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HEADSPACE GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ANALYSIS OF LIGHT HYDROCARBONS AND VOLATILE ORGANOSULPHUR COMPOUNDS IN REDUCED-PRESSURE CULTURES OF *CLOSTRIDIUM*

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SUMMARY

A static headspace gas chromatographic method for the simultaneous separation of trace light hydrocarbons and volatile organosulphur compounds in gases of nineteen *Clostridium* cultures at reduced pressure is described The separation was achieved on *n*-octane-Porasil C after sampling of the gaseous compounds in a PTFE loop without any pretreatment Most peaks were identified by gas chromatography—mass spectrometry The presence of methane and ethylene sulphide among *Clostridium* volatiles is confirmed and 3-methyl-1-butene, 2-methyl-2-butene, dimethyl trisulphide and S-methyl thioacetate are reported for the first time in the *Clostridium* group

INTRODUCTION

If headspace gas chromatographic (GC) analysis of volatiles evolved from microbial cultures is well documented [1], the sampling of microbial gaseous compounds at reduced pressure without preconcentration has been scarcely reported [2, 3], although this approach is convenient to detect trace pollutants in water [4]

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Special attention has been given to GC separation of volatile organosulphur compounds (VOSCs) from cultures of oral *Bacteroides* [5] or rumen micro-organisms [6], or possibly originating from a microbial activity, e.g. in fermented products [7], soils [8, 9] and sewage sludges [10].

Reports dealing with analysis of VOSCs by GC alone or combined with mass spectrometry (MS) [11–16] have emphasized the various problems associated with their determination With flame ionization detection, the headspace GC detection limits for VOSCs have been estimated to be in the 1–10 ppb range [17]

The low bleed rates of covalently linked Durapak make them valuable for the GC–MS analysis of a wide range of trace chemicals with temperature programming [18] Simultaneous separation of light hydrocarbons and VOSCs was achieved by using a Durapak packing, OPN-Porasil C [19], and good resolution of C_1–C_6 hydrocarbons has been reported on *n*-octane-Porasil C [20–22].

This paper describes a technique without preconcentration for reduced pressure headspace GC analysis of both light hydrocarbons and VOSCs present among gaseous compounds of several strains of *Clostridium* by using a Durapak packing, *n*-octane-Porasil C

EXPERIMENTAL

Bacterial strains

The nineteen strains of *Clostridium* included in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U S.A.), the Deutsche Sammlung von Mikroorganismen (DSM, Göttingen, F.R G.) and the Collection des Anaérobies de l'Institut Pasteur (AIP, Pasteur Institute, Paris, France) With only two exceptions, the type strain (^{T}) was studied for each species The test bacterium was *Clostridium* sp. DMS 1786, formerly known as *C hastiforme* [23]

Culture media

The culture medium, called TTY medium for thioglycolate–Trypcase–yeast extract, was composed of Trypcase (15 g/l), yeast extract (5 g/l), sodium chloride (2 5 g/l) and sodium thioglycolate (0.5 g/l) dissolved in glass-bidistilled water In special instances, D-glucose monohydrate (5 5 g/l) and sodium thioglycolate (up to 5 g/l) were added to TTY medium The medium, adjusted to pH 7.2, then filter-sterilized to avoid heat-induced modifications of its constituents and inoculated with an aliquot of an exponential-phase culture in a medium of the same composition, was dispensed (5 ml) in every sterile culture tube (Fig 1) Air was evacuated with a conventional vacuum pump (residual pressure in a water-free tube ca 0.3 kPa) Quadruplicates for each strain and duplicates for the uninoculated medium were processed in the same way.

Trypcase and yeast extract were obtained from BioMérieux (Lyon, France) Sodium thioglycolate (microbiological grade), sodium chloride (pro analysi) and D-glucose monohydrate (for biochemistry and microbiology) were from Merck (Darmstadt, F.R G).

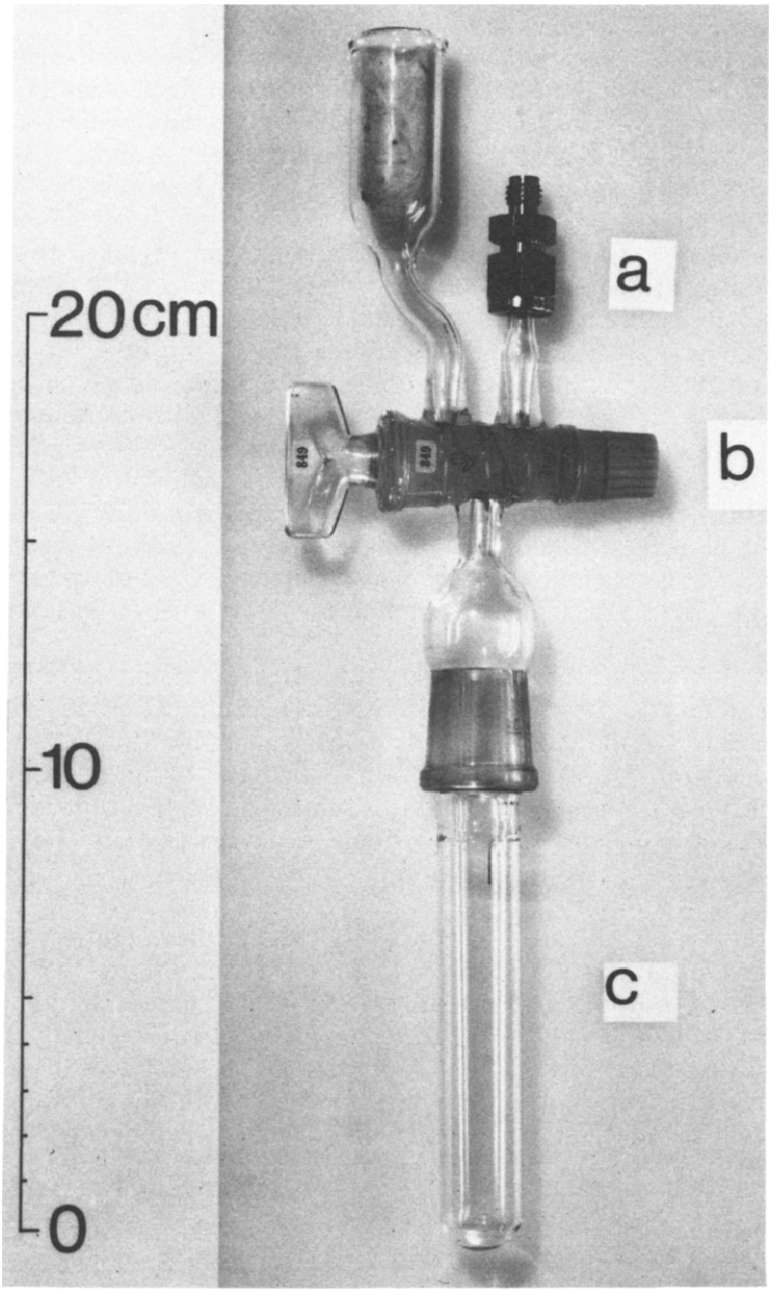

Fig 1 Tube used for bacterial culture and gas sampling at reduced pressure The culture tube (volume 28 4 ± 1 1 ml), constructed with an 18-mm O D borosilicate glass tube (c), is fitted in its upper part with a two-way stopcock (b) allowing air evacuation and, if needed, the introduction of any gaseous or liquid reagent Ground-glass parts are lubricated with Apiezon H grease (Apiezon Products, London, U K) A 6 35—3 18-mm stainless-steel Swagelok reducing union (a) with PTFE front and back ferules makes possible the junction of the culture tube to the 3 18-mm O D PTFE tubing of the GC sampling device

Reduced pressure headspace GC and gas sampling conditions

The sampling device was constructed with PTFE tubing (3 18 mm O.D.), i.e. the loop (2 80 mm I D , volume ca. 14 1 ml) of the six-way gas sampling valve and the connections (1 60 mm I.D) At one end of this sampling device, a bellows valve (Nupro Model SS 2H, Nupro, Willoughby, OH, U.S.A) was fitted and, at the other end, the culture tube was connected to the PTFE tubing

For the simultaneous separation of light hydrocarbons and VOSCs, two borosilicate glass columns (1.40 m long, 6 35 mm O.D , 2 mm I D) packed with 100—120 mesh *n*-octane-Porasil C (Alltech Assoc , Deerfield, IL, U S.A) and fitted with Vespel—graphite (85 15) ferules were run in the dual mode from 5°C for 1 min to 100°C, with an increase rate of 5°C/min The gas chromatograph (Girdel 300, Delsi, Suresnes, France) was equipped with two flame ionization detectors (hydrogen flow-rate, 25 ml/min, air flow-rate, 300 ml/min, sensitivity, $5 \cdot 10^{-11}$ A f s) and dual mass-flow controllers (helium purity > 99 995%, 17 ml/min) The detector, injector and gas sampling valve temperatures were set at 100, 100 and 140°C, respectively To prevent intersample contamination, the gas sampling device was degassed for about 40 min and the Swagelok union, the PTFE front and back ferules were flushed with air between each injection

The GC analysis of methane was also carried out on two other packings [21] in borosilicate glass columns (3 10 m long, 6 35 mm O D., 2 mm I.D) 40–60 mesh molecular sieve 5 Å and 80—100 mesh phenylisocyanate-Porasil C (both from Alltech Assoc) operated isothermally at 40°C (helium, 17 ml/min) or 10°C (helium, 32 5 ml/min), respectively As a baseline disturbance observed with the 14-ml PTFE loop perturbed the methane peak integration (Delsi ICAP 10 digital integrator), gaseous compounds were sampled in a 5-ml stainless-steel loop

After an incubation period of 168 ± 4 h at 37°C, each culture tube was vortexed, then thermostatted in a water bath at 25 0 ± 0 1°C for 60 min After expansion of the gaseous compounds for 1 min in the air-evacuated sampling device, the gas sample was injected To restore the back-pressure of helium more quickly, the gas sampling valve was turned back to the initial position 3 min after injection by convention Gas permeability of PTFE tubing was considered negligible and gas tightness of the sampling device was checked by pressure measurements

When syringe sample introduction was required, the 6-mm O.D part of the culture tube was placed in a glass tube (80 mm long, 11 mm I.D) swept with a helium stream (cylinder output pressure ca 200 kPa). Helium was allowed to get in through the two-way stopcock In the culture tube filled up to atmospheric pressure, an extra 5.5 ml of helium were introduced with a gas-tight syringe through a GC septum placed in a stainless-steel Swagelok reducing union (helium-pressurized tube). The tube was vortexed again, 5 5 ml of gaseous compounds were withdrawn and the volume was adjusted to 5.0 ml prior to injection

Chemicals

Acetylene was prepared extemporaneously from calcium carbide (Prolabo,

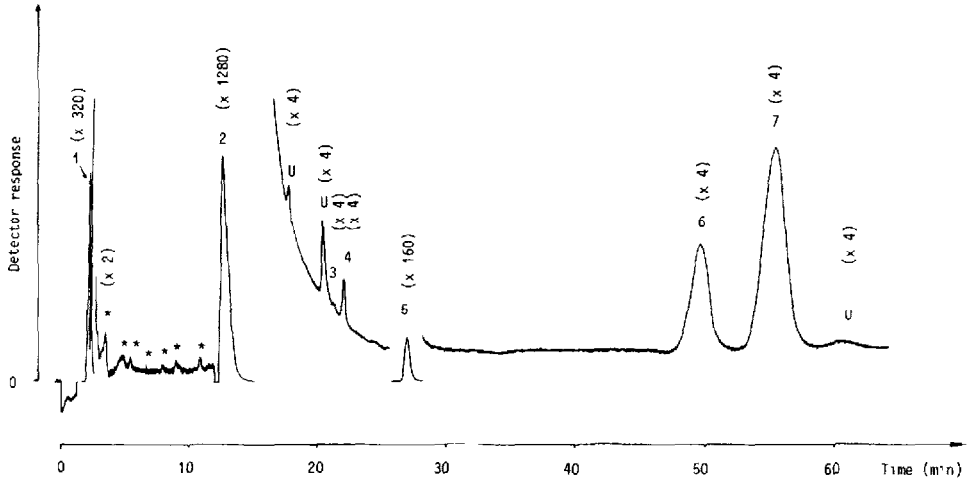

Fig 2 Gas chromatogram obtained from a reduced pressure headspace of a TTY medium culture of *Clostridium* sp DSM 1786 Dual borosilicate glass columns (1 40 m long, 6 35 mm O D , 2 mm I D) packed with 100—120 mesh *n*-octane-Porasil C Temperature programming 5°C for 1 min, 5°C/min up to 100°C Detector flame ionization (temperature 100°C, sensitivity at attenuation × 2 $5 \cdot 10^{-11}$ A f s) Carrier gas helium (flow-rate 17 ml/min) Peaks 1 = methane, 2 = methanethiol, 3 = ethylene sulphide, 4 = dimethyl sulphide, 5 = dimethyl disulphide, 6 = dimethyl trisulphide, 7 = S-methyl thioacetate, * = light hydrocarbons, U = unknown Attenuations are given in parentheses For details about the first eluting peaks, see Fig 6a

TABLE I

RETENTION TIMES OF SOME C_1—C_7 HYDROCARBON AND VOSC STANDARDS

Dual borosilicate glass columns packed with 100—120 mesh *n*-octane-Porasil C GC conditions as in Fig 2 (A) From Farmitalia Carlo Erba (Paris, France), (B) from Fluka (Buchs, Switzerland), (C) from Prolabo (Paris, France), (D) from Aldrich (Strasbourg France), (E) from Merck (Darmstadt, F R G), (F) from Eastman Kodak (Rochester, NY, U S A), for acetylene and S-methyl thioacetate, see the text

Compound	Retention time (min)	Compound	Retention time (min)
Methane (A)	2 2	2-Methyl-1-butene (A) + *cis*-2-pentene (B) + *trans*-2-pentene (B)	13 2
Ethane (A) + ethylene (A)	2 8	*n*-Hexane (C)	13 4
Acetylene	3 0	Methanethiol (B)	13 7*
Propane (A)	3 2	2-Methyl-2-butene (D)	14 5
Propene (A)	5 0	*n*-Heptane (C) + ethanethiol (D)	17 6
Isobutane (A)	5 2	Ethylene sulphide (D)	20 9
n-Butane (A)	5 6	Dimethyl sulphide (E)	21 8
1-Butene (A)	8 2	Propylene sulphide (B)	25 3
Isobutene (A)	9 0	Ethyl methyl sulphide (B)	26 0
trans-2-Butene (A)	9 2	Dimethyl disulphide (C)	27 0
n-Pentane (A)	9 4	Dimethyl trisulphide (F)	50 2
3-Methyl-1-butene (A)	11 1	S-Methyl thioacetate	57 0

*The retention time of methanethiol decreases to 12 7 min when large amounts are injected

TABLE II

SELECTED LIGHT HYDROCARBONS AND VOSCs DETECTED IN REDUCED PRESSURE HEADSPACE OF TTY MEDIUM CULTURES OF NINETEEN *CLOSTRIDIUM* STRAINS ANALYSED ON A GLASS COLUMN PACKED WITH *n*-OCTANE-PORASIL C

Results, arbitrarily expressed as peak areas in mm^2 (triangulation), are given as means (n = 3 for each strain, n = 2 for uninoculated TTY medium) with standard deviations in parentheses Headspace GC conditions as in Fig 2 Identification of compounds by retention time (RT) (co-chromatography) and/or mass spectrometry (MS), DSM = Deutsche Sammlung von Microorganismen, ATCC = American Type Culture Collection, AIP = Collection des Anaérobies de l'Institut Pasteur, ^{T} = type strain

Clostridium strain	Peak area (mm^2)							
	Methane (RT)	3-Methyl-1-butene (RT, MS)	Methanethiol ($\times 10^{-3}$) (RT, MS)	Ethylene sulphide (RT, MS)	Dimethyl sulphide (RT)	Dimethyl disulphide (RT, MS)	Dimethyl trisulphide (RT, MS)	S-Methyl thioacetate (RT, MS)
Clostridium sp DSM 1786	76 107 (3334)	47 (6)	1431 04 (58 66)	21 (12)	420 (62)	23 334 (3208)	4737 (462)	13 189 (1023)
C ghoni ATCC 25757^T	16 812 (1701)	32 (0)	1379 84 (4 43)	700 (40)	133 (16)	54 827 (12 199)	5737 (331)	3128 (180)
C bifermentans ATCC 638^T	6980 (277)	22 (3)	1424 64 (16 29)	183 (6)	67 (6)	37 867 (1665)	3979 (517)	606 (167)
C bifermentans AIP TM	20 960 (1089)	26 (3)	1361 92 (31 97)	363 (32)	106 (15)	48 533 (14 772)	6156 (983)	773 (115)
C bifermentans DSM 630	12 378 (749)	17 (2)	1397 76 (53 76)	233 (12)	96 (0)	30 000 (1455)	5227 (577)	739 (397)
C sordellii AIP 82	95 552 (7097)	61 (2)	1774 93 (20 94)	83 (12)	2228 (742)	26 333 (6807)	5058 (694)	—
C sordellii AIP 499/82	33 387 (2552)	27 (8)	1599.79 (84 00)	57 (16)	173 (17)	41 240 (2559)	4480 (848)	1302 (337)
C lituseburense ATCC 25759^T	3225 (270)	17 (6)	1752 75 (50 84)	80 (28)	135 (5)	16 353 (2366)	4139 (684)	8365 (845)

C subterminale ATCC 25774^T	14 613	37	1328 64	2196	372	26 000	13 219	1349
	(1114)	(20)	(20 32)	(221)	(73)	(16 341)	(4346)	(749)
C subterminale AIP 1992	23 440	23	1705 60	19	192	74 080	6315	937
	(1406)	(2)	(8 32)	(5)	(21)	(9061)	(591)	(88)
C hastiforme ATCC 33268^T	410 283	149	15 24	31	114	423	–	231
	(15 337)	(2)	(0 36)	(8)	(6)	(188)		(44)
C sporogenes AIP G01	2320	217	2054 83	104	212	37 640	6432	–
	(35)	(6)	(10 35)	(28)	(18)	(2140)	(869)	
C sporogenes AIP 5570	1620	37	2138 44	183	60	37 080	7808	1344
	(105)	(2)	(27 37)	(15)	(11)	(1871)	(1282)	(83)
C histolyticum ATCC 19401^T	46 784	2027	6 27	–	21	144	–	–
	(3038)	(65)	(0 31)		(5)	(24)		
C histolyticum AIP Tro 2E	55 947	1067	6 66	–	18	220	–	–
	(3989)	(94)	(0 87)		(10)	(72)		
C histolyticum AIP 654	77 888	1851	8 76	167	45	363	–	–
	(4354)	(76)	(0 20)	(73)	(13)	(69)		
C histolyticum DSM 627	69 920	2096	6 34	12	27	779	136	–
	(3728)	(112)	(0 36)	(0)	(5)	(341)	(62)	
C histolyticum DSM 1126	38 773	2688	6 37	–	28	421	122	–
	(2026)	(64)	(0 61)		(3)	(237)	(83)	
C cadaveris ATCC 25783^T	460	67	1502 72	46	27	23 160	4344	1986
	(27)	(5)	(37 88)	(15)	(5)	(2796)	(372)	(180)
Uninoculated TTY medium	529	30	2 76	–	–	620	140	502
	(86)	(3)	(1 15)			(226)	(28)	(210)

Paris, France) by hydrolysis and S-methyl thioacetate was a gift from Professor J Adda (Institut National de la Recherche Agronomique, Dijon, France) All other chemicals (listed in Table I) were, at least, of analytical grade.

GC—MS conditions

The GC separation was achieved with the *n*-octane-Porasil C glass column. Electron-impact (70 eV) mass spectra were run on a Riber R 10-10 quadrupole mass spectrometer coupled to a System Industries-Digital Equipment Corporation PDP/8M calculator and a Girdel 30 S gas chromatograph (Nermag, Rueil Malmaison, France). The mass range was 20—250 a.m.u (values 28 and 32 a.m u excluded) and the scan rate was 100 a m.u /s

Gaseous compounds were syringe-sampled from helium-pressurized tubes and, in special instances, successive injections were performed to trap trace components, e.g. ethylene sulphide and C_5 alkenes, on the first centimetre of the column cooled with carbon dioxide. Mass spectra were compared with those of MS registers

RESULTS AND DISCUSSION

A typical chromatogram for *Clostridium* sp. DSM 1786, obtained on *n*-octane-Porasil C, is shown in Fig 2 Retention times of various hydrocarbons and VOSCs on this packing are listed in Table I Results obtained for selected peaks from nineteen *Clostridium* strains are summarized in Table II The

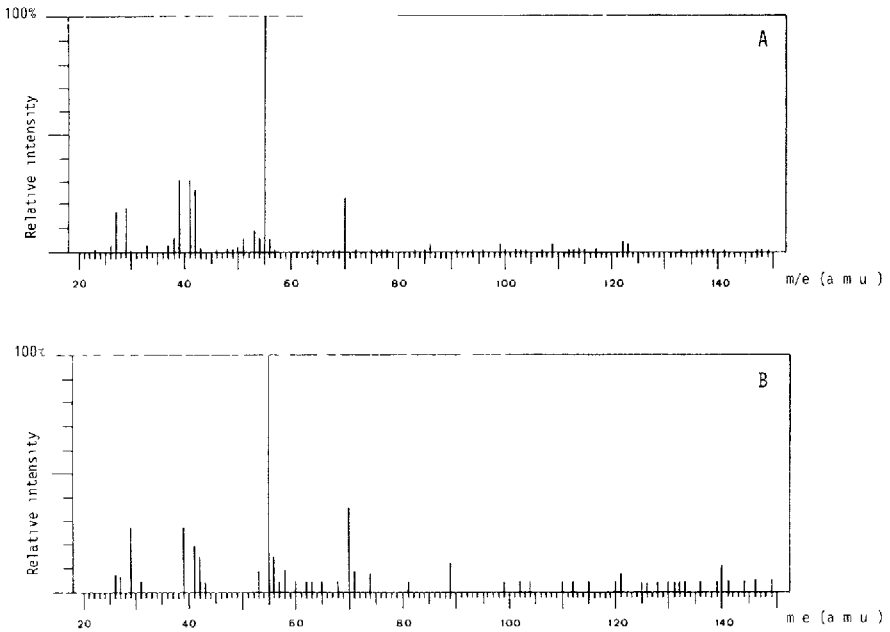

Fig 3 Electron-impact (70 eV) mass spectra of two C_5 alkenes (molecular mass M = 70) from a gas sample of *C histolyticum* ATCC 19401^T cultured in TTY medium (A) 3-Methyl-1-butene and (B) 2-methyl-2-butene Air was added 18 h prior to the GC—MS analysis to oxidize methanethiol The gaseous compounds were preconcentrated on the head of a glass column packed with *n*-octane-Porasil C

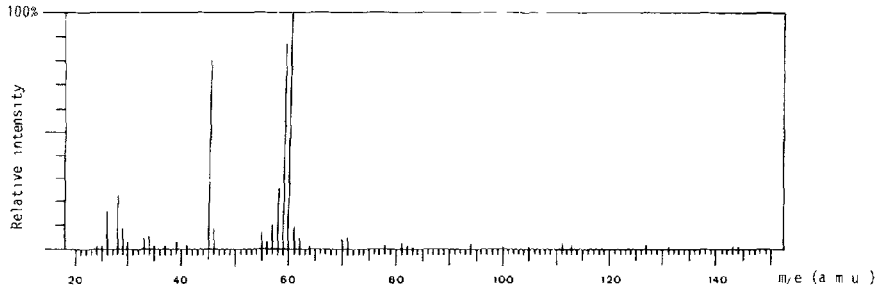

Fig 4 Electron-impact (70 eV) mass spectrum of ethylene sulphide (molecular mass M = 60) from a gas sample of *C sporogenes* AIP G01 cultured in TTY medium supplemented with D-glucose and sodium thioglycolate, after preconcentration on the head of a glass column packed with *n*-octane-Porasil C

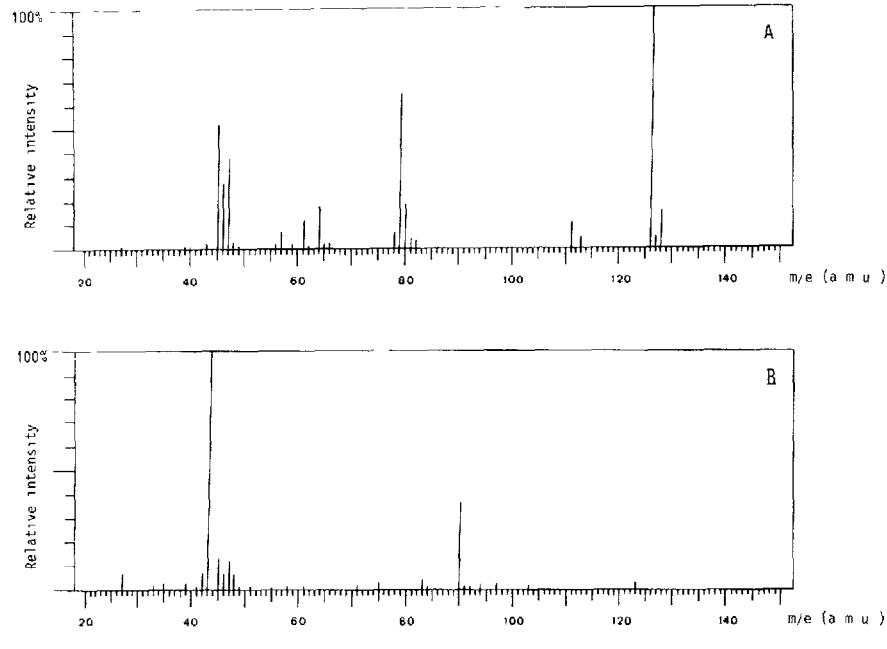

Fig 5 Electron-impact (70 eV) mass spectra of (A) dimethyl trisulphide (molecular mass M = 126) and (B) S-methyl thioacetate (molecular mass M = 90) from a gas sample of *Clostridium* sp DSM 1786 cultured in TTY medium Separation was achieved on a glass column packed with *n*-octane-Porasil C

identity of the peaks was determined by co-chromatography with authentic standards and confirmed by GC—MS for 3-methyl-1-butene (Fig 3A), 2-methyl-2-butene (Fig 3B), methanethiol, ethylene sulphide (Fig 4), dimethyl disulphide, dimethyl trisulphide (Fig 5A) and S-methyl thioacetate (Fig 5B). For methane, C_3—C_4 hydrocarbons and dimethyl sulphide, the identity of the peak was deduced from co-chromatography experiments on *n*-octane-Porasil C and, for methane, on two additional packings, molecular sieve 5 Å and phenylisocyanate-Porasil C (Table III)

To avoid any contamination and/or chemical modifications prior to the GC analysis, 6 *M* sodium hydroxide [2, 3] was not added to the culture and there

TABLE III

ANALYSIS OF METHANE IN REDUCED PRESSURE HEADSPACE OF TTY MEDIUM CULTURES OF *CLOSTRIDIUM* sp DSM 1786

Glass columns packed with either molecular sieve 5 Å (oven temperature 40°C, helium flow-rate 17 ml/min) or phenylisocyanate-Porasil C (oven temperature 10°C, helium flow-rate 32 5 ml/min) were used Results are arbitrarily expressed in μV·s (integration)

Headspace sample (μV s)	Peak area (mean ± S D) (μV/s)	
	Molecular sieve 5 Å (retention time, 5 85 min)	Phenylisocyanate-Porasil C (retention time, 2 10 min)
Clostridium sp DSM 1786	842 182 ± 4451	789 473 ± 21 816
Uninoculated TTY medium	5274 ± 1719	6978 ± 755

was no pretreatment of the gas sample (e g with chemical traps) As moisture in the gas was not removed, ethane was not separated from ethylene [20] and the corresponding peak, very narrow but discernable, was not easily quantitated in the presence of large amounts of methane (Fig. 6) The length of the loop (2 50 m in contrast to 1 40 m for the GC column) caused a slight disturbance of the baseline (Fig 6) but did not perturb the detection of methane As ghosting phenomenon was not observed, the position of a sample

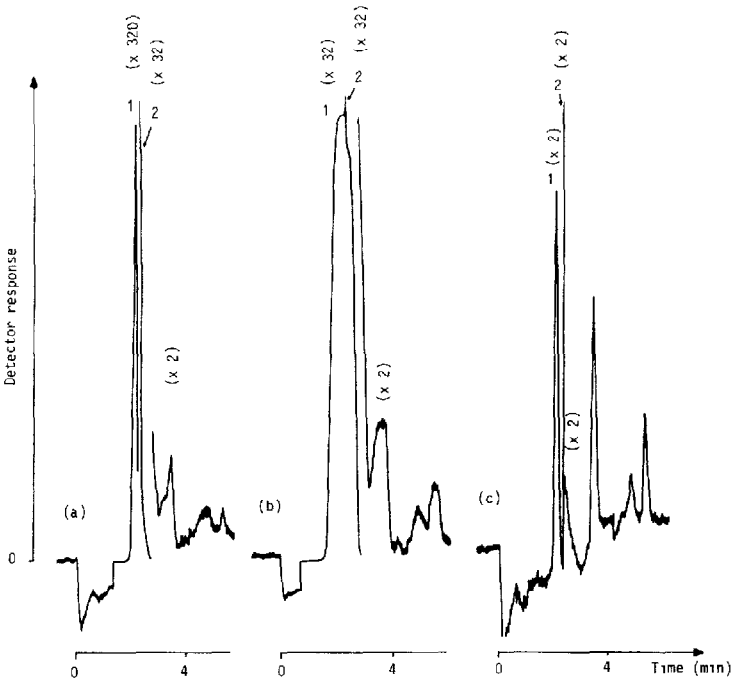

Fig 6 Influence of helium addition and amount of methane injected on the shape of the first peaks eluting on *n*-octane-Porasil C Headspace GC conditions as in Fig 2 (a) *Clostridium* sp DSM 1786 cultured in TTY medium without addition of helium, (b) after addition of helium, (c) *C cadaveris* ATCC 25783^T cultured in TTY medium without addition of helium Peaks 1 = methane, 2 = ethane + ethylene

in the sequence of analyses was not judged important However, the peak of dimethyl disulphide was always more important with the first bacterial gaseous sample and generally decreased slowly with subsequent ones, whereas no variation was observed for other compounds, especially methanethiol and bacterial gas pressures This could be due to the adsorption of dimethyl disulphide on the GC system and/or its reduction to methanethiol in the presence of hydrogen, a gas produced in large amounts by *Clostridium* sp DSM 1786 [23] For this reason, in each quadruplicate, the first chromatogram was put aside for peak area determinations Peak areas have been arbitrarily expressed in mm^2 or, in some instances, in $\mu V \cdot s$ as, in headspace quantitative studies, problems are encountered for calibration and initial concentration determination when gas pressures are in the sub-atmospheric range [24, 25]

In a gas—liquid system, the ratio R, given by the formula $R = (A_G^n - A_G^{n+1})/A_G^n$ where A_G is the peak area corresponding to a gaseous compound G for successive $(n, n+1)$ injections, is constant if this compound is distributed only in the gas phase Consequently, its initial amount can be calculated by extrapolation [24] For methane, results obtained from multiple injections on molecular sieve 5 Å for the same culture tube showed that this analyte behaved in such a way (Table IV) Contrastively, for VOSCs analysed on *n*-octane-Porasil C, especially methanethiol, an enrichment of the gas phase from the liquid phase was observed.

Temperature and gas pressure are major thermodynamic parameters in headspace—GC analysis [25] and must be perfectly controlled. Results could be subtracted only if all thermodynamic parameters (chiefly temperature and gas pressure) are identical for the uninoculated TTY medium and the bacterial culture For this reason, a blank would be needed for each different bacterial culture These virtually ideal blanks would be prepared by introducing hydrogen and carbon dioxide (the main gases produced by *Clostridium*

TABLE IV

ANALYSIS OF METHANE AFTER SUCCESSIVE INJECTIONS OF A REDUCED-PRESSURE HEADSPACE OF A TTY MEDIUM CULTURE OF *CLOSTRIDIUM* sp DSM 1786

The GC separation was achieved on a glass column packed with molecular sieve 5 Å (oven temperature 40°C, helium flow-rate 17 ml/min) Results are arbitrarily expressed in $\mu V \cdot s$ (integration) For the definition of R, see text

Injection step	Methane peak area ($\mu V \cdot s$)	R
1	846 316	
2	660 339	0 219749
3	515 891	0 218748
4	402 602	0 219599
5	316 600	0 213615
6	248 582	0 214839
7	193 197	0 222804
8	149 803	0 224610
9	117 240	0 217372
10	89 388	0 237564

bacteria) to an uninoculated TTY medium, their partial pressures and the pH of the medium being those of a 168-h bacterial culture They have not yet been achieved and further improvements would be needed to conciliate bacteriological and quantitative trace headspace GC analysis requirements. Nevertheless, this series of results showed the presence, at low levels, of some hydrocarbons and VOSCs in the headspace of uninoculated TTY media. Furthermore, to have good resolution of the first eluting peaks, it is obviously advantageous to use reduced-pressure culture tubes rather than the pressurized vials often used in anaerobic microbiology and generally fitted with rubber septa, a source of organic contamination and adsorption

In an attempt to compare the results obtained from bacterial cultures and uninoculated TTY media, another series of experiments was carried out, in which all the tubes were filled up to atmospheric pressure with the carrier gas, helium, before headspace GC analysis on *n*-octane-Porasil C The contribution of light hydrocarbons and VOSCs detected in the headspace of an uninoculated TTY medium to gaseous compounds of *Clostridium* sp DSM 1786 was negligible, except for C_3—C_4 hydrocarbons (Table V) The only drawback was the broadening of the methane peak, a consequence of the injection of a larger volume of gas via the valve (Fig 6) For this reason, the difficulty of determining, with precision, the area of the methane peak did not allow the construction of a calibration curve for methane—helium mixtures at atmospheric pressure. However, when bacterial gaseous compounds were analysed for methane on molecular sieve 5 Å, the shape and the area of the methane peak were the same, whether helium was added or not, thus, it was possible to construct the calibration curve The appropriate amount of methane was introduced into a large dilution tube (volume ca 560 ml) fitted with an

TABLE V

SELECTED LIGHT HYDROCARBONS AND VOSCs DETECTED IN HEADSPACE OF HELIUM-FILLED TTY MEDIUM CULTURES OF *CLOSTRIDIUM* sp DMS 1786

Headspace GC conditions are as in Fig 2

Compound	Peak area (mean ± S D) (mm^2)	
	Clostridium sp DSM 1786	Uninoculated TTY medium
Methane	93 120 ± 1450	203 ± 117
Propane	370 ± 225	50 ± 31
Isobutane	99 ± 29	142 ± 3
n-Butane	160 ± 8	285 ± 38
3-Methyl-1-butene	33 ± 6	—
Methanethiol	1 300 480 ± 34 631	1232 ± 396
Ethylene sulphide	21 ± 9	—
Dimethyl sulphide	296 ± 14	—
Dimethyl disulphide	23 693 ± 3900	330 ± 42
Dimethyl trisulphide	4361 ± 234	93 ± 4
S-Methyl thioacetate	9072 ± 291	—
Unknown*	403 ± 223	310 ± 127

*Retention time = 62 0 min

impinger and purged beforehand with helium, until no GC response for methane could be recorded. Calibration mixtures, realized in the same way for isobutane, were analysed on *n*-octane-Porasil C. It was calculated by extrapolation that the initial amounts of methane and isobutane in a 168-h culture of *Clostridium* sp. DSM 1786 in 5 ml of TTY medium were ca 60 nmol and 50 pmol, respectively. In comparison, they were ca. 150 and 75 pmol in the headspace of an uninoculated TTY medium.

To rule out the artifactual origin of methane, various technical parameters were modified. On the one hand, two modes of sample introduction in the *n*-octane-Porasil C column were compared with the conventional one: (i) conventional sampling, the valve being directly connected to the head of the column with PTFE tubing to shunt the metallic injection port, (ii) on-column injection with a gas-tight syringe from helium-pressurized culture tubes, the only metallic surfaces being the detector inlet and the syringe needle. In both modes, the profiles did not show major differences, although, with the second mode, the early eluting peaks were sharper and their retention times shorter On the other hand, experiments were carried out with grease-free culture tubes (fitted with two PTFE Rotaflo stopcocks) and a liquid-nitrogen trap interposed in the vacuum line during the two steps of air evacuation No noticeable interference created by the vacuum pump and/or Apiezon H grease was found

The fact that, among VOSC (mainly methanethiol)-forming strains (Table II), some produced large amounts of methane, e.g *Clostridium* sp. DSM 1786, and others formed small amounts, if any, of this hydrocarbon, e g *C cadaveris* ATCC 25783^T and *C sporogenes* AIP 55/70, made the chemical transformation of VOSCs into methane very unlikely.

The occurrence of small amounts of methane among gaseous compounds of *Clostridium* bacteria has been previously reported [2, 3, 26, 27] In an earlier report [28], the high proportion (1—5%) of methane found in cultures of *C. bifermentans* and *C. caproicum* isolated from sludges makes questionable the involvement of only these strains in the methanogenesis observed Our results clearly indicate that methane formation is not restricted to a particular strain and that methane-forming strains are distributed among human (e.g *C hastiforme* ATCC 33268^T) and soil (e.g *C ghoni* ATCC 25757^T) isolates [29].

Ethylene sulphide was identified among volatiles of *C sporogenes* AIP G01 cultured in TTY medium supplemented with D-glucose and sodium thioglycolate, conditions in which the formation of this heterocyclic compound is enhanced [3]. As no reagent was added to the sample, the hypothesis of only a sodium hydroxide induced transformation of 2-mercaptoethanol (probably originating from sodium thioglycolate by bacterial reduction) to ethylene sulphide [3] can be ruled out

In gases of several strains of *Clostridium* subjected to direct MS analysis without prior GC separation, methanethiol and dimethyl disulphide have been reported [30], along with C_2—C_4 thiols and dimethyl sulphide for *C tetani* [31], but dimethyl trisulphide and S-methyl thioacetate have not yet been reported in the *Clostridium* group.

For the five strains labelled *C histolyticum* originating from different collections, the headspace GC patterns were virtually identical and showed

two particular hydrocarbon peaks identified as 3-methyl-1-butene and 2-methyl-2-butene, the latter giving a shoulder on the small methanethiol peak

The headspace GC profile of *C hastiforme* ATCC 33268^T was unlike that of *C subterminale* ATCC 25774^T, the most striking feature being the difference in methane and methanethiol formations This can be an additional argument to protein electrophoretic patterns, morphological properties, hydrogen production [29] and citrate utilization [32] for the distinction of these two species

The description of methane and ethylene sulphide as *Clostridium* volatiles is of interest if one keeps in mind that (i) the possible involvement of *Clostridium* bacteria in the cause of some human cancers, particularly large bowel cancers, has been suggested [33], (ii) anaerobic bacteria (e.g. Clostridia) are in a higher number in colonic polyps than in the adjacent normal mucosa [34], (iii) the percentage of humans with detectable methane in their breath is higher in patients with large bowel cancer than in controls [35], and (iv) ethylene sulphide has been reported to induce sarcoma in rats [36]. Therefore, the detection by headspace GC of both methane and ethylene sulphide in biological liquids (urine, blood) and clinical material (e.g colonic polyps and faeces) incubated in suitable media could be useful to study the involvement of *Clostridium* bacteria in cancer patients The headspace GC method described in this paper would also be valuable for the chemotaxonomy of the *Clostridium* group, particularly for clinical isolates resembling *C subterminale* and *C hastiforme* [29] and for the study of environmental samples in which Clostridia are known to be present

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