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Note

Identification by gas chromatography–mass spectrometry of short-chain hydroxy acids produced by *Fusobacterium* species and *Clostridium innocuum*

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Gas chromatographic (GC) analysis of acid fermentation products formed in culture media is routinely used by many laboratories to aid in identifying anaerobic bacteria [1–3]. The non-volatile fatty acids are converted into butyl or methyl esters before GC analysis [1, 4]. While studying the fermentation patterns of anaerobic strains, we observed two unknown components in some methyl ester samples of spent media of the genus *Fusobacterium*. These components were eluted between lactic acid and succinic acid. Peak areas were distinctly different from one *Fusobacterium* species to another. Moreover, one of these unidentified products was also present in spent medium of *Clostridium innocuum*.

The aim of the present study was to identify these metabolic products by gas chromatography–mass spectrometry (GC–MS) and to determine their taxonomic value in identification of these bacteria.

EXPERIMENTAL

Organisms and cultures conditions

The following type strains (T) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.), National Collection of Industrial Bacteria (NCIB, Aberdeen, U.K.) and the "Collection des Anaérobies de l'Insti-

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tut Pasteur" (AIP, Paris, France): *F. russii* ATCC25533, *F. nucleatum* AIP1612A (ATCC25586), *F. mortiferum* AIP350A (ATCC25557), *F. gonidiaformans* AIP3554A (ATCC25563) and *C. innocuum* NCIB10674. The other strains employed for this study were isolated from clinical material; these isolates, 59 *Fusobacterium* and 33 *C.innocuum,* were identified using the procedures described in the VPI Anaerobe Manual [1]. Organisms were maintained anaerobically in TGY broth (pH 7.4–7.6) containing (g/l): trypticase, 30; yeast extract, 20; D(+)-glucose (anhydrous), 5; cysteine hydrochloride, 1. Culture techniques, media and samples for chromatographic studies have been described previously [5]. Trypticase and yeast extract were obtained from Difco Labs. (Detroit, MI, U.S.A.), D(+)-glucose (anhydrous) and cysteine hydrochloride were from Prolabo (Paris, France).

Gas chromatography

Non-volatile fatty acids were converted into methyl esters, which were extracted into chloroform. The chloroform extract of methyl esters was analysed by injecting 1 μl into a glass column (2 m$\times$4 mm I.D.) packed with 10% SP1000 and 1% phosphoric acid on 100–120 mesh Chromosorb W AW (Supelco, Gland, Switzerland). The gas chromatograph was a Model DFL 121 instrument (Intersmat, Suresnes, France) equipped with a hydrogen flame-ionization detector and connected to a Model CR3A integrator (Shimadzu, Tokyo, Japan). The operating conditions were: injector and detector temperature, 200°C; oven temperature, 135°C; carrier gas (nitrogen) flow-rate, 30 ml/min; attenuation, 1; sensitivity, 10^{-10} A/mV.

Gas chromatography–mass spectrometry

The GC–MS system consisted of a Nermag R10-10C quadrupole mass spectrometer (Nermag, Rueil-Malmaison, France) controlled by a digital PDP 11-23 system and interfaced with a Girdel Series 32 gas chromatograph. Electron energy, emission current and electron multiplier voltage were 70 eV, 200 mA and 2 kV, respectively, and the temperature of the ion source was 120°C. The instrument was operated in electron impact (EI) and in positive chemical-ionization (CI) modes. Either ammonia [6] or [$^2H_3$]ammonia (Commissariat à l'Energie Atomique, Saclay, France) was used as the reagent gas in CI mode, with an ion-source pressure of 0.1 Torr and electron energy of 90 eV. The gas chromatograph was equipped with a 25 m$\times$0.23 mm I.D. fused capillary column coated with SP1000 phase (Chrompack-France, Les Ulis, France). The injector, oven and interface temperatures were 210, 110 and 210°C, respectively. The carrier gas was helium.

Reagents

All reagents and carboxylic acids were of analytical purity. Pyruvic, lactic, DL-2-hydroxybutanoic[2(OH)-B], DL-2-hydroxypentanoic[2(OH)-P], fumaric, succinic and phenylacetic acids were purchased from Sigma (St. Louis, MO, U.S.A.). Chloroform and methanol were from Merck (Darmstadt, F.R.G.). *o*-Toluic acid (internal standard) was from Prolabo.

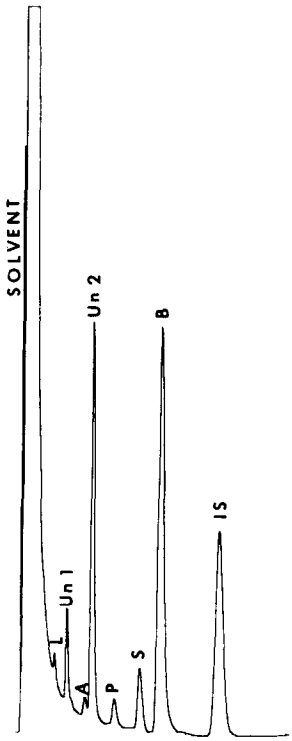
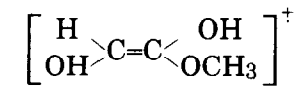


Fig. 1. Gas chromatogram of esterified short-chain acids from *F. nucleatum* AIP1612A$^{(T)}$. Peaks: L = lactic acid; Un1 and Un2 = unknown components; S = succinic acid; A = acetic acid; P = propionic acid; B = butyric acid; IS = internal standard (*o*-toluic acid, final concentration 10 m*M*).

RESULTS

A typical chromatogram of the esterified acid products from *F. nucleatum* strain AIP1612A$^{(T)}$ is shown in Fig. 1. Acetic, propionic and butyric acids produced by *F. nucleatum* were also extracted by chloroform and were detected under our analysis conditions. The unknown components labelled Un1 and Un2 produced significant peaks. Table I shows the relative retention time (RRT) to *o*-toluic acid (internal standard) of some non-volatile acids and these unknown components separated on SP 1000/phosphoric acid phase.

The mass spectral data obtained in EI mode confirmed the presence of lactic and succinic methyl esters, but the unknown components showed no molecular ion M^+ and could not be determined with this method. However, an ion fragment peak at *m/z* 90 was present in the component Un2 (RRT 0.3959) and may be represented by:



In order to determine the molecular masses of these organic acids, we used CI with ammonia as reagent gas. We found the following ions: component Un1 *m/z*

TABLE I

RELATIVE RETENTION TIME (RRT) TO *o*-TOLUIC ACID OF METHYL ESTERS OF ORGANIC ACIDS AND UNKNOWN COMPOUNDS

GC conditions as described in the text; *o*-toluic acid retention time 7.658 min.

Compounds	RRT
Pyruvic	0.16
Lactic	0.2141
Unknown 1	0.2688
Unknown 2	0.3959
Fumaric	0.53
Succinic	0.6211
Phenylacetic	1.2785

136 $[M+NH_4]^+$ corresponding to M=118 and component Un2 *m/z* 150 $[M+NH_4]^+$ corresponding to M=132, which were the base peaks. The hydroxyl group suggested by fragment *m/z* 90 in EI mode was confirmed when $[^2H_3]$ammonia was used instead of ammonia. We observed the presence of an ion at *m/z* 141 $[M'+ND_4]^+$ for component Un1 (RRT 0.2688) and at *m/z* 155 $[M'+ND_4]^+$ for component Un2 (RRT 0.3959). In these two molecules the hydrogen atom of the hydroxyl group was exchanged with one deuterium atom. These results were confirmed by the "new molecular mass" M′ due to the exchange of only one hydrogen atom belonging to the hydroxyl group of the compound. This suggested that compound Un1 was a 2-hydroxybutanoic methyl ester and compound Un2 a 2-hydroxypentanoic methyl ester. MS and GC RRT data were compared with authentic standards to confirm the identities of these acids. The RRTs were identical: 0.2707 and 0.3914, respectively. Comparisons of mass spectra are shown in Figs. 2 and 3. 2-Hydroxypentanoic acid was also identified by this procedure in *C. innocuum*. To our knowledge, these acids have never been reported or identified as fermentation products of anaerobic bacteria, except for *C. difficile* after derivatization with heptafluorobutyric anhydride [7].

Production of hydroxy acids

The uninoculated media (control) contained 2-hydroxybutanoic acid, 0.14 ± 0.08 m*M* (mean ± S.D. from ten samples). This value was taken into account and deducted from final quantity.

C. innocuum. The 33 strains of *C. innocuum* tested produced 2-hydroxypentanoic acid. The amounts ranged from 0.49 to 1.8 mmol/l and varied among strains. The type strain NCIB 10674$^{(T)}$ produced 0.67 mmol/l.

Fusobacterium species. The production of hydroxy acids by various *Fusobacterium* species is shown in Table II. 2-Hydroxybutanoic acid was produced by all species tested, except for *F. mortiferum* and some strains of *F. naviforme*. Relatively large amounts of 2-hydroxypentanoic acid were found in *F. nucleatum*. Traces of this metabolic product were also detected in *F. gonidiaformans, F. necrophorum,* and *F. varium,* but none in *F. mortiferum, F. naviforme* and *F. russii*. In order to test the reproducibility of these chromatographic patterns, ten inde-

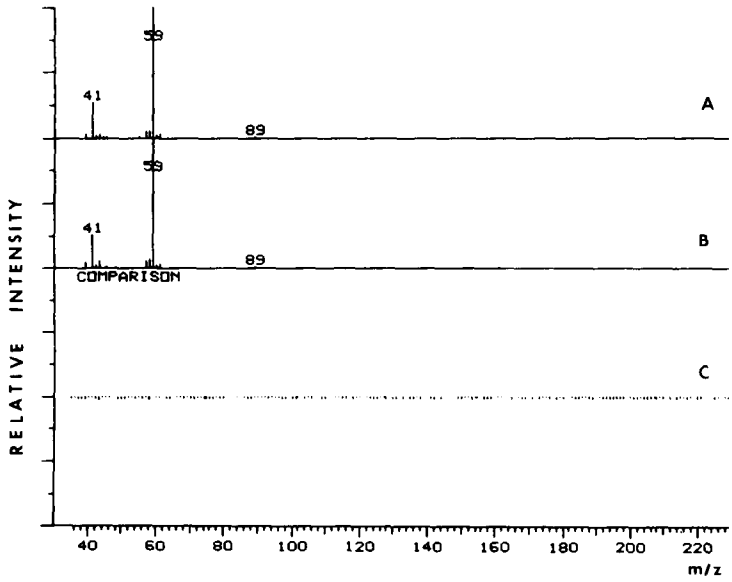


Fig. 2. Comparison of EI mass spectrum of the authentic methyl ester of 2-hydroxybutanoic acid (A) with EI mass spectrum of compound Un1 (B). (C) The difference in relative intensity between A and B.

pendent experiments were performed with each reference strain of *Fusobacterium*. Results are shown in Table III. The reproducibility of the analysis was rather poor for traces of hydroxy acids (less than 0.5 m*M*), but better for higher

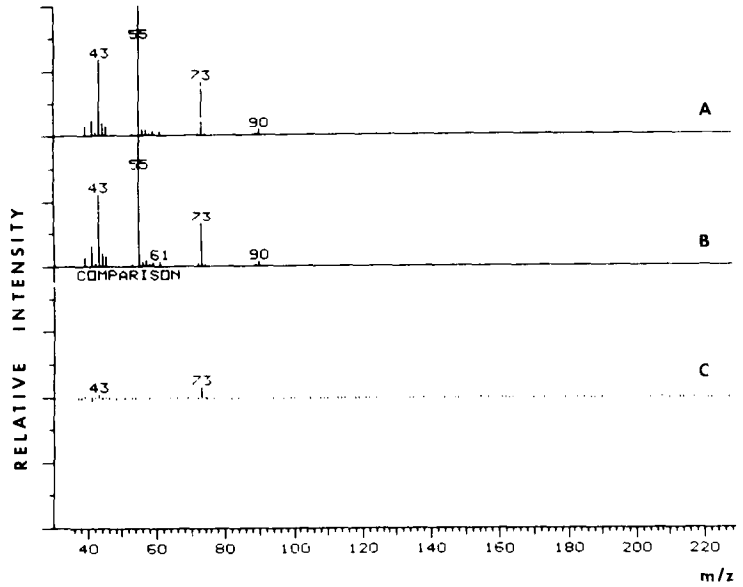


Fig. 3. Comparison of EI mass spectrum of the authentic methyl ester of 2-hydroxypentanoic acid (A) with EI mass spectrum of compound Un2 (B). (C) The difference in relative intensity between A and B.

TABLE II

PRODUCTION OF HYDROXY ACIDS BY *FUSOBACTERIUM* SPECIES

N.D. = not detectable (less than 0.02 m*M*).

Fusobacterium species	Number of strains	Concentration (m*M*)	
		2-Hydroxybutanoic	2-Hydroxypentanoic
F. gonidiaformans	1	5.83	0.25
F. mortiferum	8	N.D.	N.D.
F. naviforme	5	0 -4.4*	N.D.
F. necrophorum	23	1.02-5.45	0.02-0.28
F. nucleatum	15	1.58-5.21	0.86-3.63
F. russii	1	1.85	N.D.
F. varium	8	0.93-1.58	0.04-0.34

*Two strains produced 3.3 and 4.4 m*M*, respectively.

concentrations. In the latter case, the maximum variation from the mean value was 5–11%, for each strain and each hydroxy acid.

DISCUSSION

Results of the present study indicate that, in CI-MS, use of $[^2H_3]$ammonia as a reagent gas is particularly useful to determine the presence or absence of groups, such as OH, NH and NH_2, which contain one of more hydrogen atoms exchangeable with deuterium. In both compounds, we observed a pseudo-molecular ion $[M' + ND_4]^+$.

The production of hydroxy acids may be of interest in clinical bacteriology for two reasons. First, 2-hydroxybutanoic acid is produced by several species of anaerobes and, in particular, the *Clostridia*, e.g. *C. perfringens, C. barati, C. diffi-*

TABLE III

REPRODUCIBILITY OF THE GC ANALYSIS OF HYDROXY ACIDS WITH REFERENCE STRAINS OF *FUSOBACTERIUM* SPECIES

N.D. = not detectable (less than 0.02 m*M*).

Fusobacterium strain*	Concentration (mean ± S.D., $n = 10$) (m*M*)	
	2-Hydroxybutanoic	2-Hydroxypentanoic
F. gonidiaformans AIP 3554A$^{(T)}$	5.86 ± 0.2	0.26 ± 0.02
F. mortiferum AIP 350A$^{(T)}$	N.D.	N.D.
F. naviforme AIP 3657	N.D.	N.D.
F. necrophorum AIP 696D	2.28 ± 0.24	0.18 ± 0.05
F. nucleatum AIP 1612A$^{(T)}$	1.45 ± 0.02	2.81 ± 0.19
F. russii ATCC 25533$^{(T)}$	1.91 ± 0.09	N.D.
F. varium AIP 5009	1.12 ± 0.08	0.06 ± 0.01

*AIP = Collection des anaérobies de l'Institut Pasteur; ATCC = American Type Culture Collection; (T) = type strain.

TABLE IV

INCREASE IN THE AMOUNT OF 2-HYDROXYBUTANOIC ACID OBTAINED INTO THE PRESENCE OF METHYL 2,2-DIMETHOXYPROPANOATE

Data calculated from different concentrations of standard pyruvic acid.

Concentration of pyruvic acid (m*M*)	Concentration of 2(OH)-B (m*M*)
1	0.097
2	0.209
4	0.407
6	0.622
8	0.819
10	1.027
20	2.050

cile and *C. sporogenes* (data not shown). We did not find this acid in gram-negative bacteria, except for the *Fusobacterium* genus. However, some bacteria produce pyruvic acid, and several authors [8, 9] have shown that two major products are formed through esterification of this acid: methyl pyruvate and methyl 2,2-dimethoxypropanoate (MDMP).

Under our GC conditions, MDMP was eluted at the same retention time as 2-hydroxybutanoic acid. Thus, when pyruvic acid is present in the medium, the amount of 2-hydroxybutanoic acid is increased (due to MDMP) from a value corresponding to 10% of the amount of pyruvic acid (see Table IV for results obtained with standard solutions of pyruvic acid). Secondly while low concentrations of 2-hydroxypentanoic acid seem to be present in spent media of some *Clostridia*, higher concentrations are produced only by *F. nucleatum* and *C. innocuum*. 2-Hydroxypentanoic acid can thus be used as a chemical marker of *C. innocuum*.

Differentiation of the *Fusobacterium* species can be easily performed by detection of these acids. *F. nucleatum* and *F. naviforme* are phenotypically very similar and can be distinguished from one another only in their ability to convert threonine into propionate [1]. However, this test requires an additional GC analysis. Our results indicate that *F. nucleatum* can be differentiated from *F. naviforme* by its production of 2-hydroxypentanoic acid using a single chromatographic analysis.

In conclusion, qualitative analysis of hydroxy acids down to a concentration of 0.05 m*M* is thus feasible. Quantitative analysis is fairly accurate at concentrations up to 0.5 m*M*. The determination of these organic compounds may be an additional argument for identification and taxonomy of obligate anaerobic bacteria.

ACKNOWLEDGEMENT

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REFERENCES

1 L.V. Holdeman, E.P. Cato and W.E.C. Moore, Anaerobe Laboratory Manual, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA, 4th ed., 1977.
2 U.L. Sutter, V.L. Vargo and S.M. Finegold, Wadsworth Anaerobic Bacteriology Manual, Extension Division, University of California, Los Angeles, CA, 2nd ed., 1975.
3 V.R. Dowell, Jr. and T.M. Hawkins, Laboratory Methods in Anaerobic Bacteriology, CDC Laboratory Manual, Publication No. (HSM) 73.8322, Center for Disease Control, Atlanta, GA, 1973.
4 J.P. Salanitro and P.A. Muirhead, Appl. Microbiol., 29 (1975) 374.
5 J.P. Carlier, Bull. Inst. Pasteur, 83 (1985) 57.
6 B. Munson, Anal. Chem., 49 (1977) 772A.
7 J.B. Brooks, O.L. Nunez-Monteil, B.J. Wycoff and C.W. Moss, J. Clin. Microbiol., 20 (1984) 539.
8 P.G. Simmonds, B.C. Pettitt and A. Zlatkis, Anal. Chem., 39 (1967) 163.
9 E. Hautala and M.L. Weaver, Anal. Biochem., 30 (1969) 32.