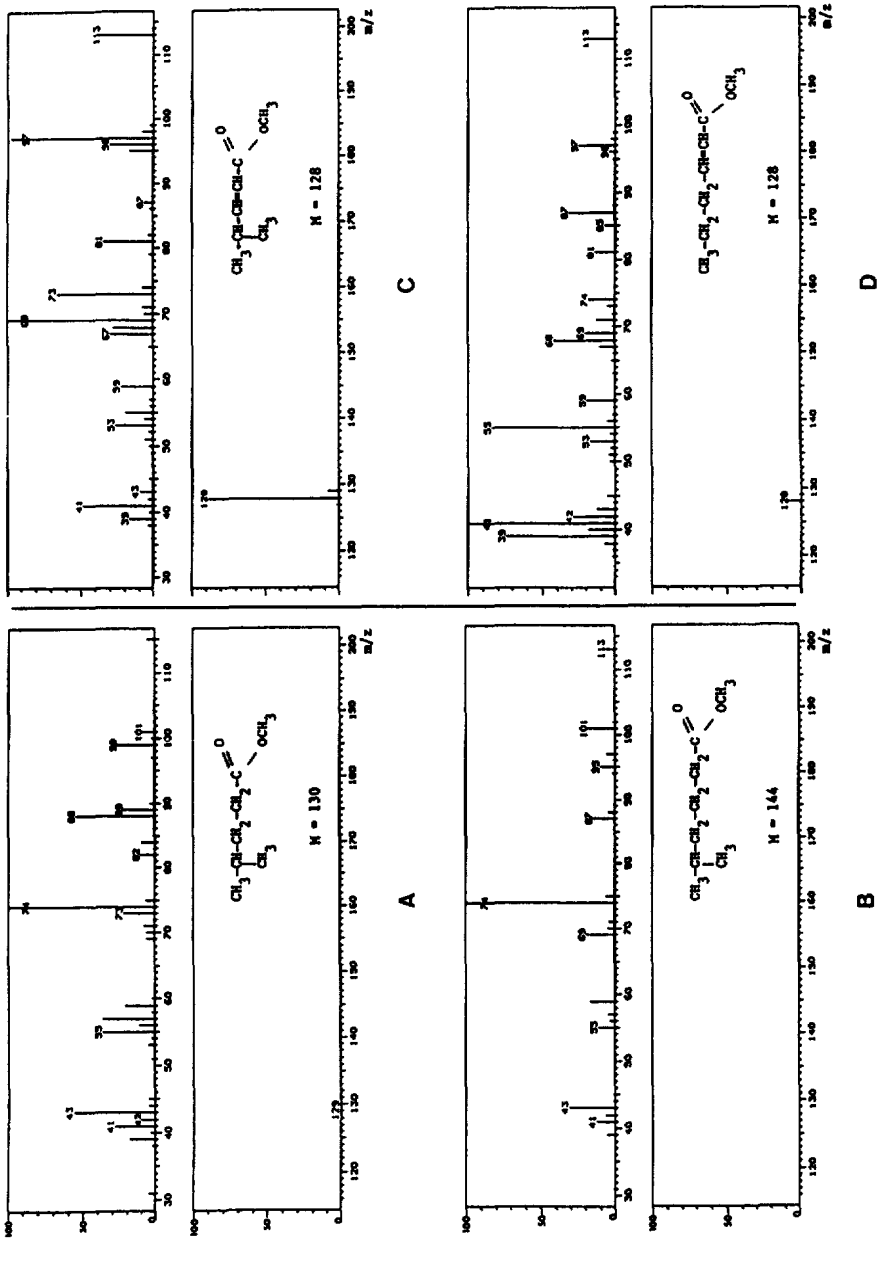


Fig. 2. EI mass spectra of (A) methyl isocaproate, (B) compound I, (C) 4-Me $C_{5:1}$ and (D) compound II from cultures of *Clostridium difficile*.

either as VFAs or NVFAs. Other peaks, which were not identified hitherto, have been labelled from 1 to 11, which will correspond to compounds I to XI. The chemical names of these compounds and their relative retention times (RRT) with respect to the internal standard (*o*-toluic acid) on the SP-1000 packed column are listed in Table II.

Identification of volatile fatty acids

The identities of VFAs were confirmed by determination of the RRT of each acid (aqueous standard solution containing 10 mmol l^{-1} of each VFA) before and after methylation on the SP-1000 packed column.

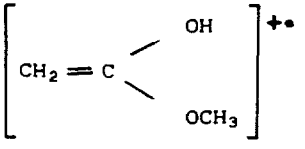
After methylation, each $C_{2\ 0}$–$C_{5\ 0}$ VFA gave only a minor peak with an RRT similar to that obtained after injection of the acidic diethyl ether extract containing VFAs in acidic form [8]. As has already been shown [9], two peaks corresponding to isocaproic acid MeE and caproic acid MeE were observed, eluting immediately after the solvent peak at RRT 0.1225 and 0.1380, respectively. Moreover, two additional peaks, corresponding to non-esterified isocaproic and caproic acids, were also observed with RRT 1.5096 and 1.8064, respectively. In Fig. 1A, the methyl isocaproate peak overlaps with the solvent peak and, therefore, it cannot be seen as the recording integrator was set at minimum sensitivity (1 mV full-scale).

The identity of these methyl ester peaks was confirmed by GC–MS. The EI mass spectrum of methyl isocaproate for a *C. difficile* culture is shown in Fig. 2A. In addition, the free acid peaks disappeared when the solution containing the methyl ester derivative was made basic with sodium hydroxide solution (pH ca. 7.5) before chloroform extraction. These data provide evidence that VFAs are only partially esterified. The methyl esters of $C_{2:0}$–$C_{5:0}$ VFAs probably elute with chloroform, but their less volatile free acid forms are separated and can interfere with other compounds; an important example is the interference between caproic acid and methyl 3-phenylpropionate (see Table II).

Identification of unknown compounds I–XI corresponding to peaks 1–11

Identification of peaks labelled 1 to 11 was confirmed by both GC–MS and GC (RRT) data in comparison with authentic standards processed as bacterial cultures.

Compound I (see chromatogram in Fig. 1A) and isocaproic acid MeE. The EI mass spectrum of compound I (Fig. 2B) reveals as data of 4-Me $C_{5\ 0}$ MeE (Fig. 2A) with a base peak at *m/z* 74 which indicates the possibility of a saturated



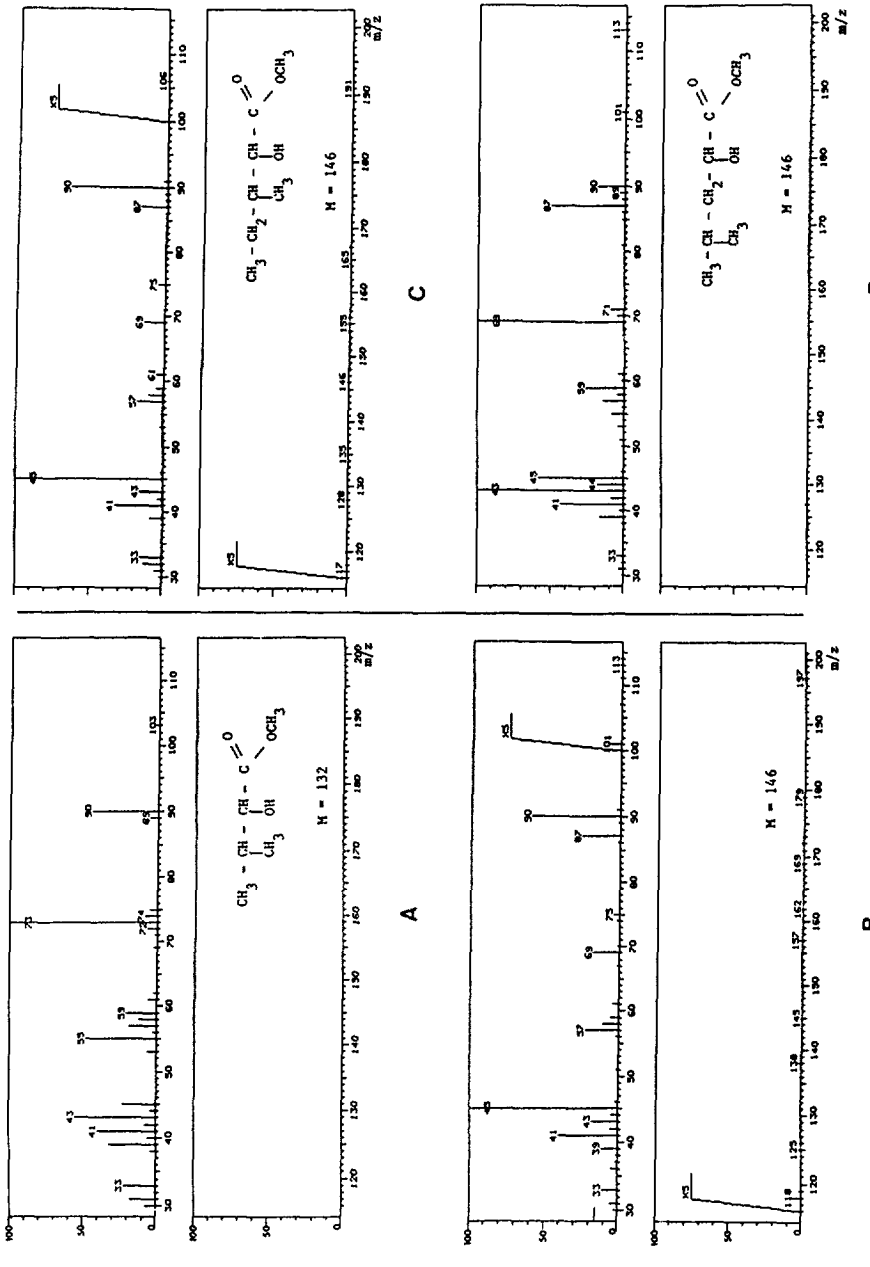


Fig. 3. EI mass spectra of (A) compound IV, (B) compound V, (C) compound VI and (D) compound VII from cultures of *P. anaerobius*.

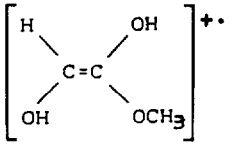
fatty acid methyl ester (McLafferty rearrangement) and a peak at *m/z* 43 which may be characteristic of methyl branching at the *iso* position in the chain.

The molecular masses of these methyl esters were determined by PICI with ammonia. An ion at *m/z* 148 $[M+NH_4]^+$ gives the molecular mass M = 130 corresponding to 4-Me $C_{5:0}$ MeE. For the compound I an ion $[M+NH_4]^+$ was observed at *m/z* 162; this compound I has a molecular mass of 144 and was identified as 5-methylhexanoic acid MeE.

Compound II (see chromatogram in Fig. 1A). The EI mass spectrum is shown in Fig. 2D. A molecular ion is present at *m/z* 128. A double bond at position 2 eliminates the formation of a major ion at *m/z* 74. The ion at *m/z* M − 59 confirms the structure of a methyl ester. We have identified this compound as 2-hexenoic acid MeE. In some instances, however, we found from spent media for *Clostridium difficile* cultures another peak (RRT 0.1690) having a closely related mass spectrum (see Fig. 2C). The GC retention time together with the MS data suggest that this peak corresponds to 4-methyl-2-pentenoic acid MeE.

Compound III (hydroxy acid) (see chromatograms in Fig. 1A, B and D). This compound corresponds to 2-hydroxybutanoic acid MeE and has been reported earlier [7].

Compounds IV, V, VI and VII (hydroxy acids) (see chromatograms in Fig. 1A and B). The molecular masses of these compounds determined by PICI were M = 132 for IV and M = 146 for each of V, VI and VII. The EI mass spectra (Fig. 3A–D) of these compounds show peaks at M − 59 and at *m/z* 90, which are characteristic fragments of 2-hydroxy acid methyl esters. The absence of an ion or a small ion at *m/z* 74 indicates a substitution at position 2 which, in the case of 2-hydroxy acid methyl esters, leads to the formation of a McLafferty ion rearrangement at *m/z* 90 as depicted below.



Utilization as before [7,10] of N^2H_3 as the reactant gas confirmed the presence of one hydroxy group in each molecule of compounds IV–VII. GC–MS and GC (RRT) data allowed us to identify IV as 2-hydroxy-3-methylbutanoic acid MeE and VI and VII as 2-hydroxy-3-methylpentanoic and 2-hydroxy-4-methylpentanoic acids MeE, respectively. The mass spectrum of V was very similar to those of 2-OH 3-Me $C_{5:0}$ MeE and 2-OH 4-Me $C_{5:0}$ MeE, with the exception of the presence of an ion at *m/z* 101 which was consistently observed. However, comparison of the retention time of V with those of methyl esters of 2-OH 3-Me $C_{5:0}$, 2-OH 4-Me $C_{5:0}$ and 2-OH $C_{6:0}$ standards clearly showed that this compound was not identical with any of them. Tentative identification was based on the following criteria, however: (i) the ion at *m/z* 90 gave evidence of a 2-hydroxy acid MeE structure; (ii) the presence of a hydroxy group

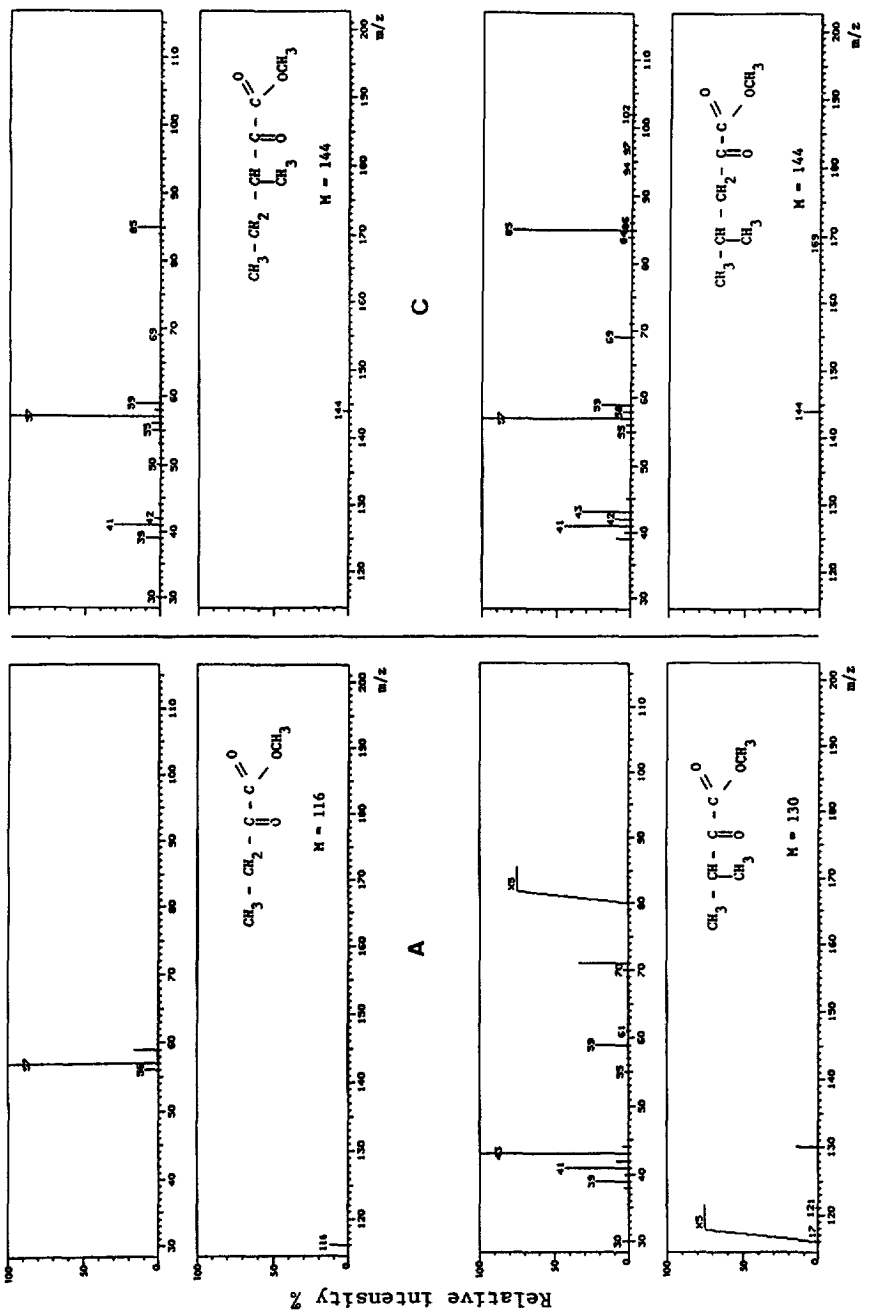
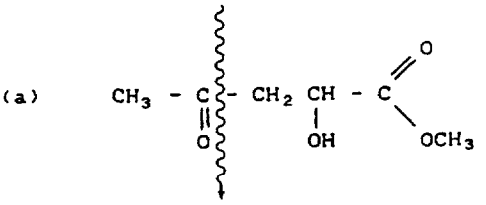


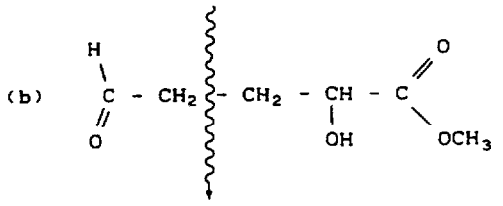
Fig. 4. EI mass spectra of (A) compound XI from cultures of *P. prevotii*, (B) compound VIII, (C) compound IX and (D) compound X from cultures of *Capnocytophaga sputigena*.

was confirmed by PICI with N^2H_3, only one proton being exchanged with one deuteron, leading to the formation of an ion at *m/z* 149 $[M' + {}^2H]^+$ and an ion at *m/z* 169 $[M' + N^2H_4]^+$; and (iii) the ion at *m/z* 43.

Thus, based on these data the two following structures are proposed for V, i.e. (a) 2-hydroxy-4-oxopentanoic acid MeE and (b) 2-hydroxypentanedioic acid 5-semialdehyde MeE. These hypotheses cannot be verified at present because neither compound is commercially available.



m/z =43



m/z =43

Compounds VIII–XI (oxo acids) (see chromatograms in Fig. 1C and D). The EI mass spectra show a molecular ion peak at *m/z* 116 for XI (Fig. 4A), 130 for VIII (Fig. 4B) and 144 for both IX and X (Fig. 4C and D) and a peak at M−59. In addition, a fragment at *m/z* 43 was observed for VIII and X, indicative of a methyl group at the *iso* position and a base peak at *m/z* 57 for IX–XI. The molecular masses of these compounds were confirmed by PICI.

Consequently, these compounds were identified as VIII = 3-methyl-2-oxobutanoic acid MeE, IX = 3-methyl-2-oxopentanoic acid MeE, X = 4-methyl-2-oxopentanoic acid MeE and XI = 2-oxobutanoic acid MeE.

Ratio and distribution of organic acids in anaerobic bacteria

We have examined the production of these newly described compounds by a range of commonly encountered anaerobes. Uninoculated TGY broth controls processed in the same way contained only traces of 2-OH $C_{4\ 0}$. The nature and amounts of these acids varied in accordance with the genera and species of anaerobic bacteria. When the organic acids are produced, with the exception of 4-Me $C_{5:1}$, $C_{6\ 1}$ and 2-oxo $C_{4.0}$, their concentrations varied in the range 0.20–2.20 m*M*. Whereas 4-Me $C_{5.1}$ and $C_{6\ 1}$ are detected at trace levels (0.10 m*M*),

TABLE III

CARBOXYLIC ACIDS NEWLY DESCRIBED IN ASSOCIATION WITH CULTURES OF SOME ANAEROBIC BACTERIA

Bacteria	Concentration[a]												Identification[b]
	5-Me $C_{6\,0}$	4-Me $C_{5\,1}$	3-Me 2-oxo $C_{4\,0}$	$C_{6\,1}$	2-Oxo $C_{4\,0}$	2-OH $C_{4\,0}$	3-Me 2-oxo $C_{5\,0}$	4-Me 2-oxo $C_{5\,0}$	2-OH 3-Me $C_{4.0}$	V	2-OH 3-Me $C_{5\,0}$	2-OH 4-Me $C_{5\,0}$	
Clostridium bifermentans	+	–	–	–	–	+	–	–	+	+	ND	ND	RRT
Clostridium botulinum A	+	ND	ND	ND	–	+	–	–	+	+	ND	ND	RRT
Clostridium botulinum B	–	ND	ND	ND	–	+	–	–	+	+	ND	ND	RRT
Clostridium difficile	+	tr	–	tr	–	+	–	–	+	+	–	+	MS
Clostridium paraputrificum	–	–	–	–	–	–	–	–	–	–	–	+	MS
Clostridium perenne	–	–	–	–	–	+	–	–	–	–	+	+	MS
Clostridium sordellii	+	ND	ND	ND	–	+	–	–	+	+	ND	ND	RRT
Clostridium sporogenes	–	–	–	–	–	+	–	–	+	+	+	+	MS
Propionibacterium avidum	–	–	–	–	–	+	–	–	+	–	+	+	MS
Propionibacterium granulosum	–	–	–	–	–	+	–	–	+	–	ND	ND	RRT
Peptostreptococcus anaerobius	–	–	–	–	–	+	–	–	+	+	+	+	MS
Peptostreptococcus asaccharolyticus	–	–	–	–	+	+	–	–	–	–	–	–	RRT
Peptostreptococcus prevotii	–	–	–	–	+	+	–	–	–	–	–	–	MS
Capnocytophaga sputigena	–	–	+	–	tr	–	+	+	–	–	–	–	MS
Capnocytophaga gingivalis	–	–	–	–	–	–	+	–	–	–	–	–	RRT

[a] +, >0.20 mmol l^{-1}; tr, 0.05 mmol l^{-1}–0.20 mmol l^{-1}; –, <0.05 mmol l^{-1}; ND, presence of a peak at the corresponding retention time but the identity was not determined by GC–MS.

[b] Identification of compounds based on GC (RRT) or GC–MS analysis.

2-oxo $C_{4:0}$ may occur in larger amounts, e.g., 4 m*M* for *Peptostreptococcus prevotii*. Table III gives the distribution of these newly described NVFAs among some anaerobes.

Anaerobe spore-forming bacteria, restricted to Clostridium genus. *Clostridium* species included in this study could be divided into two groups, according to their ability to form isobutyric up to isocaproic acids (first group) or not (second group).

Representative strains of the first group, i.e., *Clostridium bifermentans, Clostridium botulinum* types A and B (proteolytic strains), *Clostridium difficile, Clostridium sordellii* and *Clostridium sporogenes*, gave equally their corresponding 2-hydroxy acids, with the exception of 2-OH 2-Me $C_{3\ 0}$ and 2-OH $C_{5\ 0}$. So far 2-OH $C_{5\ 0}$ has only been found for *Clostridium innocuum* [7]. In addition, GC patterns for some species (*Clostridium bifermentans, Clostridium botulinum* type A, *Clostridium difficile, Clostridium sporogenes*) show a prominent peak of 5-Me $C_{6\ 0}$ MeE.

Bacteria of the second group, i.e., *Clostridium paraputrificum, Clostridium perenne, Clostridium perfringens, Clostridium baratii* and *Clostridium tertium* (data not shown for *Clostridium tertium*), which form acetic, propionic and butyric acids, produced certain 2-hydroxy acids (i.e., 2-OH $C_{4\ 0}$, 2-OH 3-Me $C_{5:0}$ and 2-OH 4-Me $C_{5:0}$, but in *Clostridium paraputrificum* cultures only 2-OH 4-Me $C_{5:0}$ was found.

Gram-positive non-sporing rods. GC analysis of NVFAs for these bacteria made possible the identification of 2-OH $C_{4:0}$, 2-OH 3-Me $C_{4\ 0}$ and 2-OH 4-Me $C_{5\ 0}$ mainly for *Propionibacterium avidum* and *Propionibacterium granulosum*. 2-OH 3-Me $C_{4:0}$ is an important product for both species. Other species within the *Propionibacterium* genus and also members of the *Actinomyces* genus either did not produce or produced only minor amounts of these acids. Although bifidobacteria showed a peak with the same RRT as 2-OH 4-Me $C_{5:0}$, the identity of this compound could not be established with certainty. Indeed, the mass spectrum of this compound was seemingly contaminated with other compounds and was not interpretable.

Peptostreptococcus. Chromatograms for cultures of *Peptostreptococcus anaerobius* strains were identical with those obtained from cultures of *Clostridium sporogenes*, whereas the GC profiles for *P. prevotii* and *P. asaccharolyticus* were nearly identical although clearly different from the GC patterns for other members of the *Peptostreptococcus* genus. In particular, representatives of these two species produced relatively large amounts of 2-oxo $C_{4\ 0}$ (1.2–4.0 m*M*) and moderate amounts of 2-OH $C_{4\ 0}$.

Gram-negative non-sporing rods. In this group, the production of these new organic compounds seems restricted to certain species. In addition to *Fusobacterium* species, which produced 2-OH $C_{4\ 0}$ and 2-OH $C_{5:0}$ [7], several 2-oxo acids may be detected for members of the *Capnocytophaga* genus, especially for *Capnocytophaga sputigena*. With the exception of the presence of 2-OH 3-Me

$C_{5:0}$ and/or possibly 2-OH 4-Me $C_{5:0}$ in spent media of *Bacteroides bivius*, we have not found any of these acids for bacteria referred to at the present time as *Bacteroides* genus such as the *B. fragilis* group or black-pigmented *Bacteroides*.

Effects of addition of glucose and/or amino acids to the culture medium on the formation of new organic acids

It is recognized that the glucose concentration in media has an influence on the amounts of VFAs produced by proteolytic clostridia [11]. Several workers have also shown that some *Clostridium* species gave increased yields of VFAs when grown in a medium supplemented with amino acids [12,13]. Therefore, we examined the effects of glucose and/or amino acids on the production of the newly described organic acids in all strains of *Clostridium botulinum* types A and B, *Clostridium difficile, Clostridium sporogenes, Clostridium sordellii* and *Peptostreptococcus anaerobius* listed in Table I. When these strains were grown in TY broth (i.e., TGY broth without glucose), the yield of acids was relatively unaffected. However, *Peptostreptococcus anaerobius* produced 5-Me $C_{6:0}$ in TY medium and, in contrast, the amount of this acid was reduced by half for *Clostridium sordellii*. In TY medium supplemented with 0.2% (w/v) of one of several amino acids (L-2-aminobutyric acid, L-norvaline, L-valine, L-leucine [13]), the production of each homologous 2-hydroxy acid was at least doubled. Such an effect was not observed after addition of either of two amino acids (L-isoleucine or L-norleucine). Enrichment with L-norleucine resulted in the production of 5-Me $C_{6:0}$ for *Clostridium sporogenes* and *Clostridium botulinum* B. In contrast to the results obtained by other workers for *Clostridium difficile* after growth in a trypticase–yeast–salt medium supplemented with L-isoleucine [14], we did not find any significant changes in 2-OH 3-Me $C_{5:0}$ or 2-OH 4-Me $C_{5:0}$ levels in any of the strains of the different species tested. However, the strains used by those investigators were not studied here. On the other hand, L-isoleucine greatly increased the production of compound V in all species examined that were shown to produce this compound (the increase was calculated from the peak areas).

DISCUSSION

The results presented in this work indicate that analyses of NVFAs based on a simple procedure (i.e., methylation and extraction with chloroform) allowed the identification of several acids that were not reported in previous GC studies [1,15]. These included mainly 2-hydroxy and 2-oxo acids.

These 2-hydroxy and 2-oxo acids were present at relatively low concentrations (0.05–4 m*M*) in spent media. However, their detection could be of significant interest, as fermentation patterns within each species are often similar; further even the relative proportions of distinctive low-yield products could

be extremely helpful. Some hydroxy acids have already been detected by frequency-pulsed electron-capture GC analysis of cerebrospinal fluid of patients with Nocardia infection [16] or diarrhoea diseases caused by facultative bacteria such as *Shigella* spp. [17]. In these investigations, the hydroxy acids were pointed out as important markers for identification of the aetiological agents. This in itself represents a very important advance, although it requires a more specific and sensitive type of detection system. It is therefore useful to know that these compounds might be identified by a simpler procedure, i.e., as methyl derivatives with flame ionization detection, when the level of metabolic product falls within the micromolar range, as could be expected for identification of pure cultures. It would therefore be of interest to examine the body fluids by this simpler system, notwithstanding the questions of detectability, as this could then be introduced for routine analyses.

Several of the products described above have the same or close retention times and may be erroneously identified. This is particularly true for the methyl esters of 5-Me $C_{6:0}$ and pyruvic acid, 2-oxo $C_{4:0}$ and lactic acid, and 2-OH $C_{4\ 0}$ and 3-Me 2-oxo $C_{5\ 0}$ (see RRT values, Table II). Consequently, although moderately polar phases are mainly used to separate NVFAs, we found that these substances were better resolved on a very polar phase. An example of a chromatogram obtained on CP-Sil 88 for a *Capnocytophaga sputigena* strain is given in Fig. 5. This phase allows an excellent separation of hydroxy and oxo acids methyl esters, even for 2-OH 3-Me $C_{5\ 0}$ MeE (not shown in Fig. 5) and 2-OH 4-Me $C_{5:0}$ MeE.

Originally, an attempt was made to separate these compounds using a non-polar stationary phase (SE-54), but it was found that there was considerable tailing. As the generally used moderately polar stationary phase did not give sufficient resolution, for these reasons, it was decided to try a very polar column recommended for the separation of methyl esters of fatty acids (see Experimental).

In addition, we examined some other NVFAs that have been suggested as metabolites of anaerobic bacteria [1] and specifically *Clostridium bifermentans* [9], i.e., oxaloacetic, oxalic, malonic and methylmalonic acids. It has been reported that oxaloacetic acid decomposes to pyruvic acid in both acidic and basic solutions [18], and that the extent of decomposition of oxaloacetic acid in 0.01 *M* sulphuric acid was 75% after 20 h at 21 °C. For further confirmation of these observations, a 5 m*M* standard solution of oxaloacetic acid (from either Sigma or Merck) was methylated and analysed by GC–MS. The mass spectra obtained corresponded to pyruvic acid MeE and 2,2-dimethoxypropanoic acid MeE but, as expected, neither oxaloacetic acid diMeE nor 2,2-dimethoxysuccinic acid diMeE was found. The purity of the commercial free acids was verified by direct introduction into the MS system. Hence it seems impossible to analyse by GC oxaloacetic acid after methylation, as it is destroyed during the procedure. In other respects, in all our GC–MS analyses we did not detect any

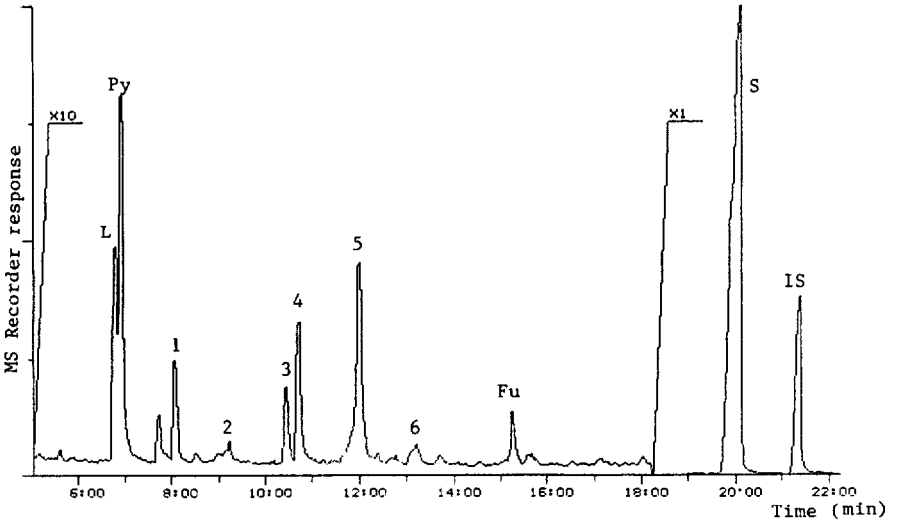


Fig. 5. Gas chromatographic separation of methylated TGY broth culture of *Capnocytophaga sputigena*, obtained with a capillary column coated with CP-Sil 88 stationary phase. Peaks: IS = internal standard; 1 = 3-Me 2-oxo $C_{4\ 0}$ MeE; 2 = 2-oxo $C_{4\ 0}$ MeE; 3 = 2,2-dimethoxypropanoic acid MeE; 4 = 3-Me 2-oxo $C_{5\ 0}$ MeE; 5 = 4-Me 2-oxo $C_{5\ 0}$ MeE; 6 = 2-OH 4-Me $C_{5\ 0}$ MeE. The peak numbers do not correspond to those in Fig. 1. For other abbreviations, see Table II. GC conditions as described under Experimental.

of oxalic, malonic and methylmalonic acids. As the media and growth conditions were very similar to those used for the earlier studies on *Clostridium bifermentans* [9] and other anaerobes [1], it is not likely that those factors could account for the differences in the metabolic patterns that were observed. On the other hand, their RRT values are closely related to those of 2-OH 3-Me $C_{4\ 0}$ MeE, compound V and free acetic acid, respectively. This feature may constitute a possible error in interpretation.

In conclusion, although a simple methylation and chloroform extraction procedure such as that described in the VPI Anaerobe Laboratory Manual [1] allows the identification of several unusual acidic compounds from spent media of anaerobes, the use of moderately polar stationary phases sometimes renders the interpretation of the chromatograms doubtful because of interference between compounds with the same RRT. A moderately polar stationary phase is most often used for the general resolution of short-chain fatty acids, but there are instances, as seen here, where a very polar stationary phase is required in order to resolve these methyl esters successfully. Data obtained with CP-Sil 88 indicate that this type of very polar stationary phase might allow the detection of the greatest number of methyl esters. In this study, the bacteria tested represent those most commonly encountered in the clinical laboratory, but the number of genera and species were relatively small. Therefore, a de-

tailed study of many more strains must be performed in order to determine the taxonomic value of these new NVFAs, and work in this direction is continuing.

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