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Fatty Acids in the Lipids of Marine and Terrestrial Nitrifying Bacteria[1]

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Fatty acids in the lipids of 19 marine and terrestrial nitrifying bacteria have been analyzed. Ammonia-oxidizing bacteria have a very simple acid composition; palmitic and palmitoleic acid account for 96 to 100% of the total acids. The fatty acids of nitrite-oxidizing bacteria cover a wider range, from C_{14} to C_{19}, but from two to four acids still account for more than 80% of the total acids. Branched iso- and anteiso-acids are present in traces only in 2 of the 19 bacteria. The chemical and morphological similarity between blue-green algae and these bacteria is discussed.

The geochemical role played by bacteria is poorly understood, although it is tacitly assumed that they synthesize and transform significant amounts of organic compounds both in the waters and in the sediments of the ocean. Numerous authors have shown that bacteria of different taxonomic positions show diagnostically useful differences in their lipid chemistry (5). The long persistence of lipids in the sea and in marine sediments might enable us to determine analytically the relative contribution of different bacteria to the lipid content of a particular environment. Thus, it has been suggested that the relatively high proportion of iso- and anteiso-acids in marine sediments (4) and in sea water (M. Blumer, *unpublished information*) reflects the activity of marine and sedimentary bacteria. Unfortunately, with the exception of scattered analyses (9, 10), little systematic research has been done on the fatty acid composition of marine bacterial lipids. Considerable work in this area is needed for the future application of bacterial chemotaxonomy to marine studies.

In our laboratory, marine and selected nonmarine autotrophic nitrifying bacteria are under intensive investigation. We have studied the fatty acids in the lipid fraction of some of these organisms to provide a basis for further work on their role in organic geochemistry.

MATERIALS AND METHODS

Organisms. Except where indicated otherwise in Table 1, all of the organisms were isolated in this laboratory by enrichment and serial dilution techniques. Because these organisms are autotrophs, no organic nutrients were included in the medium. The mineral salts medium used for enrichment, isolation, and maintenance of cultures included: water, 1 liter; K_2HPO_4, 10 to 150 mg; $CaCl_2 \cdot H_2O$, 2 to 5.4 mg; $MgSO_4 \cdot 7H_2O$, 10 to 20 mg; chelated iron [Sequestrene (13% Fe), Atlas Chemical Industries, Inc., Wilmington, Del.], 130 μg; $Na_2MoO_4 \cdot 2H_2O$, 40 μg; $MnCl \cdot 4H_2O$, 20 to 55 μg; $CoCl_2 \cdot 6H_2O$, 0.4 μg; $CuSO_4 \cdot 5H_2O$, 5.2 μg; $ZnSO_4 \cdot 7H_2O$, 22.6 μg; and 0.25 ml of 0.5% phenol red. For marine organisms, the above mineral salts medium was made up with sea water, whereas distilled water was used for terrestrial organisms. The lower salt concentrations were used for nitrite-oxidizing bacteria and the higher concentrations, with the exception of phosphate, were used for ammonia-oxidizing bacteria. For these, the phosphate level was decreased to 50 μg/liter to avoid precipitation. For ammonia-oxidizing bacteria, $(NH_4)_2SO_4$ (10 mM) served as the substrate, whereas $NaNO_2$ was the substrate for the nitrite-oxidizing bacteria. Nitrite was added in increments of 3 mmoles per liter since it is toxic in high concentrations. When the culture had nearly depleted the nitrite, another increment was added until the total addition was approximately 30 mmoles per liter.

Cultures were grown in 15-liter carboys aerated with air sterilized by passage through two 0.45-μm filters (Millipore Corp., Bradford, Mass.) arranged in series. During growth of the ammonia oxidizers, the *p*H dropped, it would eventually fall from 7.8 to 5.5 if not adjusted. Therefore, each carboy was connected to a reservoir of sterile K_2CO_3 (0.4 M) from which periodic additions were made whenever the color of the phenol red changed. During the early growth stages, the *p*H had to be adjusted rarely, but just before harvesting adjustment was required form four to six times daily.

Special caution was taken to insure pure cultures. All cultures were inoculated periodically into several heterotrophic media, including 0.1% Tryticase (BBL), 50% strength AC medium (Difco), and thioglycolate (BBL). If growth was found on any medium, the pres-

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TABLE 1. *Source and taxonomic position of nitrifying bacteria*

Strain	Source	Taxonomy
	Ammonia-oxidizing bacteria	
	Terrestrial or fresh-water strains	
57	Soil of Galapagos Island	*Nitrosolobus multiformis*[a]
22	Soil of Southwest Africa	*N. multiformis*
71	Soil of Surinam, South America	*N. multiformis*
51	Received from Dr. Schmidt, University of Minnesota (original source of culture unknown)	*Nitrosomonas europaea*
31	Received from Dr. Pramer, Rutgers University (original source unknown)	*N. europaea*
49	Soil of Surinam, South America	*Nitrosomonas sp.*
91	Chicago sewer	Unidentified
101	Chicago sewer	Unidentified
	Marine strains	
107	Gulf of Maine	*Nitrosocystis oceanus*
27	Barbados Harbor	*N. oceanus*
29	Pacific Ocean (exact source unknown)	*N. oceanus*
19	Pacific Ocean, off the continental shelf along the coast of Peru	*Nitrosomonas* sp.
15	Pacific Ocean, on the continental shelf along the coast of Peru	*Nitrosomonas* sp.
40	Atlantic Ocean, twelve miles from the mouth of the Congo River	*Nitrosomonas* sp.
25	Atlantic Ocean, 200 miles from the mouth of the Amazon	*Nitrosomonas* sp.
	Nitrite-oxidizing bacteria	
	Terrestrial strains	
213	Received from Dr. Alvin Nason, Johns Hopkins University original source unknown	*Nitrobacter agilis*
106	Received from Dr. Helen Funk, Goucher College; American Type Culture Collection ATCC 14123	*Nitrobacter agilis*
	Marine strains	
231	Off Fernandino Islands (100 yrd), Galapagos Archipelago	*Nitrococcus mobilis*[b]
211	Atlantic Ocean, 200 miles from the mouth of the Amazon River	*Nitrospina gracilis*[b]

[a] Tentative classification (Watson, *unpublished data*).
[b] Tentative classification (Waterbury and Watson, *unpublished data*).

ence of heterotrophic bacteria was assumed and the culture was discarded. Since many contaminants do not grow in the above and in many other heterotrophic media, cultures which failed to produce growth on these were not judged to be free of contaminants until examined by phase-contrast and by electron microscopy. The morphology of many of the nitrifying bacteria is sufficiently distinctive to permit the detection of most heterotrophic contaminants with the phase-contrast microscope.

Each species of nitrifying bacteria has a characteristic fine structure. To make certain that a culture consisted of only one species, all cultures were fixed, embedded, sectioned, and examined by use of an electron microscope.

Cultures were harvested with a continuous-flow centrifuge (Sorvall) at 12,000 × *g* and at 5 C and were washed twice with sterile mineral salts medium without substrate. Subsequently, cells were frozen in 5-ml glass vials and kept at −20C.

Extraction and esterification. Frozen bacteria (0.05 to 0.5 g, wet wt) were weighed and ground in a tissue grinder in 2 ml of methanol-benzene (1:1, v/v) for 5 min. The homogenate was centrifuged and the supernatant fluid was decanted and dried at 15 C on a rotary evaporator. The residue was taken up in 1-ml *n*-pentane and a sample corresponding to 0.01 g of bacteria (wet wt) was saponified in 1 ml of 0.5 M KOH in methanol and then esterified with 1.2 ml of saturated BF_3 in methanol, according to the method of Metcalfe et al. (6). The product was diluted with 2 ml of saturated NaCl and extracted three times with 1.5 ml of *n*-pentane. The combined extracts were dried on a rotary evaporator.

Gas chromatography. Gas chromatographs used were Aerograph models 600 D and 1200 (Varian Aerograph, Walnut Creek, Calif.) with flame detector, linear temperature programmer, automatic attenuator (model 50 B, Hewlett-Packard, Avondale, Pa.), and a 1-mv recorder. Operating conditions were: injector temperature, 200 C; detector temperature, 250 C; and nitrogen flow at maximal plate efficiency (10 to 12 ml/min). Packings were 12.5% FFAP (Varian Aerograph) and 0.84% Apiezon L on Chromosorb W and G, 80 to 100 mesh, acid-washed, and siliconized. Columns were stainless steel (10 to 12 ft), 0.12 inch outside diameter, with a 0.03-inch wall.

Samples of the methyl esters corresponding to 2 mg of bacteria (wet wt) were injected as CS_2 solutions. The columns were held at constant temperature until complete elution of the CS_2; they were then programmed at 2 C/min from 120 to 290 C (Apiezon) and from 175 to 250 C (FFAP). Columns were held at maximal temperature until elution of the C_{22} methyl ester.

Chromatograms were recorded from both columns in the presence and in the absence of normal C_{12} to C_{22} methyl esters as internal standards. Equivalent chain lengths (7) were measured, relative to the internal standard, for the unknown acids from the bacteria and for methyl esters prepared from cod liver oil. The use of this as a secondary standard for lipid analysis has been proposed by Ackman et al. (1). Quantitative determinations were made by estimating peak areas from the product of height and width at half-height. All values were normalized to 100%.

Reagents. All solvents were redistilled as previously described (3). Reference compounds were normal C_{12} to C_{22} fatty acids (Matheson, Coleman, and Bell, Norwood, Ohio) and cod liver oil (E. R. Squibb & Sons, New York, N.Y.). Both were converted to methyl esters as described here. The reagents for esterification were freshly prepared.

RESULTS AND DISCUSSION

The straight-chain saturated acids are identified by their retention behavior on a polar and a nonpolar column. The C_{16} and C_{18} monoethylenic acids are tied to those present in cod liver oil (1; *see* Table 2). Minor monoethylenic acids at C_{14}, C_{15}, C_{17}, C_{19}, and possibly at C_{20} are tentatively identified as belonging to the same series, having a double bond in 9-position from the carboxyl group; their equivalent chain lengths fall into the same series as those of the major unsaturated acids.

Iso- and anteiso-acids are easily resolved from the saturated and monoethylenic acids by gas chromatography on FFAP. Only strain 211 (Table 3) contains a measurable amount of the i-16:0 acid; this and a-17:0 are present in traces in strain 106. [The shorthand notation of chain length:double bond (e.g., 16:0) is used throughout.] It is estimated that, if present, their individual concentrations in the other bacteria must lie below 0.5%. Branched acids are common in the *Bacillaceae* (5), but it is interesting to note that gram-negative bacteria producing iso- and anteiso-acids have been isolated from lagunal muds (10). According to P. L. Parker and C. Van Baalen (*personal communication*), these small rods are aerobic heterotrophic bacteria, probably belonging to the order *Pseudomonadales*. It is clear from the analyses presented here that the nitrifying bacteria are not the source of the branched acids in sea water and marine sediments. In view of their geochemical prominence, the search for iso- and anteiso-acids should be extended to other marine bacteria, including other representatives of the *Pseudomonadales*.

TABLE 2. *Equivalent chain lengths of methyl esters from cod liver oil*[a]

Fatty acid	Double-bond position[b]	Apiezon L	FFAP
16:1	9	15.70[c]	16.32
17:1	9	—	7.35
18:1	9	17.65	18.27
20:1	11	19.65	—

[a] Unknown methylesters from the bacteria were considered identified if their equivalent-chain-length values agreed with those of esters prepared from cod liver oil to ±0.01 (Apiezon) and ±0.03 units (FFAP).

[b] From carboxyl group.

[c] Not detectable.

Several interesting facts emerge from the analytical data of Table 3. The lipid composition of the nitrifying bacteria, especially of the ammonia oxidizers, is less complex than that found in most other groups of bacteria (5). The 16:1 acid accounts for 60 to more than 80% of the total acids, whereas the sum of 16:0 and 16:1 makes up 96 to 100% of the acids in the lipids of the cell.

One or two fatty acids have not before been found to comprise such a large percentage of the total fatty-acid content of a bacterial cell with the exception of *Mycoplasma* (11). The latter organism contained as much as 97% of the 18:1 acid, although this varied with the culture medium. Whether any evolutionary significance can be attached to the simple fatty acid composition of the nitrifying bacteria remains an open question.

Significant differences in the fatty acid composition exist even among the nitrifying bacteria. The sharpest demarcation exists between the ammonia and nitrite oxidizers. The 18:1 acid, although present in minor quantities in some ammonia-oxidizing bacteria, assumes a greater importance in the nitrite oxidizers. In these we find, in addition, a wider molecular-weight range,

TABLE 3. *Fatty acids in the lipids of nitrifying bacteria*

Fatty acid	Double-bond position[a]	Ammonia-oxidizing bacteria															Nitrite-oxidizing bacteria			
		Terrestrial or fresh-water strains								Marine strains							Terrestrial strains		Marine strains	
		57	22	71	51	31	49	91	101	107	27	29	19	15	40	25	213	106	231	211
14:0		0.3	TR[c]		TR		TR			0.2	0.7	0.3	0.2		0.3	TR	TR	0.3	0.3	13
14:1	9[b]	0.7	TR		TR	1.6	TR	1.3	1.5	TR	0.6	0.9	0.2		0.2	TR	TR	0.2	0.4	16
15:0		TR		TR	TR		TR			0.3	0.6	0.3	0.2		0.2	TR	TR	0.2	TR	TR
15:1	9[b]	TR			0.7		TR			0.1	TR		0.2		0.3	TR		TR		TR
i16:0																		TR		1.39
16:0		25	25	18	25	17	25	23	25	31	33	39	30	26	27	39	5.4	7.3	20	12
16:1	9	74	75	81	75	82	75	76	74	66	65	59	66	74	72	61	1.4	1.1	43	48
a17:0																		TR		
17:0													TR				0.3	0.2	0.4	
17:1	9[b]												0.1				0.9	0.7	2.1	
18:0				TR									0.3				0.7	1.0	0.8	
18:1	9			TR						2.6			1.1				63	54	34	8.5
19:1	9[b]																28	35	TR	
20:1	(?)[d]												2.1							

[a] From carboxyl group.
[b] Monoethylenic acids, presumably unsaturated in 9-position. Equivalent-chain-length values: 14.25, 15.30, 17.31, and 19.29, all on FFAP.
[c] Trace present, too small for quantitative measurement.
[d] Minor peak, partly obscured by column bleed; estimated equivalent-chain-length value, 20.25.

extending from the 14:0 to the 19:1 acid in significant amounts.

In general, the degree of saturation is higher in the marine than in the terrestrial ammonia-oxidizing bacteria. It is interesting to note that the two marine organisms, no. 40 and 15, showing a lesser degree of saturation, were isolated from low-saline waters. Also of interest is the fact that strain 71 had a slightly higher degree of unsaturation than did 57 and 22, although these three organisms appear to be morphologically identical. The significance of this observation merits additional study.

A difference was also found between marine and terrestrial nitrite-oxidizing bacteria. The terrestrial strains had a higher content of 18:1 and 19:1 than did the marine strains. However, the two terrestrial strains were both *Nitrobacter agilis* which, although grown autotrophically in this investigation, can be grown heterotrophically (12). Whether the observed differences reflect the differences in environment or whether they stem from the fact that *N. agilis* is a facultative autotroph still need exploration before any definite conclusions can be drawn.

Since the nitrifying bacteria comprise less than 0.002% of the bacterial biomass in the oceans, it is obvious that they play a minor role in the production of the residual fatty acids found in the oceans. Even if their biomass was larger, it would be difficult to ascertain their contribution to the residual fatty acids in the oceans because they synthesize chiefly those C-16 fatty acids which are common and most abundant in other living cells.

However, the nitrifying bacteria are of interest since all are obligate chemoautotrophs with the exception of *N. agilis* (12). Thus, in contrast to heterotrophic bacteria, the nitrifying bacteria must synthesize all of their fatty acids from CO_2 rather than from organic compounds supplied in the medium.

It is also of interest to compare the fatty-acid composition of the nitrifying bacteria to that of blue-green algae. In both classes of organisms, the predominance of the 16:0, 16:1, and 18:1 acid is pronounced. The acids of blue-green algae cover a wider molecular-weight range. Polyunsaturated acids are absent in these as in other bacteria, but they are common constituents of blue-green algae. Both groups of organisms share many physiological and morphological features, although the former group are chemoautotrophs and the latter photoautotrophs. The morphological similarity between some of the members of the two groups is especially striking. For example, on the basis of electron micrographs, the blue-green alga *Anacystis nidulans* (2) is difficult to differentiate from *Nitrosomonas europaea* (8). The most distinguishing feature of both organisms is their cytomembrane system. Since the major fraction of cellular lipids is associated with membranes, it is interesting that the membranes in these two groups of organisms appear so similar morphologically.

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