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Influence of Hydrocarbons and Derivatives on the Polar Lipid Fatty Acids of an *Acinetobacter* Isolate

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The effects of hydrocarbons and hydrocarbon derivatives as growth substrates on the polar lipid fractions of an *Acinetobacter* isolate were studied. Tetradecane, hexadecane, and octadecane resulted in the incorporation of substantial quantities of equivalent-chain-length fatty acids into cellular lipids. Cells cultured on nonane, the only odd-numbered alkane tested, contained both odd- and even-chain fatty acids. The *n*-alkane dotriacontane (32 carbons), 1-chlorohexadecane, 1-chlorododecane, 1-chlorodecane, and 1-phenyldodecane yielded significant amounts of odd-chain fatty acids. A subterminal oxidative pathway is believed to account for these results. Cells grown on long-chain alcohols exhibited fatty acid profiles nearly identical to those of cells grown on the corresponding alkanes.

Microbial oxidation of hydrocarbons has received wide interest in recent years due to the potential utility of microorganisms as degradative agents of unwanted oil spillage. Also of particular interest are the oxidative pathways enabling such microorganisms to utilize hydrocarbons, initially devoid of functional groups, as a source of energy and cellular carbon. A variety of bacteria and fungi have been shown to be capable of hydrocarbon oxidation (10). Several have been investigated for the potential influence that hydrocarbon substrates might exhibit upon cellular constituents. Current studies have particularly emphasized the relationship between the hydrocarbon utilized and lipids occurring within the resulting cells. These observations indicate that certain *n*-alkanes and alkenes are oxidized to the equivalent-chain-length fatty acid and as such are directly incorporated into lipid fractions (5, 14, 15). The result is an enrichment in fatty acids of carbon length equal to that of substrate. Evidence further suggests the existence of a process by which hydrocarbon skeletons may be utilized for the synthesis or elongation of fatty acids without prior degradation to acetate (6). The purpose of this investigation was to examine the fatty acids from polar lipid fractions of an *Acinetobacter* isolate when cultured on *n*-alkanes and related compounds.

MATERIALS AND METHODS

Organism. A bacterium was isolated from Lake Erie by enrichment on hydrocarbon and identified as an *Acinetobacter* sp. (1, 2, 12), coded P-1 in our collection. The isolate, which appears quite similar to descriptions of *Micrococcus cerificans* (1, 14, 15), was maintained on Trypticase soy agar and subcultured weekly.

Media. Organisms were grown in a mineral salts medium of the following composition (in grams per liter): KH_2PO_4, 1.36; $Na_2HPO_4 \cdot 12H_2O$, 1.79; $MgSO_4 \cdot 7H_2O$, 0.12; $(NH_4)_2SO_4$, 0.66; $CaCl_2 \cdot 2H_2O$, 0.07; $FeCl_3 \cdot 6H_2O$, 4.8×10^{-3}; $MnCl_2 \cdot 4H_2O$, 1.8×10^{-3}; $ZnSO_4 \cdot 7H_2O$, 4.4×10^{-4}; H_3BO_4, 2.9×10^{-3}; $CuSO_4 \cdot 5H_2O$, 3.9×10^{-4}; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 3.7×10^{-4}; $CoCl_2 \cdot 6H_2O$, 8.1×10^{-4}, pH 6.6. Liquid hydrocarbons were filter-sterilized (0.45-μm filter; Millipore Corp.) and added to the medium at a concentration of 1% (vol/vol). Solid hydrocarbons and derivatives were autoclaved separately and then added to sterile salts solution.

Culture conditions. Cells were grown in 1-liter Erlenmeyer flasks at 25 ± 3 C on a rotary shaker. Experiments were initiated by inoculation of one loopful of actively growing bacteria from Trypticase soy agar plates. Cultures were harvested 1 day after appearance of maximal growth (turbidity) by centrifugation and washed twice with 15 mM phosphate buffer.

Lipid extraction. Wet cell pellets were suspended in 125 ml of chloroform-methanol (2:1, vol/vol) and shaken for 18 h at 25 ± 3 C. The extracts were passed through a sintered-glass filter and washed according to Folch et al. (7). The chloroform layer was condensed in a rotary evaporator and stored at −20 C.

Separation of polar lipids. Approximately 50 to 100 mg of lipid was applied to an 8-g column of prewashed, activated silica gel. Neutral lipids and residual hydrocarbon were eluted with chloroform and discarded, followed by elution of polar lipids with methanol.

Saponification and methylation. Polar lipids were saponified in 2 ml of 2 N KOH in ethanol-water (1:1). Non-saponifiable material was extracted with petroleum ether, and the aqueous phase was acidified with H_2SO_4. Fatty acids were extracted with petroleum ether, which was washed with distilled water. Methylation was accomplished by adding 0.5 ml of BF_3 (14% in methanol) to a dried fatty acid sample (less than 1 mg) and then heating for 2 min at 100 C. Methyl esters were stored at −20 C in methanol.

Gas-liquid chromatography. Fatty acid separation was carried out with a Varian Aerograph series 200 dual-channel chromatograph equipped with flame ionization detectors. Stainless-steel columns (5 ft by 0.12 in; about 1.5 m by 0.3 cm) were packed separately on Chromosorb W (Varian Aerograph, Walnut Creek, Calif.) with 15% ethylene glycol succinate, 5% silicone SE-30, or 10% diethylene glycol succinate. Injection and detector temperatures, respectively, for both ethylene and diethylene glycol succinates were 180 and 190 C; injector and detector temperatures, respectively, for SE-30 were 240 and 250 C. Column temperature was programed at a rate of 4 C/min. Carrier as was nitrogen at a flow rate of 25 ml/min. Quantitation of hydrocarbons was made on peaks from either ethylene or diethylene glycol succinate columns and was based on the average of at least two runs.

Methyl esters were identified by comparison of the relative retention times with those of standard methyl esters (Applied Science Laboratory, State College, Pa.). The area under each peak was determined by planimetry and expressed as a percentage of the total peak area.

Methyl esters were also treated with platinum catalyst and bromine by the method of Brian and Gardner (3) to assay for unsaturated and cyclopropane fatty acids, respectively.

Chemicals. Octane, nonane, decane, dodecane, tetradecane, hexadecane, octadecane, hexadecanol, tetradecanol, dodecanol, and 5% platinum catalyst on charcoal were obtained from Matheson, Coleman and Bell (Norwood, Ohio). Eicosane, dotriacontane, 1-chlorohexadecane, 1-chlorotetradecane, 1-chlorodecane, 1-phenyldodecane, and 14% BF_3 in methanol were obtained from Eastman Organic Chemicals (Rochester, N.Y.).

RESULTS

The bacteria were incubated in a mineral salts medium with various hydrocarbons, primary alcohols, chloro- and phenyl-derivatives, carbohydrates, and amino acids as carbon sources (Table 1). Carbohydrates were not capable of supporting growth even when supplemented with 0.02% yeast extract or complex vitamin mixtures, and only 6 of 16 amino acids tested provided a suitable carbon substrate. No growth was obtained on alkanes of less than 9 carbons after incubation periods of up to 6 weeks. Dotriacontane (32 carbons) was the longest-chain alkane tested and required only several days for adequate growth to appear, suggesting that even larger-molecular-weight *n*-alkanes might serve as oxidizable substrates.

The lag periods before log growth were relatively constant for the *n*-alkanes of chain lengths from 12 to 20. Shorter- and longer-length alkanes, primary alcohols, chloro- and phenyl- derivatives, all required additional time to reach log phase. No correlation between the length of the lag periods and total cell yields was evident. Although log phase for nonane-cultured cells extended several days beyond that for tetradecane-cultured cells; cell yields in both cases approached 1.5 g/liter (dry weight).

The distribution of fatty acids in cells grown on *n*-alkanes is summarized in Table 2. Analyses of cells cultured on eicosane or octadecane demonstrate similar fatty acid profiles. The predominant acid in both instances was 18:1, followed closely in abundance by 16:0. Together they comprised approximately 70% of the total fatty acids from the polar fractions. The ratio of unsaturated to saturated fatty acids (R_u/R_s) was 1.09 for eicosane-grown cells and 1.27 for cells cultured on octadecane. A ratio of 1.00 would represent 50% fatty acid unsaturation.

Cells grown on hexadecane revealed a significantly different profile. The acids 16:0 and 16:1 were major components, representing 86.2% of the total. A reduced quantity of 18:1 was present as compared with eicosane- and octadecane-cultured cells. Maximal incorporation of fatty acids of chain lengths equivalent to substrate occurred when hexadecane was the carbon source.

A substantial amount of fatty acid enrichment was also prominent with tetradecane as substrate. These cells as well as cells grown on dodecane contained marked increases in 14:0 content (over 40% in the case of both alkanes). This acid was followed in abundance by 16:0, 18:1, and 16:1 acids.

Unlike the analyses previously discussed, cells grown on decane revealed only small amounts of 14:0 (2.8%), whereas the longer-chain acids 16:0 (61.4%) and 18:1 (20.6%) predominated. These cells contained the highest percentage of 18:0 acid, although representing only 6.2%.

When cultured on nonane, the bacteria produced significant quantities of odd-chain fatty acids (Table 3). Such results suggest that long-chain fatty acids may be synthesized from the oxidative products of alkanes by addition of two carbon fragments. Although odd-carbon skeletons would be readily available from the oxidation of nonane, even-chain fatty acids still predominate, indicating the importance of de

TABLE 1. *Growth of the isolate on various substrates in a mineral salts medium*

Substrate	Lag[a] (days)	Final pH	Substrate	Lag (days)	Final pH
Dotriacontane	4	5.0	1-Chlorohexadecane	2	4.5
Eicosane	1	4.6	1-Chlorododecane	15	5.1
Octadecane	1	4.4	1-Chlorodecane	16	5.4
Hexadecane	1	3.4	1-Phenyldodecane	10	5.7
Tetradecane	1	4.1	Hexadecanol	2	4.2
Dodecane	1	4.3	Tetradecanol	2	4.1
Decane	3	4.7	Trypticase soy broth		7.2
Nonane	6	4.3			

[a] Time between inoculation and turbidity increase to 30 Klett units.

TABLE 2. *Percentage composition of polar lipid fatty acids from the oxidation of n-alkanes*

Fatty acid	Hydrocarbon substrate					
	Eicosane	Octadecane	Hexadecane	Tetradecane	Dodecane	Decane
10:0[a]	1.9			0.4	0.5	1.4
12:0	2.5	0.3	1.2	0.7	0.8	2.3
12:1					7.1	
14:0	9.6	6.9	6.5	47.2	41.2	2.8
14:1				0.6	1.4	
15:0	0.6		1.3			
16:0	29.9	36.1	50.7	29.1	19.2	61.4
16:1	14.0	14.8	35.5	9.0	10.0	5.4
18:0	2.3	Tr[b]	1.6	0.7	2.7	6.2
18:1	38.2	40.4	2.9	11.4	17.1	20.6
20:1	Tr	0.7				

[a] Chain length: number of double bonds.
[b] Tr, Trace.

TABLE 3. *Percentage composition of polar lipid fatty acids from the oxidation of substrates resulting in substantial quantities of odd-chain fatty acids*

Fatty Acid	Carbon substrate					
	Dotria-contane	Nonane	1-Chlorohexa-decane	1-Chlorodo-decane	1-Chloro-decane	1-Phenyldo-decane
10:0			0.7			Tr[a]
11:0						0.6
12:0		0.9	0.8	1.1	0.6	0.7
13:0		Tr	9.0	0.5		0.7
14:0	3.1	3.0	26.6	6.5	2.5	3.0
15:0	2.4	2.6	Tr	1.1	50.0	33.1
16:0	10.4	36.6	24.2	18.5	6.8	7.6
16:1	19.4	Tr	12.1	23.9	18.2	23.2
17:0	Tr	15.5		23.4		20.9
17:1	36.3	12.9		Tr	0.3	Tr
18:0	Tr	Tr		Tr		
18:1	28.3	28.2	16.1	25.0	21.6	5.0
Unknown			10.5			
19:1						5.3

[a] Tr, Trace.

novo fatty acid synthesis.

Interestingly, nonane was not the only substrate resulting in fatty acids of odd-numbered carbons. Cells grown on dotriacontane, chlorohexadecane, chlorododecane, chlorodecane, and phenyldodecane also produced large quantities of odd-chain fatty acids (Table 3) compared with cells grown on the substrates shown in Table 2. Present in the complex lipids of cells grown on dotriacontane were the fatty acids

17:1 (36.3%) and 15:0 (2.4%). Even-chain acids comprised the remaining constituents. The R_u/R_s was calculated to be 5.25.

All chloro- and phenyl- derivatives resulted in cells containing acids with retention times equivalent to those of odd-chain fatty acids. Cells resulting from the oxidation of 1-chlorohexadecane were characterized by the presence of 13:0 (9.0%), a trace of 15:0, and an unidentified fatty acid (10.5%) with a retention time greater than that of 18:1 and less than that of 19:0. Analysis of 1-chlorododecane- and 1-phenyldodecane-cultured cells demonstrated large quantities of 17:0 (23.4% and 20.9%, respectively) and various other odd and even fatty acids. Cells grown on 1-chlorodecane produced a similar fatty acid profile but contained no 17:0 while showing evidence of large quantities of 15:0 (15.0%).

Several long-chain alcohols served as substrates, but only two cultures were examined for their fatty acid contents (Table 4). Hexadecanol-grown cells were characterized predominantly by 16:0 (55.9%) and 16:1 (34.9%), much as when hexadecane served as substrate. The oxidation of tetradecanol revealed a profile similar but not identical to that of tetradecane, exhibiting large amounts of 16:0, 16:1, and 14:0.

For comparative purposes, cells cultivated on Trypticase soy broth were analyzed. The major component demonstrated was 18:1 (48.4%), which was present in greater amounts than found in the complex lipids of any hydrocarbon-grown cells (Table 4).

An interesting relationship often exists between the total quantities of unsaturated versus saturated lipids. It is well documented that environmental factors such as temperature can alter the relative amount of saturation in the fatty acids of microorganisms (4, 16). Another variable is the carbon source, as is the case with this hydrocarbon-utilizing bacterium. The lowest amount of unsaturation appears to be expressed when tetradecane serves as substrate (Table 5). As the length of the alkane is increased, the degree of unsaturation generally rises, reaching a maximum with the largest-molecular-weight hydrocarbon tested, dotriacontane. The only culture condition which surpassed dotriacontane in content of unsaturated fatty acids was Trypticase soy broth. However, this undefined medium may have contributed fatty acids which were incorporated directly from the external pools. All other carbon sources exhibited ratios of less than 1.00 with no readily discernable pattern.

TABLE 4. *Percentage composition of fatty acids from long-chain alcohols and Trypticase soy broth*

Fatty acid	Carbon substrate		
	Hexadecanol	Tetradecanol	Trypticase soy broth
10:0		1.5	
12:0		1.8	1.7
12:1			
14:0	2.6	34.4	0.6
14:1		3.3	
16:0	55.9	22.5	13.4
16:1	34.9	20.6	36.2
18:0	1.2		Tr[a]
18:1	5.3	14.5	48.4

[a] Tr, Trace.

TABLE 5. *Ratio of unsaturated to saturated fatty acids from the oxidation of various substrates*

Substrate	R_u/R_s[a]	Substrate	R_u/R_s
Dotriacontane	5.25	1-Chlorohexadecane	0.39
Eicosane	1.09	1-Chlorododecane	0.96
Octadecane	1.27	1-Chlorodecane	0.66
Hexadecane	0.62	1-Phenyldodecane	0.50
Tetradecane	0.27	Hexadecanol	0.67
Dodecane	0.55	Tetradecanol	0.66
Decane	0.35	Trypticase soy broth	5.50
Nonane	0.70		

[a] R_u/R_s, Ratio of unsaturated to saturated acids.

DISCUSSION

The isolate was capable of growth on a variety of hydrocarbons and related compounds. Carbohydrates and the majority of amino acids studied did not serve as a sole carbon source. Although all the compounds listed in Table 1 served as adequate growth substrates, considerable variation existed among the lag periods preceding log growth. Long induction periods appeared to be necessary for the extremely long- and short-chain alkanes as well as the majority of the chloro- and phenyl- derivatives.

A number of bacteria capable of hydrocarbon oxidation have been examined with respect to their fatty acid content. Generally, investigators use total lipid extracts for these analyses, whereas in this study only polar lipids were utilized. Polar lipids comprise a somewhat more representative view of incorporated cellular lipids, since storage compounds and initial degradative products are eliminated. This is particularly significant with hydrocarbon substrates, since large quantities of initially derived free fatty acids and alcohols may exist within the cells.

The major fatty acids in the polar lipid fractions of this organism were 14:0, 16:0, 16:1, and 18:1. Significant incorporation of fatty

acids reflecting the parent substrate chain length occurred when cells were grown on the alkanes octadecane, hexadecane, and tetradecane. Cells cultured on the odd-carbon alkane nonane revealed polar lipids enriched in odd-chain fatty acids, indicative of synthesis or elongation of carbon chains without prior degradation to acetate, which has been suggested previously (6, 14).

Observation of fatty acids from the oxidations of long-chain alcohols resulted in patterns similar to those of the corresponding-chain-length alkanes. Thus, alcohols apparently are physiologically analogous to their equivalent but more reduced parent hydrocarbon. Any minor differences in fatty acid content might be explained as an alteration in environmental conditions, i.e., addition of the hydroxyl function, which could elicit physiological changes within the organism.

An unexpected finding was the presence of odd-chain fatty acids after the oxidation of dotriacontane and the chloro- and phenyl-derivatives. It has been suggested that the rate-limiting step during the oxidation of extremely long-chain paraffins is the net oxidation of hydrocarbon to terminal alcohol (11). Thus, using this assumption, it is predicted that as soon as long-chain alcohols are produced, they are further oxidized to the corresponding acid and readily degraded via beta-oxidation. The lack of these long-chain oxidative products from extracts of microorganisms grown on such paraffins might then be explained in terms of the relative conversion rates of hydrocarbon metabolites. However, the presence of odd-chain fatty acids in large quantities can not be explained by this proposed scheme when the substrate is an even-chain hydrocarbon such as dotriacontane.

To explain the incorporation of odd fatty acids into the polar lipid fraction of this isolate after oxidation of dotriacontane, another degradative pathway must be proposed. Forney et al. (8) have presented evidence for metabolism of alkanes by means of subterminal oxidation. Knowing that various ketones and secondary alcohols have been demonstrated as metabolites of hydrocarbon oxidation, a scheme was suggested by which alkanes could be oxidized through a ketone and then ester, proceeded by cleavage to the alcohol and acid (9, 13). Such a pathway could account for odd-chain fatty acids from the utilization of dotriacontane if an ester bond were formed with odd-chain acid and alcohol constituents. However, no direct evidence was obtained to document this.

Chloro- and phenyl- derivatives of alkanes also resulted in cells which exhibited large amounts of odd-chain fatty acids. These data lead us to speculate, as was the case with dotriacontane, that some form of cleavage mechanism is present in which the substituted group is removed together with an odd-number carbon fragment, thereby resulting in the incorporation of odd-chain fatty acids.

The results of this study have led to the conclusion that this hydrocarbon-utilizing bacterium is capable of incorporating initially derived fatty acids from substrates directly into lipid material. This is true not only for total lipids, but for phospholipids as well. The data also provide us with a means to speculate on the mechanisms involved in the oxidation of long-chain alkanes and of terminally substituted chloro- and phenyl- derivatives.

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