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Effect of Temperature on the Fatty Acid Composition of *Thermus aquaticus*

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Thermus aquaticus contains four major fatty acids, iso-C_{15} (28%), iso-C_{16} (9%), normal-C_{16} (13%), and iso-C_{17} (48%), when grown at 70 C, as determined by gas chromatography and mass spectrometry. Small amounts of iso-C_{12}, normal-$C_{12:1}$, iso-C_{13}, normal-C_{14}, iso-C_{14}, and normal-$C_{15:1}$ were also detected. A change in growth temperature (50 to 75 C at 5-C intervals) affects a shift in the proportions of some of the fatty acids. The proportions of the monoenoic and branched-C_{17} fatty acids decreased and the proportions of the higher-melting iso-C_{16} and normal-C_{16} fatty acids increased. Cells grown at 75 C contained 70% more total fatty acids than cells grown at 50 C. The largest increases, in absolute amounts, were in the content of iso-C_{16} and normal-C_{16} fatty acids, with only a 1.6-fold increase in the major iso-C_{15} and iso-C_{17} fatty acids. There was a 2.5-fold decrease in normal-$C_{15:1}$ and at least a 24-fold decrease in anteiso-C_{17}, which is present at 50 and 55 C but not at higher temperatures. There was no difference in proportion or amount of fatty acids between exponential and stationary-phase cells grown at 70 C. When cells were grown on glutamate instead of yeast-extract and tryptone at 70 C, the total fatty acid content remained constant, but there was an increase in the proportions of iso-C_{16} and normal-C_{16} fatty acids concomitant with a decrease in the proportions of the iso-C_{15} and iso-C_{17} fatty acids.

The molecular mechanism of thermophily has been attributed to (i) the stability of the lipids in the membrane, (ii) a high rate of breakdown and synthesis of the macromolecules, and (iii) a physicochemical difference between the macromolecules of thermophiles and mesophiles (17). Brock (3) hypothesized that the integrity of the cell membrane may be the limiting factor in thermal death, thus suggesting that the membrane components of mesophiles and thermophiles should differ. The fatty acid composition of some mesophiles has a progressively higher melting point as the temperature of growth increases (14). In these organisms, the progressively higher melting point corresponds to decreasing proportions of monounsaturated fatty acids in the lipids.

Recent work has shown that thermophiles contain larger proportions of branched-chain saturated fatty acids than mesophiles. *Bacillus stearothermophilus* presumably grown at high temperature contains 64% branched-chain fatty acids (7). Other thermophilic sporeforming bacilli contain higher proportions of branched-chain and straight-chain saturated fatty acids and lower proportions of unsaturated fatty acids than mesophilic strains (8). A comparison of the fatty acid differences between thermophilic and mesophilic strains of sporeforming bacilli indicated that higher temperatures of growth correlate with higher proportions of iso-branched-chain isomers (17).

Thermus aquaticus, a gram-negative extreme thermophile, reportedly contains very little unsaturated fatty acids and contains a predominance of branched-chain fatty acids (10). As part of a study on the effect of temperature on the lipid components of the membrane of *T. aquaticus*, the major fatty acids have been identified, and differences in fatty acid composition when cells were grown at temperatures between 50 and 75 C have been examined.

MATERIALS AND METHODS

Materials. Authentic fatty acid methyl esters were supplied by Applied Science Laboratories, Inc., State College, Pa. All chemicals were of the best grade commercially available and were essentially as described previously (20).

Growth of T. aquaticus. *T. aquaticus* (ATCC 25104) was grown in a basal salts medium (5) containing 0.2% yeast extract (Difco) and 0.2% Tryptone (BBL) or, in one experiment, in the basal salts medium containing 20 mM glutamic acid. The *p*H was adjusted to 7.6 with 12 N KOH. Cultures were grown in 1-liter flasks containing 400 ml of medium in a Constant Temperature Water Bath Shaker (Fermentation Design, Allentown,

Pa.) at the temperatures indicated (±1 C) and shaken at 250 rev/min. Growth was measured turbidimetrically at 700 nm by using a Beckman DBG spectrophotometer. Dry weights were determined as previously described (20).

Fatty acid isolation and methylation. Fatty acids were isolated after saponification in 3 N KOH containing 50% ethyl alcohol for 12 hr and methylated as described (19), or the total lipids were extracted by the method of Bligh and Dyer (2), and the fatty acid methyl esters formed were extracted by mild alkaline methanolysis and determined directly.

Fatty acid determination. Fatty acids were determined colorimetrically by the method of Rapport and Alonzo (16) as described (9) with tripalmitin used as the standard. Fatty acids are designated as follows. The number indicates the number of carbon atoms in the chain; the prefix a- (e.g., a-15) indicates the anteiso isomer; the prefix i- (e.g., i-15) indicates the iso-branched isomer; the prefix n- (e.g., n-16) indicates an unbranched fatty acid, and the suffix :1 (e.g., 15:1) indicates a monounsaturated fatty acid.

Gas chromatography. A dual-column gas chromatograph (model 402, F & M Scientific, Division of Hewlett-Packard Co., Avondale, Pa.) was used in this study. The nonpolar column (0.25-inch by 6 ft) contained 3% SE-30 (General Electric silicone oil) on 80/100 mesh Diatoport S. The polar column (0.25 inch by 11 ft) contained 15% ethylene glycol succinate on 60/80 mesh gas chrome P prepared by Applied Science Laboratories Inc. The analysis with the polar column was run isothermally at 170 C, and the analysis with the nonpolar column was run isothermally at 156 C under the conditions described previously (19). The percentage composition was calculated from the areas of each component in the chromatogram (19).

Mass spectra. Mass spectra were determined with an LKB-9000 combined gas chromatograph-mass spectrometer as described (21) by R. K. Hammond and C. C. Sweeley (Department of Biochemistry, Michigan State University).

Phosphate analysis. Lipids were digested with 23% perchloric acid for 1 hr at 200 C and were analyzed for phosphate by the method of Bartlett (1) as adapted for the Technicon Auto-analyzer by R. L. Lester. Carbohydrate was measured colorimetrically by the method of Radin et al. (15) as described (9), with glucose used as the standard.

RESULTS

Identification of the fatty acids. The fatty acids of *T. aquaticus* were tentatively identified by comparison of the retention times on gas chromatography of their methyl esters on both polar and nonpolar columns to those of authentic standards. Figure 1 illustrates such a comparison of fatty acid methyl esters from cells grown at 70 C on the polar ethylene glycol succinate column. The total fatty acid preparation was hydrogenated exhaustively in methanol to saturate the enoic methyl esters (11); the results indicated that exponentially growing *T. aquaticus* contains less than 1% unsaturated fatty acids. Hydrogenation

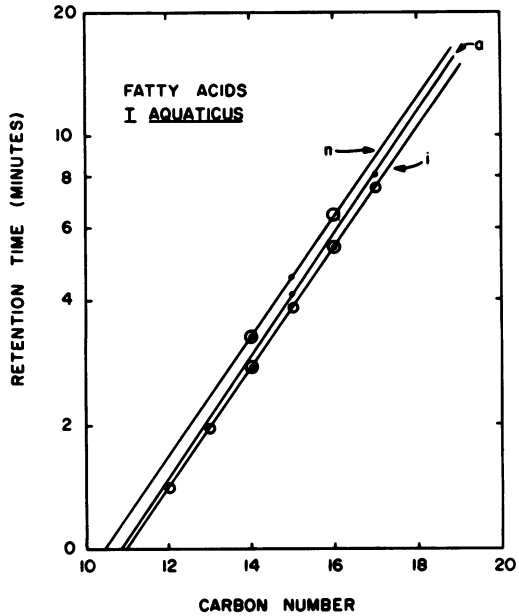

FIG. 1. *James plot of the retention times during gas-chromatographic analysis of fatty acid methyl esters from exponentially growing Thermus aquaticus incubated at 70 C. Esters were separated on a glass column (0.25 inch by 11 ft) containing 15% ethylene glycol succinate on 60/80 mesh gas chrome P analyzed isothermally at 170 C as described (19). Symbols: ●, authentic standards; ○, esters from T. aquaticus.*

in acetic acid indicated that no detectable cyclopropane fatty acids were present in these cells. Finally, by comparing the retention times on both the polar and nonpolar columns, there was no evidence for hydroxy fatty acid methyl esters. The tentative identification of the esters as i-12, 12:1, i-13, i-14, n-14, i-15, 15:1, i-16, n-16, and i-17 was compatible with the retention times after chromatography on the nonpolar and polar columns.

In *T. aquaticus* grown at 70 C, i-15, i-16, n-16, and i-17 account for 95% of the total fatty acids. The tentative identification of the major fatty acids was confirmed by mass spectrometry. The mass spectra of the two major fatty acid methyl esters are shown in Fig. 2. The parent ions detected at *m/e* 256 (lower) and 284 (upper) correspond to the pentadecanoic and heptadecanoic methyl esters. Ions at M-65 representing the combined loss of a methylene (M-14), hydrogen (M-1), methanol (M-32), and water (M-18) groups were detected at *m/e* 191 and 219 in the esters with retention times corresponding to i-15 and i-17, respectively. The M-65 fragmentation ion is characteristic of all iso-branched saturated fatty acid methyl esters examined (19). The other

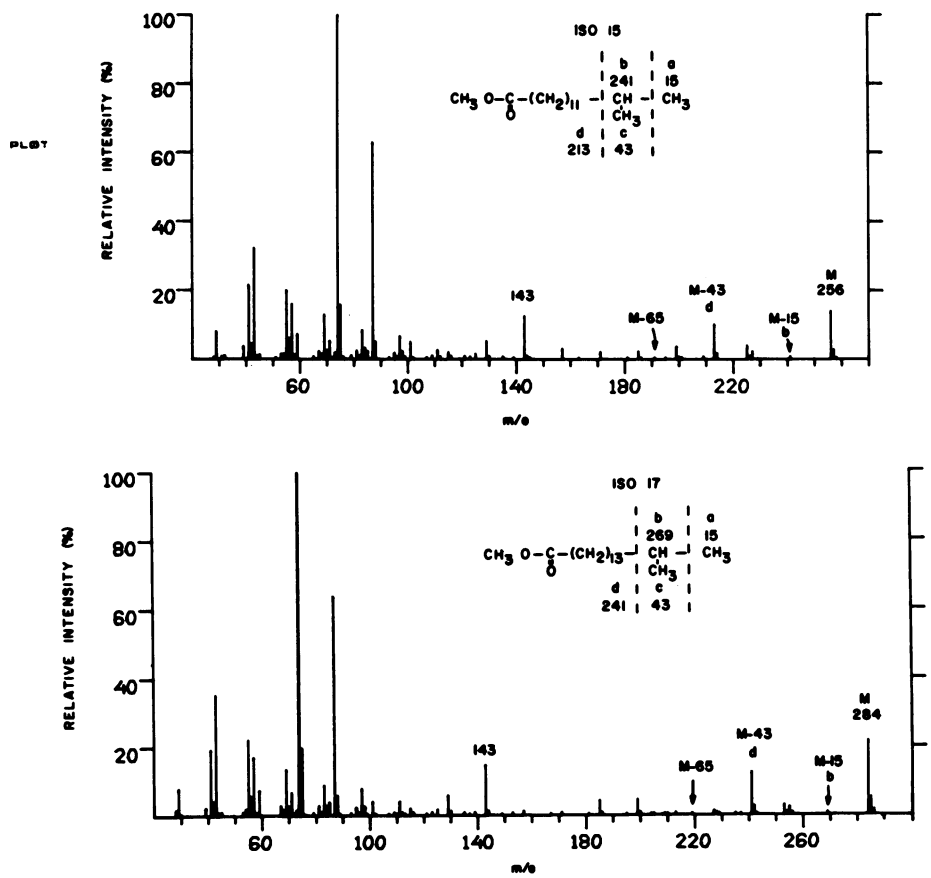

FIG. 2. *Mass spectrum of methyl 13-methyltetradecanoate (upper) and of methyl 15-methyl hexadecanoate (lower).*

fragmentation ions at M-15 at *m/e* 269 and 241 and at M-43 at *m/e* 241 and 213 correspond to the loss of the terminal methyl or isopropyl group from the iso-methyl esters (Fig. 2). The mass spectra for the i-16 and n-16 fatty acids were identical to authentic standards.

Effect of temperature. The proportions of some of the fatty acids in exponentially growing *T. aquaticus* change as the growth temperature is increased (Table 1). The proportions of i-12, i-13, i-14, n-14, and i-15 remain relatively constant in cells at growth temperatures between 50 and 75 C. There was a decrease in the proportions of 12:1, 15:1, i-17, and a-17 and an increase in the proportions of i-16 and n-16 as the temperature of growth was increased. The major shifts to note are the decrease in the content of branched-C_{17} fatty acids, especially the anteiso isomer and the increase in the amount of i-16 and n-16 (5- and 10-fold, respectively). The total fatty acid content per gram (dry weight) was 109 μmoles at 50 C, 135 μmoles at 60 C, 155 μmoles at 65 C, and 187 μmoles at 75 C. This represents an increase of 71% as the temperature was raised from 50 to 75

TABLE 1. *Effect of growth temperature on the proportions of fatty acids of exponentially growing Thermus aquaticus*[a]

Fatty acid methyl esters	Growth temp (C)					
	50	55	60	65	70	75
i-12	0.5	0.5	0.3	0.4	0.4	0.4
12:1	1.5	1.4	1.0	0.5	0.5	0.6
i-13	0.5	0.5	0.4	0.7	0.5	0.5
n-14	0.4	0.3	0.5	0.5	0.7	1.4
i-14	0.6	0.4	0.4	0.4	0.4	0.4
i-15	33.0	31.4	27.6	29.5	28.2	30.5
15:1	2.1	2.0	1.6	1.4	1.0	0.5
i-16	3.1	3.8	6.5	7.3	8.8	14.0
n-16	1.6	2.0	7.8	10.6	12.7	16.7
i-17	37.8	45.0	55.8	52.4	48.1	35.8
a-17	21.6	14.8	0.0	0.0	0.0	0.0

[a] Fatty acids were extracted, methylated, and analyzed by gas chromatography as in Fig. 1. The numbers are expressed as the percentage of the total at that temperature. The fatty acids are abbreviated as the number of carbon atoms prefaced with i for iso-branching, a for anteiso-branching, n for unbranched, or followed by :1 for monoenoic fatty acids.

C.

In absolute terms, the total amount of n-14 increased 6-fold, the i-15 increased 1.6-fold, i-16 increased 7.8-fold, and n-16 increased 18.5-fold. The a-17 decreased at least 24-fold and the 15:1 decreased 2.5-fold between 50 and 75 C. The other fatty acids remained at about the same concentrations in the lipids.

The total amount and identity of fatty acids in exponentially growing *T. aquaticus* incubated at 70 C were the same in cells incubated in the basal salts medium plus Tryptone and yeast extract as from cells grown in the basal salts medium plus 20 mM glutamate. However, the fatty acid distribution from cells grown in glutamate was altered: i-15, i-16, n-16, and i-17 accounted for 12, 23, 20, and 40% of the fatty acids, respectively, indicating an increase in hexadecanoic fatty acids and decreases in pentadecanoic and heptadecanoic fatty acids.

Extraction of the lipids. Preliminary analyses of the membranes of *T. aquaticus* have indicated that the complex lipids comprise phospholipids and glucose-containing glycolipids. The Bligh and Dyer extraction procedure (2) yielded the maximum amounts of total fatty acid, total lipid phosphate, and total lipid glucose of the modifications tried (Table 2). Modifications of the Bligh and Dyer extraction procedure are necessary with some bacteria for maximum yield (20), but with *T. aquaticus* the fatty acids extracted by the Bligh and Dyer procedure represented 92% of the total fatty acids present in the cells. The total fatty acids present in the cells were determined after direct saponification of the cells (19).

Effect of growth cycle. Since it has been shown that the content and composition of lipids vary in some bacteria according to their stage of growth (12), the lipids of *T. aquaticus* were extracted at various stages of the growth cycle. The proportions of i-15, i-16, n-16, and i-17, the total lipid glucose, the total lipid phosphate, and the total lipid dry weight do not change as the cells go from exponential to stationary growth phase at the optimum growth temperature of 70 C (Fig. 3).

TABLE 2. *Extraction of the lipids of Thermus aquaticus*[a]

Method	Micromoles of P/g[b]	Micromoles of glucose/g[c]	Micromoles of fatty acid/g[d]
A	22.3	3.2	100
B	21.7	3.0	111
C	25.3	4.1	100
D	25.3	3.1	109
E	28	4.4	112
F	22.7	4.4	100
G	28	4.4	112

[a] A culture of *T. aquaticus* grown at 50 C was divided into seven equal portions containing 85 mg (dry weight) and extracted as follows: (A) culture medium was brought to *p*H 2.0 with concentrated HCl and centrifuged; the pellet was suspended in 25 ml of isopropanol and boiled for 10 min. After this, the cells were transferred to a separatory funnel, and 12.5 ml of CH_3OH, 15 ml of 0.05 M phosphate buffer, and 18.75 ml of $CHCl_3$ were added and allowed to extract overnight. Then 18.75 ml of buffer and 18.75 ml of $CHCl_3$ were added, and the extraction system was allowed to separate. (B) Same as A except that the pellet was sonic treated for 45 sec in isopropanol. (C) The isopropanol step was eliminated and 37.5 ml of CH_3OH was added at the first CH_3OH addition instead of 12.0 ml. (D) Same as C except that the pellet was sonic treated in phosphate buffer. (E) Bligh and Dyer extraction as reported (13). (F) Boiled in isopropanol then followed by Bligh and Dyer extraction. (G) Sonic treated in buffer followed by Bligh and Dyer extraction.

[b] Lipid phosphate was determined colorimetrically by the method of Bartlett (1).

[c] Glucose was determined colorimetrically by the method of Radin et al. (9)

[d] Fatty acids were determined as the ferric hydroxymate (9)

DISCUSSION

From the response of mesophilic organisms to increasing growth temperatures, one might expect that the fatty acids of *T. aquaticus* would be completely saturated since the insertion of one double bond greatly decreases the melting temperature of a (free) fatty acid [e.g., n-16 melts at 63.1 C whereas 16:1 melts at 0.5 C (18)]. Also, one might expect that the chain length of the fatty acids would be increased over that found in mesophilic bacteria, since another important factor affecting the melting point is the chain length. Heinen et al. (10) reported that *T. aquaticus* contained n-15 as one of its major fatty acids. In our study of this organism, n-15 was a trace component and i-15 was a major fatty acid. The fatty acids of *T. aquaticus* were shown to possess very few double bonds (Table 1), as predicted, but the chain lengths of the fatty acids are surprisingly short. The longest chain length of the fatty acids in *T. aquaticus* was 16 carbon atoms (i-17), which means that its chain length is that of palmitic acid, the predominant fatty acid found in mesophiles. The thermophiles examined (7, 8, 17) have shown a predominance of branched-chain fatty acids and particularly that of the iso isomer. In *T. aquaticus*, raising the growth temperature produced a shift in the fatty acid composition; there was an increase in the proportion of i-16 (melting point 62.4 C) and n-16 (melting

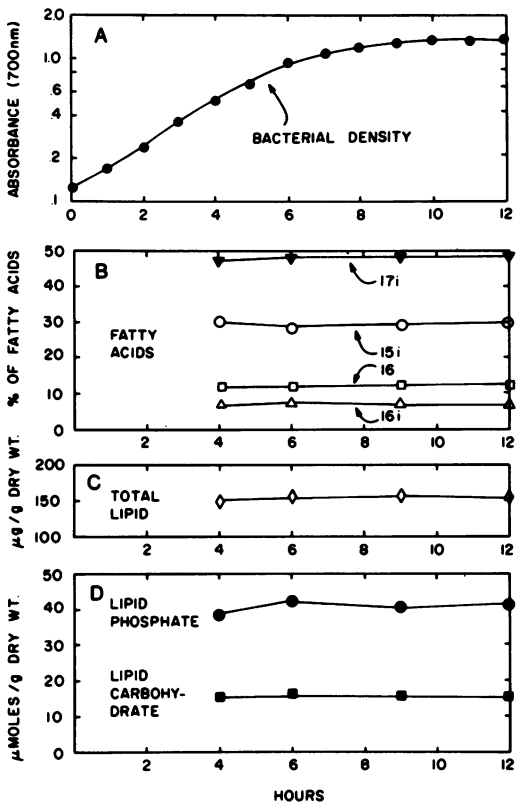

FIG. 3. *Lipid composition of Thermus aquaticus during the growth cycle at 70 C. (A) Bacterial density; (B) percentage of the i-15, n-16, i-16, and i-17 fatty acids in the lipids; (C) total dry weight of the lipid per gram (dry weight) of T. aquaticus; (D) total lipid phosphate and total lipid glucose per gram (dry weight). Lipids were extracted by the Bligh and Dyer procedure (2).*

point 63.1 C), and a concomitant decrease in the proportion of a-17 (m.p. 38 C) and i-17 (m.p. 60.5 C) (18), thus suggesting a shift to a more thermally stable composition. Kodicek (13) suggested that the iso and anteiso fatty acids make the membrane more fluid than the corresponding straight-chain fatty acids.

The fatty acid composition of *T. aquaticus* is radically different from most gram-negative bacteria (12). It seems that *T. aquaticus* resembles the gram-positive bacteria at least with respect to fatty acid composition, since the majority of the fatty acids are branched, and *T. aquaticus* contains no cyclopropane fatty acids which are found in some gram-negative bacteria. However, electron micrographs of *T. aquaticus* show a typical gram-negative cell wall (4).

The total lipids of *T. aquaticus* account for 20% of the total dry weight in cells grown at 70 C, of which the phospholipids account for only 30% of the total lipids (*manuscript in preparation*). Card et al. (6) reported that the phospholipids of *B. stearothermophilus* account for only 50 to 60% of the total extractable lipids. In both these thermophiles, the amount of neutral lipids is much greater than the amount found in mesophiles. Carotenoids account for 60% of the total lipids in *T. aquaticus* grown at 70 C; perhaps these neutral lipids help account for the stability of the membrane.

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