JOURNAL OF BACTERIOLOGY, May 1969, p. 528-531
Vol. 98, No. 2

Fatty Acids and Polar Lipids of Extremely Thermophilic Filamentous Bacterial Masses from Two Yellowstone Hot Springs

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Received for publication 7 January 1969

The fatty acid composition of filamentous bacterial masses from two very hot Yellowstone Park springs is not unusual despite the extreme environment. Both populations have a series of C_{14} to C_{20} straight-chain acids with a maximum at C_{18}, and a series of saturated iso acids with a maximum at C_{17} in one case and C_{19} in the other. The fatty acid pattern of this anomalous group of organisms is like that of bacteria but not of blue-green algae. Both populations have similar polar lipids and identical carotenoids. It is speculated that these organisms may be adapted to their high-temperature environment by means of stable lipoprotein membrane systems.

Microorganisms of extreme environments, such as the thermophilic bacteria and algae of hot springs and geysers, are of particular interest to biologists because the molecular basis of thermophily is not fully understood (9, 10); they are also of interest to planetary exploration biologists because their existence implies that there is a poorly defined "upper temperature limit" for life. Filamentous flexibacteria-like organisms can apparently grow at any temperature at which there is liquid water, even in pools which are above the ambient boiling point. These organisms have been discussed and photomicrographs have been published by Brock (5). Such organisms might thus occur on other planets, either in restricted ecological niches or more widely distributed as a consequence of some early high-temperature stage of planetary evolution. The flexibacteria, to which these organisms may be related, are a little-studied group of motile gliding organisms which have been classified morphologically as either apochlorotic blue-green algae or as bacteria (15). Both bacteria and blue-green algae are found in hot springs, but the algae are limited to growth at temperatures of 73 to 75 C or less, evidently because of evolutionary limits to their photosynthetic mechanism (5). We felt that a study of the lipids of extremely thermophilic flexibacteria-like organisms might contribute toward an understanding of both their taxonomic relationships and their adaptations to thermophily.

In general, bacteria may be distinguished from blue-green algae in terms of their fatty acids because algal acids are usually straight-chain saturated and unsaturated (11, 20), whereas the bacterial acids contain branched and normal chains but rarely contain significant amounts of polyunsaturated acids (13, 17, 19, 23, 24). In addition, the polar lipids of the bacteria include phosphatidylethanolamine and occasionally lecithin (12), whereas these substances are absent in blue-green algae (18). To the best of our knowledge, no comprehensive work on lipid analysis has yet been reported for any *Flexibacterium* (T. D. Brock and R. A. Lewin, *personal communication*), and this report is also the first on the chemistry of such an extreme thermophile collected from a natural source. The few reports on the fatty acids of thermophilic bacteria (7) and algae (11, 20) published thus far deal with laboratory cultures grown at 39 C or less.

MATERIALS AND METHODS

Filamentous bacterial masses were collected at Yellowstone National Park, Wyo. on 26 July 1968, from Firehole Pool and "Pool A" in the area of Firehole Lake Loop Road in the Lower Geyser Basin area studied by Brock and Brock (6). The gelatinous pink masses were taken from the water with tweezers, placed in Aclar fluoropolymer clean room bags to ensure freedom from lipid contamination (3), and frozen in dry ice within a few minutes for subsequent transportation to the laboratory. The colonies thus contained entrapped spring water and small amounts of "geyserite silica." The Pool A material was collected from the main outflow channel of Pool A, whereas that from Firehole Pool was collected from its rim at a temperature of 81.5 C. The temperature was meas-

ured by means of a model 42 SC thermistor thermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) equipped with a soil probe. Brock measured the *p*H of these pools as 8.6 for Firehole and 8.2 for Pool A, and he measured the temperature of the Pool A channel as 85 to 88 C.

About 50 g of the solidly frozen sample was placed in a stainless-steel Waring Blendor fitted with all Teflon seals. A 10-ml amount of "nanograde" solvent (Mallinckrodt Chemical Works, St. Louis, Mo.) per g of frozen tissue was added, and nitrogen which contained less than 8 ppm of oxygen was bubbled through the mixture for 10 min. The tissue was then blended for 2 min in the cold, and the mixture was filtered under nitrogen through an "M" porosity sintered-glass filter. The cake was then quickly returned to the blendor for reextraction. The sample was extracted three times with chloroform-methanol (2:1, v/v) and once with methanol. This sequence appeared to remove all lipid from the cells, as judged by weighing the residues left from evaporated 5-ml samples of the extracts (21). The pooled extracts were taken nearly to dryness in a Buchler evaporator at a temperature of no greater than 10 C. During the final stages of evaporation, the extracts tended to bump violently and a translucent coating of very finely divided silica appeared on the walls of the vessel. The total lipids were estimated by weighing evaporation residues of small samples of the clear pink extract on a Cahn microbalance. Polar lipids were examined by means of the thin-layer methods of Rouser et al. after removal of nonlipids with Sephadex (21). Preliminary extractions of the bacteria gave very low yields of lipid, probably as a result of adsorption on the finely divided silica during the concentration step. In our final procedure for analysis of fatty acids, the sample was transferred only once with exhaustive rinses of methanol into the vessel used for saponification. The red carotenoid pigment of the bacteria from both pools was extracted from the cells with acetone followed by methanol; the pigment was then concentrated and transferred into ether (2). The amounts of pigment were insufficient for characterization at this time.

Fatty acids were prepared by mild saponification of the total lipid at 60 to 62 C for 2 hr and were recovered by the extraction scheme of Van der Veen (25). Free acids were esterified by reaction with freshly prepared diazomethane (8). Controls run concurrently with identical solvents and treatment showed no trace of acids.

The mixture of fatty acid methyl esters was separated by capillary gas chromatography on columns of diethylene glycol succinate (DEGS) and Apiezon L. Chromatograms of the fatty acid methyl esters isolated from filamentous bacteria from the two pools are shown in Fig. 1. The effluent from the gas chromatograph was connected through a single-stage molecular separator (22) to the ion source of a quadrupole 300 mass spectrometer (Electronic Associates, Inc., Long Branch, N.J.). Individual esters were identified by both gas chromatography and combined gas chromatography-mass spectrometry by comparison with known fatty acid methyl esters or reference spectra.

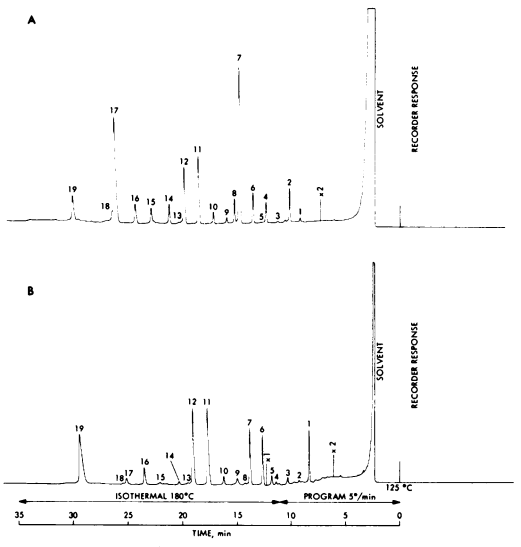

FIG. 1. *Gas chromatogram of fatty acid methyl esters from flexibacteria. (A) Firehole pool; (B) Pool A. Capillary column: inner diameter, 200 feet by 0.02 inch (60.9 m by 0.05 cm); DEGS.*

RESULTS AND DISCUSSION

The percentage composition of fatty acids is listed in Table 1. The distribution of fatty acids is qualitatively very similar for the two bacterial masses collected at different hot spring locations. The two pools are about 0.2 mile apart geographically. Both colonies contain a series of C_{14} to C_{20} straight-chain saturated fatty acids with a maximum at methyl stearate (C_{18}) and a series of C_{15} to C_{21} branched saturated iso (terminal isopropyl) fatty acids. The iso-acids of the bacteria from Firehole Pool have a maximum at methyl isoheptadecanoate (C_{17}), in contrast to those of the bacteria from Pool A, in which the most abundant acid is methyl isononadecanoate (C_{19}). Relatively small amounts of the anteiso (terminal sec butyl) branched saturated fatty acids were detected in the two samples, although approximately 2.9% of methyl anteisoheptadecanoate was found in the bacteria from Firehole Pool. The sample from Firehole Pool contained approximately 24% of a C_{20} monounsaturated fatty acid, but, surprisingly, the sample from Pool A contained less than 2% of the same monoene. Mass spectral analysis of the methyl ester gave a molecular weight of 324, and unsaturation was confirmed by hydrogenation of the sample followed by reexamination in the mass spectrometer. Each of the samples also contained a C_{21} cyclopropane fatty acid, approximately 22% being present in the sample from Pool A and 5% in the

TABLE 1. *Percentage composition of fatty acids in bacterial masses collected at two hot spring locations*

Peak elution order	Identification	Percentage composition	
		Firehole Pool	Pool A
1	C_{14}	0.715	7.513
2	iso-C_{15}	4.102	0.520
3	C_{15}	0.413	1.664
4	iso-C_{16}	2.931	0.777
5	NI[a]	Trace	1.839
6	C_{16}	4.269	9.134
7	iso-C_{17}	21.144	9.587
8	ante-C_{17}	2.930	0.373
9	C_{17}	1.011	1.766
10	iso-C_{18}	2.011	1.943
11	C_{18}	9.756	17.77
12	iso-C_{19}	8.934	16.89
13	ante-C_{19}	0.231	0.385
14	C_{19}	2.863	0.943
15	iso-C_{20}	2.680	0.793
16	C_{20}	3.796	4.971
17	$C_{20:1}$	24.454	1.768
18	NI[a]	2.565	0.329
19	C_{21} cyclopropane	5.190	22.02

[a] Not identified.

sample from Firehole Pool. The presence of the cyclopropane ring was established by mild hydrogenation of the sample followed by bromination in cold ether (4). Unfortunately, the small sample size did not permit determination of the location of the cyclopropane ring.

The presence of a high percentage of branched iso-fatty acids in the flexibacteria is consistent with reports of similar acids in other bacteria (13, 17, 19, 23, 24). The distribution of branched-chain fatty acids for most bacteria thus far examined has a maximum at C_{15}. The maxima at C_{18} for the straight-chain acids and C_{17} or C_{19} for the iso-branched series, observed in these flexibacteria, may perhaps be attributed to their extreme thermophilic environment, but otherwise the distribution is unremarkable for organisms from such an extreme environment.

A typical analysis of the Pool A cell mass is as follows. A 100-g amount of the frozen cells in spring water yielded 23.1 g of tissue when lyophilized. The tissue on extraction yielded 48.0 mg (0.21%) of total lipid, of which 8.6 mg comprised free fatty acids. The cake which remained after the extraction step weighed about 19.3 g and left 72.5% ash after heating to constant weight at 800 C. The ash consisted largely of geyserite particles, as determined by spark emission spectrometry. The analysis of the Firehole Pool material was almost identical to that of Pool A material. In each case, the Sephadex lipid fraction (21) amounted to about 85% of the total lipid extracted.

The red carotenoid pigment appeared to be the same for the bacteria of both pools. Its visible spectrum resembled that of flexixanthin (1), but with maxima at 455, 494, and 525 nm in acetone, and it behaved chromatographically as an extremely polar xanthophyll which did not move on a column of magnesium oxide-celite (1:1) on elution with diethyl ether. This suggests that it may be a primary diol such as lycoxanthin (16). Based on the assumption of an extinction coefficient in ligroin equivalent to that of both carotenes, the unknown carotenoid represents about 0.02% of the dry weight of material from Pool A and about 0.01% of that for the material from Firehole Pool.

ACKNOWLEDGMENTS

This investigation was carried out at the Jet Propulsion Laboratory, California Institute of Technology, under contract no. NAS 7-100, sponsored by the National Aeronautics and Space Administration.

We thank T. D. Brock, N. H. Horowitz, and J. S. Hubbard for criticism of the manuscript; R. I. Lynn for invaluable help in collecting the samples; and T. Kaneda for a gift of iso-C_{18} acid. We also thank S. G. Canter and the U.S. National Park Service for permission to sample the pools at Yellowstone National Park, Wyo.

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