Fatty Acid Composition of Thermophilic, Mesophilic, and Psychrophilic Clostridia

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The fatty acid composition of several thermophilic bacteria of the genus Bacillus was recently investigated (7, 17, 21). These organisms were found to be rich in saturated straight- and branched-chain fatty acids. The latter types consisted mainly of the iso fatty acids, which have higher melting points than anteiso acids. There was a reasonable correlation between the percentage of iso fatty acids and the growth temperature of the organisms: thermophiles contained a higher percentage of iso acids than anteiso acids, whereas the converse was observed for mesophiles. As part of a program for investigating the cellular components of thermophilic anaerobes, this study was conducted to determine the fatty acid distribution pattern of two thermophilic anaerobes. For comparative purposes, we also investigated the fatty acid composition of a mesophilic and a psychrophilic anaerobe.

MATERIALS AND METHODS

Cultivation of organisms. Clostridium tartarivorum strain T9-0 and Clostridium sp. strain 69, a psychrophile (18), were kindly supplied by R. H. Vaughn and J. L. Stokes, respectively. C. thermosaccharolyticum strain 3814 and C. tartarivorum were grown at 55 C in the medium previously described (20). C. pasteurianum strain W-5 was grown at 30 C in the same medium. Because the psychrophile did not grow in this medium, it was grown at 18 C in the medium described by Sinclair and Stokes (18). All of the organisms were cultivated anaerobically at the temperatures described and harvested by centrifugation at their respective mid-exponential growth phase.

Preparation of lipids and GLC. Cells from 200-ml cultures were washed twice with water, and lipids were extracted according to Folch et al. (8). The lipids were saponified (1) and esterified with BF₃-methanol by the method of Metcalfe and Schmitz (15). The resulting methyl esters in n-hexane were subjected to gas-liquid chromatography (GLC) employing a Varian aerograph (model 1740) equipped with a hydrogen flame detector. The columns were packed with 6% diethylene glycol succinate on Diatop or S and operated at 145 C with a carrier gas (nitrogen) flow rate of 50 ml per min. Analytical determinations were made with a ¼ inch by 10 ft column and preparative runs were conducted with a ¾ inch by 10 ft column.

Identification of fatty acids. Fatty acids were identified by comparing the retention times with standard fatty acid methyl esters; in cases where standard compounds were not available, the linear relationship between the logarithm of retention time and the number of carbon atoms in homologous fatty acids (11) was used. Unsaturated fatty acids were confirmed by hydrogenation with 10% palladium on charcoal as a catalyst. The presence of cyclopropane fatty acids was established by hydrogenation followed by bromination in cold ether (3). Reductive cleavage of cyclopropane fatty acids was conducted by the method of McCloskey and Law (13) with the modification that the reaction was allowed to proceed under 60 psi of hydrogen pressure for 15 hr.

Determination of the position of unsaturation in fatty acids. The unsaturated acids were oxidatively cleaved with periodate-permanganate in water containing 30% dioxane by the method of Von Rudloff (19). The resulting monocarboxylic and dicarboxylic acids were esterified and examined by GLC.

NMR and mass spectrometry studies. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian HA-100 instrument. Mass spectra were obtained with an Atlas CH-5 mass spectrometer equipped with a gas-chromatograph inlet system. The GLC column used for obtaining the mass spectra was the analytical column described above. The transfer system
including He separators was maintained at 250 C, and the ion source was maintained at 100 C. The ion source pressure was approximately $2 \times 10^{-4}$ mm of Hg; ionizing potential was 70 ev. Mass spectral analyses were also performed by the Battelle Memorial Institute, Columbus, Ohio.

**Synthesis of 9,10-methylenetetradecanoic Acid.** The compound 9,10-methylenetetradecanoic acid was synthesized from myristoleic acid by the method of Christie and Holman (6).

Standard fatty acids were obtained from Applied Science Laboratories, Inc. All organic solvents used in this study were distilled before use. Fatty acids were quantitated with an integrator installed in the GLC recorder. Infrared spectra were obtained with a Beckman IR-8 spectrophotometer.

**RESULTS**

**Fatty acid distribution in thermophilic, mesophilic, and psychrophilic anaerobes.** The fatty acids of *C. thermosaccharolyticum*, *C. tartarivor- rum*, *C. pasteurianum* and Clostridium sp. strain 69 are presented in Table 1. Notable differences between the organisms were (i) the thermophiles contained greater amounts of iso-fatty acids, i-C_{15}, and i-C_{17}, than the mesophile or psychrophile; (ii) *C. pasteurianum* contained more cyclopropane types than any of the others; (iii) the mesophile and psychrophile had a greater total number of unsaturated fatty acids, i.e., cyclopropane and olefinic acids; (iv) in the thermophiles, the branched-chain fatty acids predominated and, of the two types, iso-and anteiso-, the iso-fatty acids were present in greater amount.

Since the psychrophile was grown in a different medium than the other organisms used in this study, its fatty acid distribution pattern (Table 1) was perhaps a reflection of the medium or the growth temperature (or both) employed. If it is assumed that the complex medium used for growing the mesophile and the thermophiles consisted of a similar composition, i.e., containing an excess of fatty acid precursors such as the complex medium employed for growing the psychrophile, then a comparison with respect to temperature differences can be justified.

During the studies on the fatty acid distribution pattern shown in Table 1, an unidentified unsaturated fatty acid was detected to be present in all four organisms. Since this was most abundant in the psychrophile, this organism was employed for the isolation and characterization of the compound.

**Unsaturated fatty acid composition of Clostridi- um sp. strain 69.** The unsaturated fatty acids from the psychrophile, comprising 50 to 55% of the total fatty acids, were separated from the saturated acids by mercuric acetate treatment and silicic acid column chromatography (9).

**Table 1. Fatty acid distribution pattern of**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Organism*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Straight chain</td>
<td></td>
</tr>
<tr>
<td>C_{10}</td>
<td>T</td>
</tr>
<tr>
<td>C_{12}</td>
<td>1.5</td>
</tr>
<tr>
<td>C_{14}</td>
<td>13.9</td>
</tr>
<tr>
<td>C_{15}</td>
<td>1.4</td>
</tr>
<tr>
<td>C_{16}</td>
<td>10.0</td>
</tr>
<tr>
<td>C_{18}</td>
<td>1.1</td>
</tr>
<tr>
<td>Branched chain</td>
<td></td>
</tr>
<tr>
<td>a-C_{13}</td>
<td>1.0</td>
</tr>
<tr>
<td>i-C_{15}</td>
<td>44.1</td>
</tr>
<tr>
<td>a-C_{17}</td>
<td>3.3</td>
</tr>
<tr>
<td>i-C_{17}</td>
<td>6.4</td>
</tr>
<tr>
<td>Cyclopropane</td>
<td></td>
</tr>
<tr>
<td>C_{13}</td>
<td>2.8</td>
</tr>
<tr>
<td>C_{15}</td>
<td>3.9</td>
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<tr>
<td>C_{17}</td>
<td>5.2</td>
</tr>
<tr>
<td>C_{19}</td>
<td>2.2</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td></td>
</tr>
<tr>
<td>C_{18:1}</td>
<td></td>
</tr>
<tr>
<td>$\Delta^2$-C_{16:1}$^a$</td>
<td></td>
</tr>
<tr>
<td>$\Delta^{11:1}$-C_{18:2}$^a$</td>
<td></td>
</tr>
</tbody>
</table>
| Unsaturated cyclo-
| propane$^b$         |   | 7.8  | 10.9 | 9.8 | 18.2 |

* A. *C. thermosaccharolyticum*; B. *C. tartarivorum*; C. *C. pasteurianum*; D. *Clostridium* sp. strain 69.

Values are expressed as per cent by weight of total fatty acids and represent averages of three separate samples.

$^a$Trace (less than 1%).

$^b$Evidence for these fatty acids is presented in the text.

Figure 1 shows the unsaturated fatty acid pattern of this organism. Fraction F1 represents the unusual fatty acid described above. Fraction F2 was present in a significant quantity; upon hydrogenation of F2a and F2b the product had a retention time corresponding to palmitic acid. This suggested that F2 consisted of two C_{16:1} compounds differing only in the position of unsaturation. By preparative GLC, fractions F1 and F2 were collected individually for characterization.

**Behavior of F1 during hydrogenation and bromi-

ation treatments.** When purified F1 was analyzed by GLC, a single peak was observed (Fig. 2A). Hydrogenation under 1 atm with a palladium catalyst caused a shift in retention time, suggesting that an unsaturated bond was reduced (Fig. 2B). Bromination of the reduced compound (3) caused the disappearance of the peak shown in Fig. 2B, indicating that a cyclopropane structure was present in the molecule. When the re-
duced product (Fig. 2B) was further hydrogenated under 60 psi for 15 hr with a PtO₂ catalyst, the cyclopropane structure was reductively cleaved into three products corresponding to i-C₁₅, a-C₁₅, and n-C₁₅ (Fig. 2C).

**Location of the olefinic bond in F₁.** Fraction F₁ was oxidized with periodate-permanganate reagent (19) and the oxidized product, after esterification, was analyzed by GLC. The only compound identified was the nine-carbon dicarboxylic acid, azelaic acid. This was unequivocal proof that the site of unsaturation was between the 9 and 10 carbon atoms of the molecule.

The data obtained from experiments which included mild hydrogenation, bromination, reductive cleavage, and oxidative cleavage reactions suggest that the F₁ is a methyl ester of a fatty acid which has the following properties: (i) a compound with 15 carbon atoms, (ii) an olefinic bond between the 9,10 carbon atoms, and (iii) a cyclopropane ring adjacent to the terminal methyl group. A tentative structure is shown below.

\[
\text{CH}_3\text{-CH-CH-CH-CH(CH(CH_2)_3)_COOH} \quad (1)
\]

**Evidence that F₁ was not a cyclopropenoic acid.** To eliminate the possibility that F₁ was a cyclopropenoic acid, several tests were performed. (i) The Halphen test, a color reaction indicating a cyclopropenoid structure (2), was negative; (ii) the compound was inert to methyl mercaptan treatment (12); (iii) the methyl ester of 9,10-methylenetetradecanoic acid (shown below) was synthesized, and its retention time differed from that of reduced F₁; (iv) NMR spectrum of F₁ showed a triplet at 5.3 ppm. This indicated that an ethylenic bond was present in the molecule (10) and eliminated the possibility that F₁ contained the structure below.

\[
\text{CH}_3\text{-CH-CH-CH-CH(CH(CH_2)_3)}\text{COOH} \quad (2)
\]

**Mass spectral analysis of F₁ and hydrogenated F₁.** Figures 3A and 3B show the mass spectra of F₁ and hydrogenated F₁, respectively. Due to the experimental design, i.e., analytical column in a gas chromatograph connected to a mass spectrometer, the parent mass peaks are not evident. However, in a separate analysis with a higher concentration of F₁, the peak m/e 252 was obtained. Both spectra are characteristic for a monounsaturated fatty acid (6). The m/e values 41, 55, 69, 83, etc., representing CH₃(CH₂)₅-CH=CH(CH₂)₇(COOCH₃) (shown below) were abundant in both cases, and the peak m/e = M-32 (loss of methanol from the ester function) was observed for F₁ and reduced F₁. In the case of F₁, Fig. 3A shows that m/e 220 = M-32 while for reduced F₁ (Fig. 3B) m/e 222 = M-32. This was evidence that 1 mole of H₂ was required to reduce 1 mole of F₁. Although a cyclopropane fatty acid is known to undergo fragmentation similar to monounsaturated fatty acids (6), a careful analysis of the fragmentation pattern can differentiate these two types of compounds. The m/e values 43, 57, and 71, with relative intensities of 100, 89, and 42%, were the most abundant species in the case of reduced F₁ (Fig. 3B). For comparative purposes, a known saturated straight-chain fatty acid, palmitic acid, was subjected to mass spectral analysis under identical conditions as stated.
The identification and by hydrogenation established compounds (standard) a retention shorter. However, this compound was shown, previously the fatty acid Fl; furthermore, in carbon tetrachloride, it is located at a site different from the ethylenic band. The proposed structure for Fl is that previously shown, structure 1. The chemical name of this compound is 12,13-methylene-9-tetradecenoic acid. Whether the compound has the cis or trans configuration has not definitely been established, however, an infrared spectrum of this compound in carbon tetrachloride showed a peak at 940 cm\(^{-1}\) which is indicative of a trans configuration.

In Fig. 1, the number of small peaks with shorter retention times than Fl contain C\(_{14:1}\) (established by hydrogenation and comparison with a standard) and lower homologues of structure 1. The identification and estimation of the latter compounds was made by observing their behavior to hydrogenation and bromination treatments. Also, when the retention time relationship between a saturated C\(_{18}\) fatty acid and saturated Fl correlated well with a saturated C\(_{14}\) fatty acid and one of the smaller peaks in Fig. 1, it was assumed that the compound was an unsaturated cyclopropane C\(_{14}\) fatty acid. By using the linear relationship for identifying fatty acids, the C\(_{13}\) unsaturated cyclopropane fatty acid was identified. Although this method was not entirely satisfactory, for the reasons presented above, we tentatively propose that the smaller peaks in Fig. 1 correspond to the structures mentioned.

The distribution pattern of the unsaturated cyclopropane fatty acids in the anaerobes investigated are presented in Table 2. Identification of fraction F2. Because fraction F2 (Fig. 1), consisting of at least two unsaturated fatty acids, corresponded to palmitic acid upon hydrogenation, we assumed that F2a and F2b were both C\(_{14:1}\) compounds differing in the position of the double bond. This mixture was oxidatively cleaved (19), and the esters of the resulting products were azelaic and 1,9-nonanedicarboxylic (undecanedioic) acids as the dicarboxylic acids and heptanoic and valeric acids as the monocarboxylic acids. Since these products arose from two unsaturated fatty acids, both con-

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**Fig. 3. Mass spectra of native and hydrogenated fraction Fl.**
taining 16 carbons, it was concluded that the F2a and F2b were $\Delta^2$-C18:1 and $\Delta^{11}$-C16:1, respectively. Evidence that F2a was $\Delta^2$-C16:1 came from comparison of this acid to a standard. The failure to detect dicarboxylic acids of shorter carbon lengths eliminated the possibility that F2 contained any polyunsaturated C16 acids. It was reported (16) that C. pasteurianum contained both $\Delta^2$-C16:1, and $\Delta^{11}$-C16:1; however, we did not detect any $\Delta^{11}$-C16:1 under the conditions of our experiment.

**DISCUSSION**

The purpose of this study was to investigate the fatty acid composition of several anaerobic bacteria having different growth temperature optima. The data show that the thermophiles produced relatively large quantities of saturated straight- and branched-chain fatty acids; of these, the predominant species was the i-C18 type. The melting points of saturated straight-chain and iso-fatty acids with chain lengths of C14 to C18 range from 41 to 107 C, whereas unsaturated, cyclopropane and anteiso acids with the same chain lengths have melting points ranging from below 0 to 40 C. Grouping the fatty acids of the organisms into a high melting class (saturated straight-chain and iso acids) and a low melting class (unsaturated, cyclopropane and anteiso acids), the distribution pattern shown in Table 3 is obtained. It is seen that the thermophiles contain a preponderance of higher melting fatty acids. In contrast, C. pasteurianum and Clostridium sp. strain 69 were richer in those acids belonging to the low melting class. Shen et al. (17) reported that the thermophilic Bacillus species were richer in iso-fatty acids than mesophilic species and attributed this finding to the higher melting point property of these acids. The fatty acid composition of C. pasteurianum is similar to another mesophilic anaerobe, Clostridium butyricum (9), containing straight chain, cyclopropane and unsaturated types.

Of considerable interest was the discovery of an unsaturated cyclopropane fatty acid (12,13-methylene-9-tetradecenoic acid) in the anaerobes studied. To our knowledge, this is the first report of the isolation of an unsaturated cyclopropane fatty acid from bacteria. Because of the unsaturated nature of this compound, one can assume that it would have a comparatively low melting point. If this were the case, it is not surprising that the psychrophile produced more of this compound than the thermophiles or the mesophile. Another possibility is that the psychrophile was grown in a different medium than the others and this may have accounted for the difference in the production of the unsaturated cyclopropane acid. Although the data in this report did not clarify this point, it is tempting to speculate that the temperature effect was responsible for the production of more unsaturated cyclopropane acid by the psychrophile. This is based on our observation (unpublished data) that C. tartarivorum, grown in the medium of Mercer and Vaughn at 55 C (14), and C. pasteurianum, grown in a synthetic medium (5) at 30 C, did not change their fatty acid composition when compared with the pattern presented in Table 1. Thus, in these cases, the growth medium did not influence the fatty acid distribution pattern.

Considering the overall fatty acid composition of the anaerobes used in this study, the general pattern indicated that organisms grown at higher temperatures contained a higher percentage of saturated acids with comparatively higher melting points. In the case of C. pasteurianum, the predominance of cyclopropane and monoenoic fatty acids was indicative of its requirement for a lower temperature optimum for growth. The psychrophilic anaerobe differed from the mesophile by being richer in monoenoic and unsaturated cyclopropane fatty acids; the latter
acids were more abundant in the psychrophile. It should be noted that the unsaturated cyclopropane fatty acids are analogous to dienoic acids. Although these compounds are rare or absent in bacteria, we believe that sufficient evidence was presented to demonstrate their presence in several anaerobic bacteria.

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LITERATURE CITED


