Biosynthesis of Branched Chain Fatty Acids

I. ISOLATION AND IDENTIFICATION OF FATTY ACIDS FROM BACILLUS SUBTILIS (ATCC 7059)*

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Saito (1) has clearly identified the branched chain fatty acids, isopentadecanoic and isohexadecanoic, in the lipids of Bacillus subtilis (natto). Another branched chain fatty acid, 12-methyltetradecanoic (sarcinic acid), has also been isolated from the lipids of a species of Sarcina by Akashi and Saito (2). It is important to note that these branched chain fatty acids occur in the bacterial lipids as the major constituent of the fatty acids.

This paper describes the isolation and characterization of the bacterial fatty acids found in a different strain of B. subtilis, B. subtilis (ATCC 7059), during the course of work on the microbial synthesis of branched chain fatty acids being carried out in this laboratory (3). Both iso and anteiso fatty acids have been found in this organism. In addition to the fatty acids mentioned above, an anteiso fatty acid isomeric with sarcinic acid, 14-antepenultimate position, and anteiso (α-) for a methyl side chain in the antepenultimate position.

EXPERIMENTAL PROCEDURE

Microorganism—B. subtilis (ATCC 7059), obtained from the American Type Culture Collection, was used throughout the present work. The cell's ability to produce fatty acids decreased somewhat 1 month after the organism was obtained from the source but remained unchanged since then.

Medium and Culture—The stock culture was maintained on an agar slant in a screw cap test tube, 20 × 150 mm. The medium contained agar, 20 g; dextrose, 10 g; yeast extract, 1 g; Bacto-peptone, 1 g; MgSO4·7H2O, 0.1 g; ZnSO4·7H2O, 1 mg; FeSO4·7H2O, 1 mg; MnCl2·4H2O, 0.45 mg; CuSO4·5H2O, 0.05 mg; K2HPO4, 1.5 g; and NH4H2PO4, 0.5 g, in 1 liter of distilled water.

Large scale preparation of B. subtilis cells was carried out in 10 stainless steel pans, 4 × 30 × 38 cm, containing 6 liters of agar medium of the same composition. The inoculum was incubated for 16 to 20 hours at 37°. Under these conditions, 55 g of wet packed cells or 9 g of dried cells, and 220 mg of acidic extracts were obtained.

Actively growing cells of B. subtilis used for inoculation were incubated for 10 hours on the slant medium at 30°.

Isolation of Bacterial Fatty Acids—At the end of the incubation time, cultures on the agar medium were suspended in 0.85% NaCl solution and filtered through glass wool on a Buchner funnel. The resulting cell suspension, free from any agar debris, was centrifuged; the cells were washed once with NaCl solution, suspended in a solution of 10% KOH in 50% aqueous methanol and digested by refluxing for 4 hours. The alkaline suspension of digested cells was extracted twice with 3 volumes of acid-washed and redistilled hexane to remove basic and neutral compounds. Then the aqueous layer was acidified to pH 2.0 with dilute H2SO4 and extracted four times with 3 volumes of purified hexane. The hexane extracts were combined, washed with a small volume of 50% aqueous methanol, and dried over anhydrous K2SO4. Acidic compounds in the hexane extract were obtained by evaporation of the hexane under a stream of purified nitrogen at room temperature.

Chromatography of Fatty Acids—The bacterial fatty acids isolated from the digested cells were fractionated by gas-liquid chromatography2 of the methyl esters. The gas-liquid chromatographic fractionations were made in an apparatus constructed in this laboratory, consisting of a column, 180 cm in length and 3.5 mm in internal diameter, packed with Craig succinate polyester packing3 (20%) and operated at 180-185° with helium flow rates of 70 to 80 ml per minute for analytical work and 110 to 120 ml per minute for preparative work. A similar column packed with Apiezon L grease (10%) coated on Anakrom ABS4 was used at 170-175° with helium flow rates of 35 to 40 ml per minute for analytical work and 100 to 120 ml per minute for preparative work. Detection was carried out by a Gow-Mac thermal conductivity cell5 operated at 240°.

Before it was injected into the partition column, each fatty acid sample was methylated with freshly prepared diazomethane (4) in ether. A sample of methyl ester up to 0.5 µl could be fractionated on these columns without showing any sign of overload. For preparative purposes, however, 20 to 30 µl of a sample of methyl esters of fatty acids were fractionated. Individual fatty acid methyl esters6 fractionated by chromatographic fractionations were obtained on these columns without showing any sign of overload. For preparative purposes, however, 20 to 30 µl of a sample of methyl esters of fatty acids were fractionated. Individual fatty acid methyl esters fractionated by chromatographic fractionations were obtained on these columns without showing any sign of overload.

The materials bled out of the succinate column were examined to determine whether there was any possibility of contamination of fatty acids prepared from methyl esters fractionated on this column. The column operated at 180° with a flow rate of 120 ml per minute bled 1 mg of the liquid phase per hour. Acidic extracts obtained from the saponified bled materials, approximately 0.25
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were recrystallized from petroleum ether or acetone. The fatty acids thus obtained were purified by recrystallization from acetone (12). Fatty esters were prepared from corresponding free acids or from methyl esters by reduction in a saturated ether solution of LiAlH₄ (5).

Fatty alcohols were prepared from corresponding free acids or from methyl esters by reduction in a saturated ether solution of LiAlH₄ (5).

TABLE I

<table>
<thead>
<tr>
<th>Product</th>
<th>Starting materials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Semiester</td>
</tr>
<tr>
<td>n-C₁₅</td>
<td>C₁₀⁺</td>
</tr>
<tr>
<td>n-C₁₇</td>
<td>C₁₀</td>
</tr>
<tr>
<td>i-C₁₄</td>
<td>C₁₀</td>
</tr>
<tr>
<td>i-C₁₅</td>
<td>C₁₀</td>
</tr>
<tr>
<td>i-C₁₇</td>
<td>C₁₀⁺</td>
</tr>
<tr>
<td>a-C₁₆</td>
<td>C₁₀⁺</td>
</tr>
<tr>
<td>a-C₁₇</td>
<td>C₁₀⁺</td>
</tr>
</tbody>
</table>

* Prepared from diethyl sebacate and sebacic acid (11).
* Prepared from the corresponding alcohols by oxidation with KMN₄ in acetone or with aqueous chromic acid. The alcohols were supplied by K and K Laboratories, Inc., Jamaica, New York.
* Prepared from methyl erucate by permanganate oxidation in acetone (12).

**Attempted Determination of Unsaturation of Fatty Acids**—The detection of unsaturated fatty acids in the bacterial fatty acid methyl esters was attempted by making use of the adduct formed between mercuric acetate and unsaturated fatty acid esters (6). The procedure described by Goldfine and Bloch (7) was followed with some modification. Saturated and unsaturated fatty acid esters (6). The procedure described by Goldfine and Bloch (7) was followed with some modification. Saturated and unsaturated fatty acid esters were also distinguished by a procedure (8) based on bromination with bromine in ethyl ether. Iodine values were measured by a procedure described by Gattemann (9).

Infrared Absorption Spectra—Either carbon tetrachloride or carbon disulfide was used as solvent for absorption spectra in 1% solution. For spectra of solids, pellets, 1 mm thick, and 3 mm in diameter, containing 0.2% of the sample in KBr, were prepared by pressing the mixture in small lead rings fitted into standard dies. The total sample required for this method was 0.1 mg.

Optical Activity—Optical activity of fatty acid samples was measured in acetone by a Rudolph No. 80 polarimeter.

X-ray Diffraction Analysis—Each fatty acid sample was dissolved in chloroform, and the solution was spread evenly on a glass slide and evaporated at room temperature. The slide was scanned at 0.25° per minute by use of copper Kα (λ = 1.5418 Å) with a Norelco diffractometer. The longest interplanar spacing (2) was obtained from the average of values corresponding to the first, second, and third order diffractions of the long spacing of the fatty acid crystal. Approximately 4 mg of the free fatty acid were used for each analysis.

Neutralization Value—The neutralization value of a fatty acid sample was obtained by titration with 0.008 N methanolic KOH; phenolphthalein was used as indicator.

Standard Fatty Acids—Laurie, myristic, and palmitic acids were commercial products, recrystallized twice from acetone. Their methyl esters, also obtained commercially, were purified by fractional distillation under vacuum. Chromatography of all fatty acid esters on succinate polyester resin gave single symmetrical peaks as indication of purity. Long chain fatty acids other than those mentioned above were synthesized by Kolbe electrolysis (9). Electrolysis of the starting materials, i.e. the semesters of a dicarboxylic acid and a free monocarboxylic acid in the ratio of 1 to 2 moles, was carried out in an alkaline methanol solution (10).

The systems used are listed in Table I. The acids synthesized were recrystallized from pentane. Chromatography of their methyl esters on a succinate column gave single symmetrical peaks.

RESULTS

Isolation and Identification of Fatty Acids by Gas-Liquid Chromatography—Gas chromatography on a succinate column, at 185°C, of the fatty acid methyl esters isolated from B. subtilis gave six distinguishable peaks as shown in Fig. 1. These are numbered in order of increasing retention times as Fractions 1, 2, 3, 5, 6, and 7, the number 4 being reserved for a component to be discussed in a subsequent paper. Fractions 1, 2, 5, and 6 were symmetrical peaks, as usually is found for pure components. Peaks of Fractions 3 and 7 showed initial shoulders, indicating the presence of a second component in each case, numbered as 3-1 and 3-2; Components 3-2 and 7-2 were the major ones in each case.

For preparative use, some separation of the components of the mixed Peaks 3 and 7 was achieved by collecting separately the initial and major portions of each mixed fraction as obtained by chromatographing 20 µl of mixed acids. Each portion was rechromatographed, and the mixed peak was collected in two portions as before; by a process of successive fractionations in volving at least one more chromatographic step, nearly complete separation of component 3-2 from 3-1 and of 7-2 from 7-1 was achieved, although amounts of the purified components pre-

7 The author is indebted to Dr. L. B. Halferdahl of this institution for this analysis.
8 Adapted to the terminology used in the x-ray diffraction powder data file of the American Society for Testing Materials. They used the word "largest," instead of "longest," which is used in this paper.
9 The author is indebted to Dr. R. M. Elofson of this institution for carrying out these electrolyses.
pared by such a procedure were limited to less than 1 mg. Separation of Components 3-1 and 7-1, the minor components of the mixed Fractions 3 and 7, from Components 3-2 and 7-2, respectively, however, was more difficult. At least five successive chromatographic fractionations were required, and amounts of the purified components were limited to less than 0.1 mg.

It was found, however, that when 10 mM α-methylbutyrate was added to the culture medium, the production of Components 3-2 and 7-2 was increased greatly, whereas Components 3-1 and 7-1 practically disappeared, thus enabling 3-2 and 7-2 to be isolated in a single chromatographic fractionation (Fig. 2). The mechanism of this process will be discussed in a subsequent paper. The purity of the fatty acid methyl esters isolated was confirmed by rechromatography. All gave single peaks with symmetrical elution curves at the position expected for each acid on the chromatogram.

In order to detect unsaturated fatty acids in the bacterial mixed fatty acids, four methods were used: formation of a mercuric acetate adduct with unsaturated fatty acids; bromination in ethyl ether of unsaturated fatty acids to bromo acids; comparison of the gas-liquid chromatographic retention volumes of the fatty acid methyl esters on two different columns, polar and nonpolar (13-15); measurement of iodine value. No mercuric acetate adduct or bromo acids were obtained from the methyl esters of the bacterial mixed fatty acids, indicating that no unsaturated fatty acid methyl esters were present. Failure to observe any differences in the chromatograms of the bacterial fatty acid methyl esters with respect to relative position and amounts of each fraction on succinate and Apiezon L columns further supported the above finding. In addition, no iodine uptake by methyl esters of the bacterial mixed fatty acids was detected.
The apparent chain length of fatty acids isolated from *B. subtilis*

The logarithm of the retention volume of the standard straight chain fatty acid methyl esters was plotted against the number of carbon atoms in the chain, giving a straight line. Since the retention volume of the bacterial fatty acid methyl esters had been measured, the apparent carbon atom number was obtained from this plot.

### Table II

**Apparent chain length of fatty acids isolated from *B. subtilis***

<table>
<thead>
<tr>
<th>Chain length of peak fraction</th>
<th>1</th>
<th>2</th>
<th>3-1</th>
<th>3-2</th>
<th>4*</th>
<th>5</th>
<th>6</th>
<th>7-1</th>
<th>7-2</th>
<th>8*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td>i-C16</td>
<td>n-C16</td>
<td>i-C15</td>
<td>a-C15</td>
<td>n-C15</td>
<td>i-C16</td>
<td>n-C16</td>
<td>i-C17</td>
<td>a-C17</td>
<td>n-C17</td>
</tr>
<tr>
<td>As methyl ester on succinate</td>
<td>15.6</td>
<td>14.0</td>
<td>14.6</td>
<td>14.8</td>
<td>15.0</td>
<td>15.6</td>
<td>18.0</td>
<td>16.6</td>
<td>16.8</td>
<td>17.0</td>
</tr>
<tr>
<td>As methyl ester on Apiezon L.</td>
<td>15.6</td>
<td>14.0</td>
<td>14.7</td>
<td>14.8</td>
<td>15.0</td>
<td>16.0</td>
<td>16.7</td>
<td>16.7</td>
<td>16.7</td>
<td>16.7</td>
</tr>
</tbody>
</table>

**Occurrence as percentage of total fatty acids...**

| Ratio* | 3 | 1 | 51 | 10 | 10 | 25 |

*These fractions occurred in the bacterial fatty acids only when propionate or valerate was added to the culture medium. On a succinate column, 180 cm in length, methyl esters of a-C15 and n-C15 were resolved partially, whereas methyl esters of a-C17 and n-C17 were resolved completely.

*An approximation is made to obtain the ratio of two fractions by a geometrical adjustment of the elution curve.*

These results are consistent with the conclusion that no unsaturated fatty acids are present in the bacterial mixed fatty acids.

The absence of unsaturated fatty acids in total fatty acids isolated from *B. subtilis* (ATCC 6653) grown on a medium containing glucose, yeast extract, minerals, and a trace amount of isovalerate has also been found (16).

From retention volumes (Figs. 1 and 3), Fractions 1, 2, 3-1, 3-2, 5, 6, 7-1, and 7-2 may be identified as i-C14, n-C14, i-C15, a-C15, i-C16, n-C16, i-C17, and a-C17, respectively. Cochromatography of the bacterial fatty acid methyl esters with methyl esters of the standard acids on succinate and on Apiezon L gave single symmetrical peaks, which indicated that they were identical. Cochromatography on Apiezon L of the fatty esters obtained by LiAlH4 reduction of the methyl esters of both bacterial fatty acids and standard fatty acids likewise gave single symmetrical peaks.

The apparent carbon atom numbers and the relative occurrence of each fraction in the bacterial mixed fatty acids are listed in Table II. In decreasing order of abundance, the fatty acids are 12-methyltetradecanoic, 14-methylhexadecanoic, isopentadecanoic, isovaleric, palmitic, isopentadecanoic, isomyristic, and myristic. The two anteiso fatty acids together make up approximately 60% of the total fatty acids occurring in *B. subtilis*.

**Infrared Spectra**—The spectra of the bacterial fatty acids and of standard acids are shown in Fig. 4. The terminal carbon atoms show characteristic absorption in the range between 1380 and 1360 cm⁻¹. Normal long chain fatty acids show a weak absorption band at 1380 cm⁻¹, iso fatty acids show a doublet in the range from 1380 to 1390 cm⁻¹, and anteiso fatty acids show a stronger absorption band in the region of 1380 cm⁻¹ than that given by normal fatty acids. In this region, the absorptions of a-C16 and n-C16 are 1400 cm⁻¹, 1380 cm⁻¹, and 1360 cm⁻¹. The absorbance of isovalerate, with a-C16 and n-C16, is 1380 cm⁻¹, 1400 cm⁻¹, and 1360 cm⁻¹. The absorption of 1380 cm⁻¹ is shown not only by iso acids but also by neo acids (17). However, the possible presence of the neo acids in Fractions 3-1 and 7-1 is excluded by crystallographic data; the longest interplanar spacing of the neo acids would be expected to be shorter than that of iso acids.

In the region of 1300 to 1100 cm⁻¹, a regular band progression is observed in the spectra of the solid acids and probably is characteristic of the orientation of the methylene groups (18). The number of progression bands is directly related to the chain length of the straight chain fatty acids (19, 20). In this region the number and the relative intensities of the progression bands of the bacterial fatty acids are identical with those of the standards.

A regular decline of progression band intensities toward lower frequencies has been observed in the spectra of straight chain fatty acids (19, 21). The same direction of decline is shown in the spectra of isopentadecanoic and isoleadecanoic acids. On the contrary, isomyristic, isopalmitic, and 14-methylhexadecanoic acids show an increase in progression band intensities toward lower frequencies. Further study on the relationship between progression band intensities and structure of fatty acids is being carried out in this laboratory.

An infrared absorption band at 1020 cm⁻¹ is characteristic of cyclopropane compounds (22-24). A cyclopropane derivative of fatty acid, lactobacillic acid, was discovered by Hofmann and Lucas in a *Lactobacillus arabinosus* (23) and also in several lactic acid bacteria (25). However, no absorption band in this region was observed in the spectra of the bacterial fatty acids (Fig. 4). This result is indicative of the absence of cyclopropane compounds in the bacterial fatty acids isolated from *B. subtilis*.

**Longest Interplanar Spacings**—The largest interplanar spacings of the bacterial fatty acids, and of the corresponding amides in the case of Fractions 3-2 and 7-2, are listed in Table III. These values are in agreement with those of the standards as well as with the values reported.

However, for the free acid from Fraction 7-2, the observed long spacing of 31.4 A is approximately 2 A less than the value of 33.4 A reported by Velick (26). This discrepancy may be due in part to the difficulties, which are referred to by Velick, in...
FIG. 4. Infrared spectra of fatty acids. In each spectrum, a indicates the bacterial sample and b is the standard sample. Solid state spectra of fatty acids were obtained with KBr pellets, except for 12-methyltetradecanoic acid, for which a liquid film on a NaCl plate was used.
The longest interplanar spacing of the fatty acid was obtained from the average values of the first, second, and third order reflections of the long spacing of the fatty acid crystal. Approximately 4 mg of fatty acid were used for each analysis. The quantity of Fraction 2 was too small to permit measurement.

\[\begin{array}{cccccc}
\text{Fatty acid} & \text{A} & \text{A} & \text{A} & \text{A} & \text{A} \\
\text{Bacterial sample} & i-C_{14} & i-C_{15} & a-C_{15} & i-C_{14} & n-C_{14} \\
\text{Standard sample} & 26.9 & 29.5 & 29.3^a & 29.3^b & 30.7 \\
\text{Reported} & 26.8^d & 29.8^c & 29.2^a & 30.7 & 35.6 \\
\end{array} \]

These fractions contained approximately 10% of Fraction 3-2 and 7-2, respectively, but the strong diffraction lines, corresponding to the major components, were easily distinguished from those weak lines given by the contaminant and were chosen for the measurement of the spacings.

a Lack of an optically active sample did not permit measurements to be made.
b By Velick (26).
c By Francis, Piper, and Malkin (28).
d By Weitkamp (30).

The melting point of the free acid from Fraction 7-2 was in good agreement with that reported for (+)-14-methylhexadecanoic acid. The 14-methylhexadecanoic acid chemically synthesized in this laboratory was optically inactive and has a higher melting point (38-39°) than the optically active acid (35.5-36°), but the mixed sample of the chemical and the bacterial fatty acid melted at 37-37.5°, a value falling between the individual melting points, as usually observed in a compound that has optical isomerism.

Neutralization Values—Neutralization values of the free acid from Fractions 3, 5, and 6 are in good agreement with those of the standard fatty acids (Table IV). No depression in melting point was observed in mixed samples of bacterial and standard fatty acids, which indicates that they are identical.

Optical Activity—The free acids from Fractions 3-2 and 7-2, in 2% acetone solution, were dextrorotatory, but the amount of material available did not permit an accurate measurement.

Naturally occurring 14-methylhexadecanoic acid isolated from wool fat (30) gives a specific rotation of +5.0° in acetone. Velick and English (32) have synthesized optically active 14-methylhexadecanoic acid with the use of D-2-methylbutanol prepared from fractional distillation of fuel oil. The specific rotation of their acid was +5.23°, which indicated that the absolute configuration of the natural fatty acid is the same as those of D-2-methylbutanol and L-2-isoleucine.

DISCUSSION

Saito (1) has found i-C_{15} and i-C_{17} to be the major constituents of fatty acids in the lipids of B. subtilis (natto); in the present work, however, a-C_{15} and a-C_{17} were found to be the major components of the total fatty acids in B. subtilis (ATCC 7059). Three possible explanations of this difference may be con-
considered (a) The natto strain produces lipids in which \( \text{i-C}_{17} \) and \( \text{i-C}_{19} \) are the major components, but in the total acid fraction \( \alpha-	ext{C}_{13} \) and \( \alpha-	ext{C}_{17} \) are the most abundant. (b) The culture medium used by Saito was rich in isoleucine, isovaleric acid, or a compound that is biologically equivalent to these compounds, since the presence of these substrates in the culture medium increased greatly the production of \( \text{i-C}_{17} \) and \( \text{i-C}_{19} \) (33). (c) The natto strain is quite different in metabolic activity from the ATCC 7059 strain; the former contains enzyme systems favorable to \( \text{i-C}_{17} \) and \( \text{i-C}_{19} \) production, whereas the latter contains enzymes producing \( \alpha-	ext{C}_{13} \) and \( \alpha-	ext{C}_{17} \) under identical culture conditions.

Anteiso fatty acids have been reported in other organisms. In the case of \( \textit{Micrococcus lysodeikticus} \), Macfarlane (34) has found that anteiso fatty acids, \( \alpha-	ext{C}_{13} \) and \( \alpha-	ext{C}_{17} \), are the most abundant in the bacterial lipids, and in the total fatty acid fraction Lennarz (33) has also found the anteiso fatty acids to be the major components.

Preliminary experiments\(^{27}\) in which three different strains of \( \textit{B. subtilis} \) supplied by the Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan, were used, showed that in all three strains anteiso fatty acids, \( \alpha-	ext{C}_{13} \) and \( \alpha-	ext{C}_{17} \), occur in most abundance and that all other fatty acids found in \( \textit{B. subtilis} \) (ATCC 7059) are also present, although the relative proportions of each fatty acid are somewhat different among the strains. Recently Allison et al. (35) have reported that an anteiso fatty acid, \( \alpha-	ext{C}_{19} \), is a major fatty acid in \( \textit{B. subtilis} \). \( \text{ATCC 6653} \).

These findings suggest that the natto strain would be expected to have a system for fatty acid synthesis similar to those of the other five strains and, hence, that Saito’s observation of iso fatty acids is not uncommon, however, and it would be desirable to investigate the differences in metabolic activity between the two strains of \( \textit{B. subtilis} \), natto and ATCC 7059, under identical conditions.

SUMMARY

Six branched chain fatty acids and two straight chain fatty acids have been isolated from \( \textit{Bacillus subtilis} \) (ATCC 7059) grown on a glucose-yeast extract-Bacto-peptone medium. The identification of the fatty acids was carried out by gas-liquid chromatography and by their infrared spectra, x-ray diffraction, melting points, and neutralization values. The fatty acids identified are 12-methyltetradecanoic, 14-methylhexadecanoic, isopentadecanoic, isopalmmitic, palmitic, isoleptadecanolic, isomyristic, and myristic, in order of abundance.

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\(^{27}\) T. Kaneda, unpublished experiments.

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