The Lipid Fatty Acids of Proteolytic Clostridia

By SIDNEY R. ELSDEN,*† MARTIN G. HILTON,
KEITH R. PARSLEY AND RONALD SELF

ARC Food Research Institute, Colney Lane, Norwich NR4 7UA

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Capillary gas-liquid chromatography (g.l.c.) and g.l.c./mass spectrometry (g.l.c./m.s.) were used to study the fatty acid composition of the lipids of the following 23 species of proteolytic clostridia grown in a Trypticase/yeast extract medium: *Clostridium bifermentans*, *C. cadaveris*, *C. caloritolerans*, *C. cochlearium*, *C. difficile*, *C. ghoni*, *C. glycolicum*, *C. histolyticum*, *C. lentoputrescens*, *C. limosum*, *C. lituseburense*, *C. malenominatum*, *C. manganotii*, *C. propionicum*, *C. putrefaciens*, *C. putrificum*, *C. scatologenes*, *C. sordellii*, *C. sporogenes*, *C. sticklandii*, *C. subterminale*, *C. tetani*, *C. tetanomorphum*. These species contained a total of 55 fatty acids in the range C_{12} to C_{18} . The methyl esters were separated on a 152 m stainless steel capillary column coated with poly(1,4-butanediol succinate) and characterized by their equivalent chain lengths and by g.l.c./m.s. The predominant acids were n - $C_{14:0}$ and n - $C_{16:0}$. There were two groups of organisms: those which contained fatty acids of the n , iso and $anteiso$ series and those which contained only those of the n series. All the organisms which contained iso and $anteiso$ acids also oxidized valine, leucine and isoleucine to the corresponding branched-chain volatile fatty acids. The iso acids found had both even and odd numbers of carbon atoms and were probably derived from valine and leucine, respectively; the $anteiso$ acids all had odd numbers of carbon atoms and were probably derived from isoleucine via 2-methylbutyric acid. These organisms also produced small amounts of monoenoic iso acids. Of the seven species which contained only acids of the n series, *C. histolyticum* produces acetic acid as its only end-product whereas the other six ferment glutamic acid and produce acetic and n -butyric acids; they also form propionic acid from threonine.

INTRODUCTION

Moss & Lewis (1967) studied the fatty acid composition of strains of 13 species of the genus *Clostridium* and showed that there were considerable differences between the species. It was possible on this basis to differentiate *C. perfringens* from *C. sporogenes* and from *C. bifermentans* and also these three from the other ten species. The presence of branched-chain fatty acids in the lipids of *C. sporogenes* and *C. bifermentans* was consistent with their ability to oxidize valine, leucine and isoleucine to 2-methylpropionic acid, 3-methylbutyric acid and 2-methylbutyric acid, respectively (Elsden & Hilton, 1978); in addition, the CoA derivatives of these acids are the chain initiators in the synthesis of the iso and $anteiso$ fatty acids – see the excellent review of the fatty acids of the genus *Bacillus* by Kaneda (1977).

The work of Moss & Lewis (1967) encouraged us to make a systematic study of the fatty acid composition of the proteolytic clostridia, particularly since we had considerable information on their catabolic activities. These organisms obtain energy for growth by the anaerobic catabolism of amino acids and it seemed probable that the various catabolic patterns (Mead, 1971) would be reflected in the fatty acid composition of the lipids.

† Present address: School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ.

This paper is concerned with the nature and the amounts of the fatty acids found in the representative strains of 23 species of proteolytic clostridia. The fatty acids were characterized, in so far as is possible, by capillary gas-liquid chromatography and by gas-liquid chromatography/mass spectrometry. The 23 species contained a total of 55 fatty acids in the range C_{12} to C_{18} .

METHODS

Organisms. As in our previous work (Elsden *et al.*, 1976; Elsdén & Hilton, 1978), we used the type strains of Smith & Hobbs (1974) supplemented where necessary with organisms from our own collection. The organisms used were *Clostridium bifermentans* NCIB 10716, *C. cadaveris* NCIB 10676, *C. cochlearium* ATCC 17787, *C. difficile* NCIB 10666, *C. ghoni* NCIB 10636, *C. glycolicum* ATCC 14480, *C. histolyticum* NCIB 503, *C. lentoputrescens* NCIB 10629, *C. limosum* NCIB 10638, *C. lituseburense* NCIB 10637, *C. malenominatum* ATCC 25776, *C. manganotii* NCIB 10639, *C. propionicum* NCIB 10656, *C. putrefaciens* NCIB 9836, *C. putrificum* NCIB 10677, *C. sordellii* NCIB 10717, *C. sporogenes* NCIB 10696, *C. sticklandii* NCIB 10654, *C. subterminale* NCIB 9384 and *C. tetani* NCIB 10628; the non-reference strains used were *C. caloritolerans* NCIB 9360, *C. scatologenes* NCIB 8855 and *C. tetanomorphum* NCTC 500.

Reagents. All chemicals and solvents were of reagent grade quality. The chloroform used to extract lipids was freed from ethanol by washing with water; 2,6-di-*tert*-butyl-*p*-cresol (BHT) was added as an antioxidant at a concentration of 3 mg (100 ml)⁻¹.

Media and cultural conditions. Stock cultures were maintained in cooked-meat medium. The medium used for the growth of the culture to be analysed contained: BBL Trypticase, 2% (w/v); Difco yeast extract, 0.2% (w/v); sodium thioglycollate, 0.05% (w/v); the pH was adjusted to 7.2 to 7.4. The Difco yeast extract was washed with an equal volume of chloroform/methanol (2:1, v/v) to remove its lipids, and then air-dried. In contrast to Moss & Lewis (1967), we did not add glucose to the medium because it was known to distort the pattern of amino acid fermentation and, in particular, to decrease the amounts of the branched-chain amino acids oxidized (Saissac *et al.*, 1948), a finding which we have confirmed. The inocula were grown in 20 ml medium in 28 ml screw-capped bottles. Bulk cultures were grown in 1 l medium in screw-capped bottles of 1 l nominal capacity. The media were sterilized by autoclaving for 15 min at 121 °C.

Immediately before use the small bottles of medium were heated in a boiling water bath for 15 min, then cooled rapidly, inoculated with 0.5 ml of a cooked-meat culture and incubated for 18 h at 37 °C (30 °C for *C. putrefaciens*). This 20 ml culture was used to inoculate 1 l of freshly autoclaved medium which was then incubated for 24 h at the appropriate temperature.

Extraction of lipids. The cells were harvested by centrifuging at 10000 *g* for 20 min, suspended in 30 ml distilled water, transferred to a 50 ml stainless steel centrifuge tube and centrifuged again. The supernatant was removed as completely as possible and the cell paste was mixed with 30 ml chloroform/methanol (2:1, v/v) using a high-speed stainless steel homogenizer. After 5 min the suspension was filtered through a sintered glass filter (porosity G1) and the filtrate was set aside. The residue was re-extracted with 30 ml of the solvent mixture as above, filtered and the filtrate retained. Finally, the residue was homogenized with 30 ml of the solvent mixture, left overnight and then filtered. The three filtrates were combined and taken to dryness under an atmosphere of oxygen-free nitrogen. The residue was dissolved in 5 ml chloroform/methanol/water (120:60:9, by vol.) and the non-lipid contaminants were removed by passage through a column of Sephadex G-25 as described by Wells & Dittmer (1963). The eluate (approximately 10 ml) was taken to dryness under nitrogen and the residue was dissolved in a 1 ml toluene containing 0.005% (w/v) BHT as an antioxidant.

Esterification of fatty acids. The fatty acids in the toluene/BHT mixture (1 ml) were esterified by refluxing for 4 h with 5 ml methanol containing 5% (w/v) H_2SO_4 at 90 °C. The mixture was cooled by adding 15 ml distilled water, transferred to a test tube and the methyl esters were extracted by shaking with 3 × 5 ml petroleum spirit (b.p. 40 to 60 °C). The combined extracts were treated with 4 g Na_2SO_4 and 1 g $NaHCO_3$ to remove water and free acids. The supernatant was then transferred to a small round-bottom flask. The salts were washed twice with 5 ml petroleum spirit (b.p. 40 to 60 °C) and the combined extracts were taken to dryness under nitrogen. The methyl esters were dissolved in a small volume of chloroform and transferred quantitatively to a 2 ml screw-capped vial. The chloroform was removed under nitrogen and the esters were dissolved in 100 μ l chloroform. This solution was used for analysis, but if not required immediately it was stored at -18 °C.

Capillary gas-liquid chromatography (g.l.c.). Initially a stainless steel column (4.88 m × 3 mm o.d.) packed with 10% (w/w) poly(ethylene glycol adipate) on Diatomite C-AW, 100 to 120 mesh (J.J.'s, King's Lynn, Norfolk) was used. The carrier gas was argon (flow rate 25 ml min⁻¹). This column, operated isothermally at 190 °C, provided a great deal of information, including the distribution of the *iso* and *anteiso* acids amongst the species. However, a number of the peaks were obviously mixtures and so a column with greater resolving

Fig. 1. Stages in the analysis of fatty acid methyl esters by capillary g.l.c.: (a) preparation of short packed column; (b) collection of methyl esters in capillary trap; (c) preparation for transfer of esters; (d) transfer of esters to capillary column. 1, G.l.c. oven; 2, injection port; 3, detector; 4, short packed column; 5, stainless steel capillary trap; 6, direct current heater; 7, first liquid nitrogen trap; 8, second liquid nitrogen trap; 9, 152 m stainless steel capillary column wound on two formers; $\rightarrow$, direction of flow of helium.

power was needed. A wall-coated open tubular capillary column, operated isothermally with helium as the carrier gas, gave the required resolution but unfortunately its performance deteriorated rapidly due to the presence of impurities in the chloroform solution of the methyl esters. A preliminary purification of the esters on a short packed column prevented deterioration of the capillary column.

The final procedure (see Fig. 1) was as follows. The capillary column consisted of 152 m $\times$ 0.6 mm i.d. stainless steel tubing, coated with a 10% (w/v) solution of poly(1,4-butanediol succinate) in dichloromethane as described by Mon *et al.* (1967). It was operated isothermally at 170 °C in a Pye series 104 chromatograph with helium as the carrier gas (9 ml min⁻¹) and a flame ionization detector. The small stainless steel column (0.5 m $\times$ 6.35 mm o.d.) was packed with 10% poly(ethylene glycol adipate) on Diatomite C-AW, 100 to 120 mesh. It was fitted in the g.l.c. oven and the exit end was coupled directly to a loop of stainless steel capillary (200 mm $\times$ 0.6 mm i.d.), located outside the oven. The capillary loop was used to trap the methyl esters as they emerged from the column and immediately before each analysis it was washed with 0.5 ml reagent grade chloroform, dried, attached to the packed column, and a direct current heater was fitted, as shown in Fig. 1(a). The oven was then heated to 170 °C, the current was applied (8 A, 5 V) and the helium passed through the system at 40 ml min⁻¹ for 10 min to purge the system of interfering materials. The sample (10 μ l of the chloroform solution) was injected and exactly 60 s later the heater was turned off and the trap was immersed in liquid nitrogen to a depth of 80 to 100 mm. The heater was then quickly rearranged as shown in Fig. 1(b) and current was applied (8 A, 5 V); this prevented the condensation of esters between the column and the trap. The quantitative transfer of the esters to the trap was complete in 30 min. The heater was then switched off and removed. The trap, still in the liquid nitrogen, was disconnected and the packed column was removed. The capillary column was placed in the oven and connected to the flame ionization detector. The first 200 mm of the capillary column were bent into a loop and connected to the trap, as shown in Fig. 1(c). The heater was then coupled across the trap, the coolant was removed, the current was turned on (9 A, 5 V) and the methyl esters were transferred quantitatively from the trap to the cooled loop

of the capillary column; the arrangement is shown in Fig. 1(d). When the transfer was complete (10 min) the liquid nitrogen flask was removed, the oven door was closed, the heating was started and at this point the chart was marked to indicate the beginning of the analysis. Where possible, peaks were identified by their retention times relative to *n*-C_{16:0} (= 100), and by their equivalent chain length values (Miwa *et al.*, 1960). Peak areas and the amounts of each ester were calculated by triangulation and expressed as mol fatty acid per 100 mol total fatty acids.

The preliminary fractionation of the esters on the short packed column prevented the deterioration of the capillary column and its performance remained constant for more than 2 years without recoating. The increased resolution provided by the capillary column is illustrated by the analysis of the methyl esters of the fatty acids from *C. sporogenes*. The chromatogram from the 4.88 m packed column showed 18 peaks whereas the chromatogram of the same extract analysed on the capillary column showed the presence of 32 methyl esters, only a limited number of which could be identified.

Combined gas-liquid chromatography/mass spectrometry (g.l.c./m.s.). The complexity of the mixtures of the fatty acid esters made it clear that the g.l.c. by itself would not provide an identification of all the components, and the mixtures were analysed by g.l.c./m.s. For this purpose the 152 m stainless steel column was used in a Pye series 104 oven which was connected to an AEI MS30 mass spectrometer via an all-glass jet molecular separator. The chromatographic conditions, including the pre-treatment on the short packed column, were as described above. The temperatures of the separator and the ion source were 200 °C and 150 °C, respectively. Data from the mass spectrometer in the repetitive scan mode were acquired and processed by an AEI DS50 SM data system. The scanning rate was 3 s per mass decade and the interval between scans was increased linearly from 20 to 80 ms.

Mass spectrometry in the selected ion monitoring mode with the molecular masses for the saturated and monoenoic fatty acids was used to locate all the compounds studied. Further confirmation of the straight-chain monoenoic acids was obtained by simultaneously recording the *M* - 31 and *M* - 32 ion intensities along with their molecular ions. In the case of the monoenoic *iso* acids, confirmation was obtained by recording the *M* - 55, *M* - 87 and *M* - 105 ions and for the monoenoic *anteiso* acids the *M* - 69, *M* - 101 and *M* - 119 ions were recorded (see Boon *et al.*, 1977a; Boon *et al.*, 1977b).

RESULTS AND DISCUSSION

The 23 species examined contained a total of 55 fatty acids varying from C₁₂ to C₁₈. This must not be taken to imply that fatty acids of shorter or longer chain length were absent, but rather, if any such acids were present in these organisms, the amounts were very small. Table 1 gives the relative retention times and equivalent chain length (ECL) values of the 55 fatty acids and the amounts of the various acids in each species. For convenience, the esters are numbered in ascending order of ECL values with methyl dodecanoate as no. 1.

The mass spectrum produced by electron impact ionization of a methyl ester of a monoenoic acid indicated the mass of the molecular ion but not the position of the double bond. The positions of the double bonds given in Table 1 were determined on the basis of the concordance of the ECL values with those reported by Ackman & Castell (1967) for authentic compounds using g.l.c. with the same liquid phase and temperature as used in these experiments. With this system, the ECL values of the methyl esters of monoenoic acids of the *n* series were consistently larger than those of the esters of the saturated parent acids. However, Table 1 shows that some bacteria contained monoenoic fatty acids whose methyl esters behaved contrary to this generalization, viz nos 9, 10, 18, 19, 26, 27, 38, 39, 40, 49 and 50.

Kaneda (1968, 1977) showed that the ECL values of the methyl esters of monoenoic *iso* and *anteiso* acids were smaller than those of the corresponding *n*-saturated compounds but, as to be expected, were larger than those of the esters of their saturated parent acids. Whilst our g.l.c. system differed from that of Kaneda (1968), who used a 6.1 × 3 mm copper tube packed with 7% poly(ethylene glycol adipate) on Anakron ABS 60-80, the ECL values he obtained for the *iso* and *anteiso* methyl esters were similar to those in Table 1. The lipids of *Bacillus cereus* contain monoenoic *iso* and *anteiso* acids, and Kaneda (1977) reported that the ECL value of *iso*-16:1(10) was 15.9 and *iso*-17:1(10) was 16.88 (using the notation for fatty acids defined in Table 1). The m.s. data of the fatty acids of *C. caloritolerans* and *C.*

difficile had been recorded and analysis of these data using the confirmatory tests for monoenoic *iso* and *anteiso* acids, showed that no. 18 is *iso*-15:1, nos 26 and 27 are *iso*-16:1, no. 39 is *iso*-17:1 and nos 49 and 50 are *iso*-18:1. The agreement between the ECL values of Kaneda (1968, 1977) and those recorded in Table 1 is of interest. In keeping with these results it is likely that nos 9 and 10 are *iso*-14:1 and 38 and 40 are *iso*-17:1. No evidence was obtained for the presence of monoenoic *anteiso* acids in these two species. Boon *et al.* (1977*b*) showed that monoenoic *iso* and *anteiso* acids accounted for 34% of the total fatty acids of *Desulfovibrio desulfuricans*. With the exception of *C. sordellii*, which contained 8.1 mol per 100 mol total fatty acids, the amounts of these acids in the clostridia studied were small; nonetheless further investigation of these compounds would seem necessary.

The results in Table 1 showed that the species examined fell into two groups. The 16 species of the larger group contained *n*, *iso* and *anteiso* acids. The seven species of the smaller group produced only acids of the *n* series. All the larger group oxidized valine, leucine and isoleucine to 2-methylpropionic acid, 3-methylbutyric acid and 2-methylbutyric acid, respectively (Elsden & Hilton, 1978). There is much evidence (see Kaneda, 1977) to show that the CoA derivatives of these branched-chain volatile fatty acids are the chain initiators used in the biosynthesis of the *iso* and *anteiso* acids and that chain lengthening occurs by their sequential reaction with molecules of the C₂ donor malonyl-CoA. This mechanism predicts that the series of acids with an even number of carbon atoms (C-even series) will contain only *iso* acids derived from valine and that the series with an odd number of carbon atoms (C-odd series) will contain both *iso* and *anteiso* acids derived from leucine and isoleucine, respectively. The results in Table 1 show this to be the case. Some species also reduced leucine to *iso*-caproic acid (Elsden & Hilton, 1978) and there was the possibility that leucine, via *iso*-caproic acid, also acted as a precursor of the C-even series of *iso* acids. This was not tested with these organisms, but Kaneda (1971) has found that *Bacillus subtilis* does not incorporate *iso*-caproic acid into fatty acids. The results in Table 1 are consistent with those of Moss & Lewis (1967) save that they found that *C. histolyticum* made branched-chain acids whereas we obtained no evidence for the presence of these compounds in this species.

Kimble *et al.* (1969) examined the fatty acids of *C. botulinum*, proteolytic type B. This organism oxidized valine, leucine and isoleucine to the corresponding volatile fatty acids (Elsden & Hilton, 1978) and in the light of this it was surprising that Kimble *et al.* (1969) found no evidence for the presence of *iso* and *anteiso* acids in this organism.

Clostridium putrefaciens, *C. subterminale* and *C. histolyticum* do not produce propionic acid from threonine (Elsden & Hilton, 1978), yet these species produced C-odd fatty acids. Propionyl-CoA is usually the chain initiator of the C-odd fatty acids and it would be interesting to know how these acids were synthesized by these three species. Emmanuel (1978) has obtained evidence that certain rumen anaerobes oxidized hexadecanoic acid to pentadecanoic acid and octadecanoic acid to heptadecanoic acid; such reactions could account for the presence of the C-odd fatty acids in the above organisms. It is known that temperature influences, inversely, the proportion of unsaturated fatty acids in cell lipids and thus it should be noted that *C. putrefaciens* was grown at 30 °C and the other organisms at 37 °C.

The main object of these analyses was to identify and quantify the fatty acids present in established strains of proteolytic clostridia grown in a standard medium with a protein hydrolysate as the major source of energy; carbohydrates were not added because of the distorting effect these compounds have on the metabolism of amino acids. The results show that the mixtures of fatty acids, particularly those produced by organisms which make *iso* and *anteiso* acids, are more complex than previous analyses had suggested. It follows that, in any future comparative studies, the fatty acid analyses will have to be made with capillary g.l.c. of at least the resolving power of the column used in these experiments and preferably with capillary g.l.c./m.s. Whilst certain patterns of fatty acids are apparent, for example

Table 1. *Fatty acid composition of clostridia*

For each species, results are expressed as mol fatty acid per 100 mol total fatty acids.

No.	Acid*	Mean relative retention time†	Equiv. chain length	<i>C. sporogenes</i>	<i>C. caloritolerans</i>	<i>C. putrificum</i>	<i>C. ghoni</i>	<i>C. lituseburens</i>	<i>C. difficile</i>	<i>C. sticklandii</i>	<i>C. propionicum</i>	<i>C. mangenotii</i>
1	<i>n</i> -12:0	47.78 (1.61)	12.00	0.4	0.1	1.2	2.0	2.4	3.2	2.4	3.6	1.6
2	<i>n</i> -12:1	50.00 (1.41)	12.29	—	—	—	0.1	—	—	—	—	0.1
3	<i>n</i> -12:1	51.00	12.41	—	—	—	—	—	—	—	—	—
4	<i>i</i> -13:0	52.54 (1.50)	12.60	—	—	0.2	0.4	0.7	0.1	1.0	3.8	0.2
5	<i>n</i> -12:1	53.00	12.66	—	—	—	—	—	—	—	—	—
6	<i>a</i> -13:0	53.92 (1.26)	12.77	—	—	0.1	1.1	0.4	0.1	0.5	0.3	0.5
7	<i>n</i> -13:0	55.95 (1.66)	13.00	0.7	0.9	5.0	1.5	1.2	1.5	0.8	0.5	2.6
8	<i>i</i> -14:0	61.44 (1.37)	13.53	1.1	1.0	1.1	1.1	0.5	0.5	0.6	1.9	0.6
9	14:1	63.44	13.71	—	—	—	—	—	—	—	—	—
10	14:1	65.75 (1.48)	13.91	—	—	—	0.2	—	—	—	—	0.1
11	<i>n</i> -14:0	66.78 (1.18)	14.00	18	17	27	8.1	11	3.3	17	21	3.8
12	<i>n</i> -14:1	69.10 (1.15)	14.19	0.4	0.1	0.3	0.5	0.3	0.5	0.3	0.8	0.1
13	<i>n</i> -14:1(7)	70.86 (0.94)	14.32	1.4	1.5	2.1	1.4	0.5	0.6	1.6	0.7	0.9
14	<i>n</i> -14:1	72.26 (1.22)	14.43	0.4	0.3	0.6	0.2	0.4	0.5	0.2	0.2	0.2
15	<i>i</i> -15:0	73.69 (1.21)	14.54	1.9	2.2	1.6	0.5	1.5	0.8	3.8	13	0.4
16	<i>n</i> -14:1	74.56 (0.83)	14.60	—	—	0.7	—	—	—	—	2.3	—
17	<i>a</i> -15:0	75.93 (1.18)	14.70	0.6	0.5	0.7	0.8	0.4	0.3	1.2	3.6	0.2
18	<i>i</i> -15:1‡	77.70 (1.19)	14.82	—	0.1	0.1	0.1	—	0.1	0.5	—	—
19	15:1	78.50 (0.87)	14.88	—	—	0.3	0.3	0.2	—	—	1.7	0.2
20	<i>n</i> -15:0	80.22 (1.06)	15.00	17	16	15	1.4	7.0	1.1	4.1	1.4	2.2
21	<i>n</i> -15:1	83.25 (1.09)	15.18	—	—	—	0.1	0.2	—	—	—	—
22	<i>n</i> -15:1	85.05 (0.92)	15.26	1.3	1.4	1.2	0.1	0.4	0.3	0.5	—	0.4
23	<i>n</i> -15:1	86.81 (1.03)	15.36	1.1	1.9	1.5	0.2	0.6	0.1	0.1	0.3	0.2
24	<i>n</i> -15:1	88.00	15.42	—	—	—	—	—	—	—	—	0.8
25	<i>i</i> -16:0	89.07 (1.00)	15.47	3.7	2.2	1.3	2.9	1.5	7.6	0.4	0.8	3.2
26	<i>i</i> -16:1‡§	94.89 (0.74)	15.76	0.2	0.1	—	0.9	—	0.5	—	—	0.3
27	<i>i</i> -16:1‡§	96.16 (0.99)	15.82	0.3	0.5	—	1.1	—	0.6	—	—	0.5
28	<i>n</i> -16:0	100.00	16.00	27	27	23	35	35	37	45	20	36
29	<i>n</i> -16:1	103.44 (0.50)	16.14	0.2	1.1	0.4	1.1	1.0	—	—	—	—
30	<i>n</i> -16:1	104.00	16.18	—	—	—	—	0.8	—	—	—	—
31	<i>n</i> -16:1(7)	105.91	16.27	2.3	1.4	1.8	8.0	2.2	2.8	4.0	2.1	6.2
32	<i>n</i> -16:1(9)	107.91 (0.88)	16.32	5.0	5.6	4.0	6.0	2.1	3.6	5.8	0.9	5.8
33	<i>n</i> -16:1	110.22 (1.20)	16.45	1.6	1.5	1.2	0.7	0.2	1.5	0.8	0.6	1.5
34	<i>i</i> -17:0	111.40 (1.14)	16.50	0.4	0.4	0.1	1.6	2.3	2.1	1.1	1.1	0.8
35	<i>n</i> -16:1	114.67 (1.37)	16.64	1.6	0.5	0.9	0.3	0.5	—	0.2	0.3	—
36	<i>a</i> -17:0	115.53 (1.26)	16.67	0.6	0.4	0.1	1.4	0.8	1.9	0.3	0.9	1.3
37	<i>n</i> -16:1	116.00	16.69	—	—	—	—	—	—	0.3	—	—
38	17:1	117.25 (1.30)	16.74	—	—	—	0.2	—	—	—	—	—
39	<i>i</i> -17:1‡	118.81 (1.47)	16.80	—	0.1	—	0.2	—	0.6	0.3	0.3	0.2
40	17:1	121.00 (1.41)	16.88	—	—	—	0.4	—	—	—	—	—
41	<i>n</i> -17:0	124.14 (1.79)	17.00	3.5	3.9	1.4	1.4	7.2	2.7	0.6	1.1	7.5
42	<i>n</i> -17:1	129.60 (1.36)	17.12	—	—	—	—	0.2	—	—	—	—
43	<i>n</i> -17:1	132.53 (1.63)	17.18	—	1.4	0.4	0.4	0.5	0.6	0.2	—	—
44	<i>n</i> -17:1	134.09 (1.56)	17.22	0.9	—	0.4	0.6	0.6	0.5	0.6	—	1.3
45	<i>n</i> -17:1(9)	136.45 (2.01)	17.26	1.6	3.0	0.6	1.2	1.0	0.6	0.4	0.2	0.9
46	<i>n</i> -17:1	141.16 (1.07)	17.36	—	—	—	—	—	—	—	—	0.4
47	<i>i</i> -18:0	141.83 (2.19)	17.37	0.2	0.2	—	0.5	0.6	9.3	—	—	0.6
48	<i>n</i> -17:1	144.33 (2.31)	17.42	0.2	0.2	0.2	0.2	0.1	—	0.2	0.3	0.3
49	<i>i</i> -18:1‡	150.25 (2.59)	17.53	—	0.1	0.1	0.3	0.1	1.5	0.2	—	0.3
50	<i>i</i> -18:1‡	152.50 (2.00)	17.57	—	0.1	0.2	0.3	0.2	—	—	—	0.1
51	<i>n</i> -18:0	160.48 (4.08)	18.00	3.1	3.3	3.4	5.7	11	12	2.5	11	10
52	18:1	165.06	18.13	—	—	—	—	0.2	—	—	—	—
53	<i>n</i> -18:1(9 <i>cis</i>)	172.09 (4.65)	18.32	0.9	1.1	0.7	5.3	2.0	1.3	1.8	4.4	4.5
54	<i>n</i> -18:1(11 <i>cis</i>)	175.30 (4.71)	18.40	1.8	1.5	0.8	3.4	1.5	0.2	0.7	0.9	1.8
55	<i>n</i> -18:1(13)	177.88 (2.37)	18.47	0.7	0.5	0.3	0.8	0.6	—	0.3	—	1.2
No. of molecular species				32/55	36/55	37/55	45/55	40/55	34/55	35/55	30/55	40/55

* In fatty acid designations, the first number indicates the number of carbon atoms and the second the number of double bonds; the prefixes *n*, *i* and *a* indicate normal, *iso*-branched and *anteiso*-branched acids, respectively; numbers in parentheses indicate the position of the double bond.

Table 1 (cont.)

No.	<i>C. bifermentans</i>	<i>C. sordellii</i>	<i>C. glycolicum</i>	<i>C. scatologenes</i>	<i>C. subterminale</i>	<i>C. putrefaciens</i>	<i>C. cadaveris</i>	<i>C. histolyticum</i>	<i>C. tetanomorphum</i>	<i>C. tetani</i>	<i>C. limosum</i>	<i>C. lentoputrescens</i>	<i>C. malenominatum</i>	<i>C. cochlearium</i>	Frequency
1	0.3	0.4	2.1	3.5	6.5	10	1.4	9.6	0.6	7.7	2.2	2.1	1.1	4.7	23/23
2	—	—	—	—	—	—	—	0.5	—	—	—	—	—	—	3/23
3	—	—	—	—	—	0.8	—	0.6	—	—	—	—	—	—	2/23
4	0.2	1.2	2.3	0.4	2.1	—	0.1	—	—	—	—	—	—	—	13/23
5	—	—	—	—	—	0.9	—	0.5	—	—	—	—	—	—	2/23
6	0.5	1.7	1.2	—	1.4	—	0.3	—	—	—	—	—	—	—	12/23
7	0.6	2.6	1.9	0.1	0.5	—	0.3	0.5	0.1	1.0	0.6	0.6	0.3	0.8	22/23
8	1.1	1.2	2.2	0.3	0.2	0.7	0.3	—	—	—	—	—	—	—	16/23
9	—	—	0.1	—	—	—	—	—	—	—	—	—	—	—	1/23
10	0.1	—	0.1	—	—	—	—	—	—	—	—	—	—	—	4/23
11	3.8	1.8	5.7	7.8	22	5.2	21	35	26	35	28	40	38	47	23/23
12	0.1	—	0.4	0.3	0.9	0.9	0.5	2.8	2.2	2.9	0.9	2.6	1.1	—	21/23
13	0.2	0.2	0.8	0.7	1.1	3.0	0.7	5.3	1.7	3.3	1.6	4.1	4.3	—	22/23
14	0.1	0.2	0.6	0.3	0.4	—	0.2	1.3	0.6	0.9	0.2	0.8	1.0	0.5	22/23
15	2.1	4.4	3.8	1.0	4.5	0.1	0.3	—	—	—	—	—	—	—	16/23
16	—	—	—	—	—	—	—	1.2	0.7	1.0	0.2	0.8	0.8	0.4	9/23
17	1.7	2.3	2.1	0.6	2.5	—	0.8	—	—	—	—	—	—	—	15/23
18	0.2	0.5	0.5	0.2	0.6	—	—	—	—	—	—	—	—	—	10/23
19	—	0.2	0.1	—	—	—	0.1	—	—	—	—	—	—	—	8/23
20	7.8	1.1	8.3	6.7	0.6	1.1	0.9	0.9	0.6	1.3	3.6	1.5	2.7	2.7	23/23
21	0.1	—	0.4	—	—	—	—	—	—	—	—	—	—	—	4/23
22	0.2	0.1	1.3	0.3	0.1	—	0.1	0.3	0.2	0.3	0.3	0.6	0.6	—	20/23
23	0.1	—	0.8	1.7	0.2	3.2	0.2	0.6	0.3	2.0	0.5	0.9	0.7	1.7	22/23
24	—	—	—	—	—	—	—	0.3	—	—	—	—	—	—	2/23
25	9.5	8.6	2.9	0.9	0.2	—	0.4	—	—	—	—	—	—	—	15/23
26	0.6	1.0	0.7	—	—	—	—	—	—	—	—	—	—	—	8/23
27	0.3	0.5	0.4	0.5	—	0.3	0.2	—	—	—	—	—	—	—	11/23
28	27	19	30	41	39	27	52	12	18	18	39	20	20	34	23/23
29	—	0.3	1.2	—	—	—	—	1.0	1.8	—	—	—	—	—	9/23
30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1/23
31	1.5	1.6	4.9	—	4.0	4.1	2.1	8.3	7.1	4.4	6.6	10	7.7	0.4	22/23
32	0.6	0.6	2.0	15	4.1	2.0	1.3	6.6	5.6	4.0	8.6	9.6	12	0.5	23/23
33	0.1	0.4	0.6	3.3	—	3.0	0.3	1.7	1.7	1.0	1.0	1.4	0.8	0.6	22/23
34	3.4	9.4	2.2	0.9	1.6	—	0.4	—	—	—	—	—	—	—	15/23
35	0.8	—	3.0	0.5	—	3.0	—	0.5	0.8	0.5	0.6	0.5	0.8	0.3	18/23
36	4.5	7.4	1.1	0.5	1.0	—	0.8	—	—	—	—	—	—	—	15/23
37	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1/23
38	0.3	2.0	0.4	0.4	—	—	—	—	—	—	—	—	—	—	5/23
39	0.3	1.5	0.4	—	0.3	—	—	—	—	—	—	—	—	—	10/23
40	—	1.2	0.5	—	—	—	—	—	—	—	—	—	—	—	3/23
41	11	5.3	4.1	1.9	0.2	0.8	0.8	0.3	0.3	0.4	0.9	—	0.3	0.4	22/23
42	0.1	0.6	0.4	—	—	—	—	—	—	—	0.3	—	—	—	5/23
43	1.0	0.5	1.0	—	—	1.0	—	0.3	—	0.4	0.4	—	0.2	0.2	15/23
44	1.1	—	—	0.9	—	—	—	—	0.4	—	—	—	0.3	—	11/23
45	0.7	0.5	1.7	1.8	0.7	—	0.8	—	—	0.3	0.4	0.2	0.5	0.5	20/23
46	0.8	—	—	0.6	—	—	—	1.0	—	0.5	—	—	—	0.2	6/23
47	1.8	3.8	0.3	—	—	—	—	—	—	—	—	—	—	—	9/23
48	0.2	0.3	0.4	0.2	0.1	1.2	0.1	0.4	—	0.4	—	—	—	0.2	18/23
49	0.3	0.7	0.2	—	0.1	—	0.1	—	—	—	—	—	—	—	12/23
50	0.2	0.5	—	—	—	—	0.1	—	—	—	—	—	—	—	8/23
51	8.5	9.6	2.6	5.4	3.0	1.9	12	3.6	5.6	7.3	1.8	1.5	4.1	3.0	23/23
52	—	0.7	—	—	—	—	—	—	—	—	—	—	—	—	2/23
53	1.5	5.2	2.2	1.8	1.2	10	0.7	3.9	22	6.9	1.6	2.4	2.4	1.7	23/23
54	2.8	0.7	0.4	0.5	0.4	2.7	0.2	0.6	2.9	0.5	0.9	0.4	0.3	0.2	23/23
55	1.4	0.7	1.7	—	0.5	—	0.5	0.4	0.8	—	—	—	—	—	14/23
43/55	40/55	44/55	31/55	30/55	22/55	32/55	28/55	22/55	23/55	22/55	19/55	22/55	20/55		

† Figures in parentheses are standard deviations.

‡ Acid identified in *C. caloritolerans*.§ Acid identified in *C. difficile*.

C. difficile contains larger amounts of *iso*-C₁₆ and *iso*-C₁₈ than any other species studied, we do not know how consistent this pattern is in strains of this species. Similarly, the constancy of the presence, let alone the amounts, of the minor fatty acids – arbitrarily defined as those present in amounts smaller than 1 mol per 100 mol total fatty acids – needs to be examined at the strain level. Attention will also have to be paid to the monoenoic *iso* acids, and the location of the double bond in the common monoenoic *n* acids nos 14, 23 and 33 has to be established. Valine, leucine and isoleucine are the precursors of the *iso* and *anteiso* acids and since the amounts of these amino acids vary with the protein used it would be desirable in future comparative studies for agreement to be reached on the type of protein hydrolysate to be used in the medium. With these reservations in mind, certain generalizations may be made. Of the 55 fatty acids, eight were present in all 23 species, namely *n*-12:0, *n*-14:0, *n*-15:0, *n*-16:0, *n*-16:1(9), *n*-18:0, *n*-18:1(9) and *n*-18:1(11); a further seven acids were present in 22 species, namely *n*-13:0, *n*-14:1(7), *n*-14:1 (no. 14), *n*-15:1 (no. 23), *n*-16:1(7), *n*-16:1 (no. 33) and *n*-17:0. Of these acids, *n*-14:0 and *n*-16:0 were quantitatively the most important. *n*-16:0 tended to be the dominant acid in those organisms which made *iso* and *anteiso* acids, and *n*-14:0 was the major acid produced by those organisms which only made those acids of the *n* series.

The analyses showed that the qualitative composition of the mixtures of fatty acids reflected the catabolic processes of the organisms; the exception was the synthesis of C-odd fatty acids by organisms which do not make propionic acid from threonine. The mixture of fatty acids in those organisms which produce 2-methylpropionic acid, 3-methylbutyric acid and 2-methylbutyric acid were the most complex, e.g. *C. ghoni* contained 45 acids. Those organisms which made only *n* acids produced 19 to 28 acids. Of these organisms, *C. histolyticum* ferments a very small number of amino acids (Mead, 1971), and apart from ammonia and carbon dioxide, the only end-product so far identified is acetic acid (Guillaume *et al.*, 1956). The other six species, *C. tetanomorphum*, *C. tetani*, *C. limosum*, *C. lentoputrescens*, *C. malenominatum* and *C. cochlearium* ferment glutamic acid to acetic and *n*-butyric acids via the methyl aspartate pathway (Buckel & Barker, 1974; W. Buckel, personal communication); they also have in common the ability to convert tyrosine to phenol (Elsden *et al.*, 1976; M. G. Hilton, unpublished).

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REFERENCES

- ACKMAN, R. G. & CASTELL, J. D. (1967). A generalized study of the effect of structure and other factors in open tubular gas chromatography of fatty acids on a polyester liquid phase with particular reference to monoethylenic fatty acid isomers. *Journal of Gas Chromatography* **5**, 489–496.
- BOON, J. J., VAN DE GRAAF, B., SCHUYL, P. W. J., DE LANG, F. & DE LEEUW, J. W. (1977*a*). The mass spectrometry of *iso*- and *anteiso*-monoenoic fatty acids. *Lipids* **12**, 717–721.
- BOON, J. J., DE LEEUW, J. W., VAN DER HOEK, G. J. & VOSJON, J. H. (1977*b*). Significance and taxonomic value of *iso*- and *anteiso*-monoenoic fatty acids and branched β -hydroxy acids in *Desulfovibrio desulfuricans*. *Journal of Bacteriology* **129**, 1183–1191.
- BUCKEL, W. & BARKER, H. A. (1974). Two pathways of glutamic fermentation by anaerobic bacteria. *Journal of Bacteriology* **117**, 1248–1260.
- ELSDEN, S. R. & HILTON, M. G. (1978). Volatile acid production from threonine, valine, leucine and isoleucine by clostridia. *Archives of Microbiology* **117**, 165–172.
- ELSDEN, S. R., HILTON, M. G. & WALLER, J. M. (1976). The end products of the metabolism of aromatic aminoacids by clostridia. *Archives of Microbiology* **107**, 283–288.
- EMMANUEL, B. (1978). The relative contribution of propionate and long chain even-numbered fatty acids to the production of long-chain odd-numbered fatty acids in rumen bacteria. *Biochimica et biophysica acta* **528**, 239–246.
- GUILLAUME, J., BEERENS, H. & OSTEUX, H. (1956). Production de gaz carbonique et fermentation du glycocolle per *Clostridium histolyticum*. *Annales de l'Institut Pasteur* **91**, 721–726.
- KANEDA, T. (1968). Fatty acids in the genus *Bacillus*. II. Similarity in the fatty acid compositions of *Bacillus thuringiensis*, *Bacillus anthracis* and

- Bacillus subtilis*. *Journal of Bacteriology* **95**, 2210–2216.
- KANEDA, T. (1971). Incorporation of branched-chain C₆-fatty acid isomers into the related long-chain fatty acids by growing cells of *Bacillus subtilis*. *Biochemistry* **10**, 340–347.
- KANEDA, T. (1977). Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriological Reviews* **41**, 391–418.
- KIMBLE, C. E., MCCOLLOUGH, M. L., PATERNO, V. A. & ANDERSON, A. W. (1969). Comparison of the fatty acids of proteolytic type B and non-proteolytic types E and F *Clostridium botulinum*. *Applied Microbiology* **15**, 883–888.
- MEAD, G. C. (1971). The amino acid-fermenting clostridia. *Journal of General Microbiology* **67**, 47–56.
- MIWA, T. K., MIKOLAJCZAK, K. L., EARL, F. R. & WOLFF, I. A. (1960). Gas chromatographic characterization of fatty acids. Identification constants for mono and dicarboxylic methyl esters. *Analytical Chemistry* **32**, 1739–1742.
- MON, T. R., FORREY, R. R. & TERANISHI, R. (1967). Effects of adsorption-reducing material with open tubular and packed column gas chromatography. *Journal of Gas Chromatography* **5**, 497–500.
- MOSS, C. W. & LEWIS, V. J. (1967). Characterization of clostridia by gas chromatography. I. Differentiation of species by cellular fatty acids. *Applied Microbiology* **15**, 390–397.
- SAISSAC, R., RAYNAUD, M. & COHEN, G.-N. (1948). Variation du type fermentaire des bactéries anaérobies du groupe de *Cl. sporogenes* sous l'influence du glucose. *Annales de l'Institut Pasteur* **75**, 305–309.
- SMITH, L. D. & HOBBS, G. (1974). Genus III *Clostridium*. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 551–572. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins.
- WELLS, M. A. & DITTMER, J. C. (1963). The use of Sephadex for the removal of non-lipid contaminants from lipid extracts. *Biochemistry* **2**, 1259–1263.