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Lipids of *Pseudomonas aeruginosa* Cells Grown on Hydrocarbons and on Trypticase Soy Broth[1]

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Lipids were extracted from cells of *Pseudomonas aeruginosa* grown on a pure hydrocarbon (tridecane), mixed hydrocarbons (JP-4 jet fuel), and on Trypticase Soy Broth. Total lipids produced from each substrate represented from 7.1 to 8.2% of cellular dry weight, of which 5.0 to 6.4% were obtained before cellular hydrolysis (free lipids) and 1.7 to 2.0% were extracted after cellular hydrolysis (bound lipids). Free lipids from cells grown on each medium were separated into four fractions by thin-layer chromatography. All fractions were present in cells from each type of medium, and the "neutral fraction" constituted the largest fraction. The fatty acid composition of free lipids was determined by gas-liquid chromatography. Cells grown on each medium contained saturated and unsaturated C_{14} to C_{20} fatty acids. Trace amounts of C_{13} fatty acids were found in tridecane-grown cells. Saturated C_{16} and C_{18} were the major acids present in all cells. Quantitative differences were found in fatty acids produced on the three media, but specific correlations between substrate carbon sources and fatty acid content of cells were not evident. Tridecane-grown cells contained only traces of C_{13} acid and small amounts of C_{15} and C_{17} acids, suggesting that the organism's fatty acids were derived from de novo synthesis rather than by direct incorporation of the hydrocarbon.

The distribution of fatty acids in cellular lipids in *Sarcina lutea* (10, 27), *Rhodomicrobium vannielii* (21, 22), *Bacillus* species (12, 13, 14), and many other species of microorganisms has been investigated extensively. However, most studies of the composition of microbial lipids were conducted with cells grown on synthetic media. Relatively little information is available on the lipid composition of cells grown on hydrocarbons.

Raymond and Davis (23) observed a marked increase in the lipid content of a *Nocardia* species grown on *n*-alkanes. Cells grown on *n*-hexadecane and on *n*-octadecane contained about 70% lipid material. In contrast, cells grown on *n*-hexane and *n*-tridecane contained about the same amount of lipids as glucose-grown cells. Davis (3) grew a *Nocardia* species on *n*-alkanes with 13 to 20 carbon atoms; he found fatty acids of cellular triglycerides and aliphatic waxes with carbon skeletons analogous to the carbon skeleton of the *n*-alkane substrate on which the organism was grown. Similar findings were observed in cellular lipids of a *Mycobacterium* grown on *n*-alkanes with 13 to 17 carbon atoms (4). However, other organisms show only a partial correlation between substrate chain length and chain length of cellular fatty acids (7, 8, 17). A *Mycobacterium* species did not show a correlation when grown on C_{11} or C_{12} alkanes, but it did show a correlation when longer-chain alkanes were the substrate (5).

This report compares the cellular lipids and fatty acids of *Pseudomonas aeruginosa* grown on mixed hydrocarbons (JP-4 jet fuel), on a pure hydrocarbon (*n*-tridecane), and on a nonhydrocarbon medium [Trypticase Soy Broth (TSB)].

MATERIALS AND METHODS

Organism. A hydrocarbon-utilizing strain of *P. aeruginosa* was used in all experiments. This organism was isolated from a JP-4 jet fuel-water sample (6) and has been maintained in a hydrocarbon-water medium by periodic subculture for more than 4 years.

Chemicals. JP-4 jet fuel was obtained from the Aero Propulsion Laboratory, Wright-Patterson Air Force Base, Ohio; 2,2 dimethoxypropane (practical) was obtained from Matheson, Coleman and Bell, Norwood, Ohio; and *n*-hexane and *n*-tridecane were obtained from Phillips Petroleum Co., Bartlesville, Okla. Tripalmitin, α-hydroxystearic acid, and DL-α-lecithin were obtained from Calbiochem, Los Angeles, Calif. Octadecane was a gift of the Monsanto Research Corp., Dayton, Ohio.

[1] This paper represents part of a thesis submitted by the senior author to the Graduate School, University of Dayton, in partial fulfillment of the requirements for the M.S. degree.

Culture systems. Three media were used: (i) a mineral salts solution (2) overlaid with mixed hydrocarbons (JP-4 jet fuel), (ii) a mineral salts solution overlaid with a pure hydrocarbon (*n*-tridecane), and (iii) TSB (BBL) prepared in the mineral salts solution. Five-liter round-bottom flasks were used as culture vessels. Each vessel was closed with a neoprene stopper, through which three glass tubes were inserted: one was used for aerating the culture, one was used for sampling, and one was used as an exhaust port. Four liters of the appropriate aqueous medium was added to each vessel, the *p*H was adjusted to 7.0, and each vessel was sterilized by autoclaving at 121 C for 1 hr. For hydrocarbon media, the salts solution was cooled to room temperature and overlaid with 5% (v/v) filter-sterilized JP-4 jet fuel or *n*-tridecane.

Culture procedures. The test organism was transferred to 4-oz (118 ml) bottles containing 30 ml of: (i) mineral salts-JP-4, (ii) mineral salts-tridecane, or (iii) TSB. Subsequently, three separate stocks were maintained on these media by periodic subculture. Cultures in the hydrocarbon media were incubated for 4 days, and cultures in TSB were incubated for 2 days at 37 C under static conditions. Samples from the aqueous phase of each culture were adjusted to 85% transmission with a Spectronic-20 colorimeter set at a wavelength of 420 nm. Then each 4-liter batch of medium was inoculated with 10 ml of the appropriate standardized suspension. All cultures were incubated in a water bath at 37 C and aerated with filter-sterilized air at a rate of 0.35 ft^3 per hr per liter.

Each culture was sampled immediately after inoculation and at appropriate intervals thereafter. The *p*H of each sample was determined, a smear was prepared and Gram-stained to check for contamination, and a viable cell count was made by plating appropriate dilutions on Trypticase Soy Agar.

Cells were harvested by continuous flow centrifugation at 27,000 × *g*. Each culture was passed through the continuous flow unit twice, and then the cells were washed three times in saline. Cell mass was determined by placing 5.0-ml samples of the washed suspension on tared planchets which were then dried to constant weight at 100 to 110 C.

Lipid extraction. Dried cells were ground with a mortar and pestle to a fine powder and were extracted with acetone and chloroform-methanol as described by Huston and Albro (10). The extracts were combined and evaporated to dryness under reduced pressure in a rotary evaporator. The residue was dissolved in a known volume of chloroform. The yield of free lipids was estimated by drying aliquot samples to constant weight.

The cell residue was refluxed for 2 hr with 2 N KOH. The extract was acidified and then extracted with cholroform to obtain "bound lipids."

Thin-layer chromatography (TLC). Crude lipids were fractionated by a combination of TLC procedures (18, 19, 26). TLC separations were made on glass plates (20 by 20 cm) coated with a 250- to 275-μm layer of Silica Gel G. Plates were activated at 100 to 110 C for 1 hr before use, and were developed in unlined chambers with *n*-hexane-diethyl ether-glacial acetic acid (90:10:7.5, v/v) as solvent. Lipids were detected by exposing plates to iodine vapors for 5 min and outlining the spots with a sharp instrument. Several plates were developed so that ninhydrin, rhodamine B, or 2,7-dichlorofluorescein could be applied to separate plates after spots were located with iodine. Sprayed plates were viewed under ultraviolet light. In some cases, plates were sprayed with aqueous H_2SO_4 and charred at 100 to 110 C for 1 hr.

R_F values of all components were compared with R_F values of reference compounds separated on the same plate. Standards, chosen to represent different classes of lipids, were octadecane, tripalmitin, α-hydroxystearic acid, and DL-α lecithin.

Relative amounts of the fractions resolved were determined by photodensitometry with a densitometer, model 501 A, and a light source unit, model 52-C, equipped with a mechanical stage (Photovolt Corp., New York, N.Y.).

Gas-liquid chromatography (GLC). Methyl esters of the extracts were prepared as described by Huston and Albro (10). Esters from each extract were evaporated to dryness in a rotary evaporator and dissolved in a known volume of *n*-hexane for GLC. After each experiment, *n*-hexane was evaporated with nitrogen, and methyl esters were stored at 10 C under nitrogen.

Methyl esters were chromatographed on a gas chromatograph equipped with a dual flame ionization detector (model 700; F & M Scientific Corp., Avondale, Pa.). A recorder with disc integrator was employed to measure the area under each peak. A stainless-steel column [6 ft by $\frac{1}{8}$ inch (182.8 by 0.32 cm)] packed with 10% diethylene glycol succinate (DEGS) on Gas Chrom W was used for separations. The carrier gas was helium at a flow rate of 50 to 60 ml/min. Column temperature was maintained at 140 C. Temperatures of injection port and detector were 260 and 255 C, respectively.

Esters were also separated on a nonpolar column with a gas chromatograph (Varian Aerograph, series 1200) equipped with a single flame ionization detector. Separations were made on a stainless-steel column [6 ft by $\frac{1}{8}$ inch (182.8 by 0.32 cm)] packed with 5% silicone rubber (SE-30) on Gas Chrom W. The carrier gas was helium at a flow rate of 30 ml/min, and column temperature was maintained at 170 C. Temperatures of injection port and detector were 310 and 230 C, respectively.

Sample sizes varied from 1 to 10 μliters, and column efficiency and detector response were determined with National Institutes of Health reference mixture (NIH-D) as described by Horning et al. (9). Peak identifications were made by co-chromatography with single methyl ester reference standards (Hormel Institute, University of Minnesota, Austin). Mono-unsaturated methyl esters were differentiated from saturated methyl esters after bromination (11) and by noting their reverse positions when separated on a nonpolar column (SE-30). Esters present in each extract were quantitated by calculating the area

under individual peaks as a percentage of total peak area.

RESULTS

P. aeruginosa grew well in each medium. Growth curves from a typical experiment are shown in Fig. 1. Initial populations ranged from 2.0×10^5 to 2.0×10^6 cells/ml. TSB cultures had a maximal population of approximately 5.0×10^9 cells/ml after 72 hr of incubation, but JP-4 and tridecane cultures developed more slowly, reaching populations of 5.0×10^8 and 10^9 cells/ml, respectively, after 120 hr of incubation. Cells were harvested at 96 or 120 hr of incubation, but in each experiment analyses were performed on cells of the same age from the three media.

During the growth period, the *p*H of each culture increased from 6.9 to 7.1 (JP-4), 6.9 to 7.2 (tridecane), and 6.8 to 7.9 (TSB), indicating that acids did not accumulate in the culture systems.

The average total lipid content of cells (per cent dry weight), as shown in Table 1, was 7.95 (JP-4), 7.08 (tridecane), and 8.22 (TSB). Variations were noted, but the trend of the data was the same in five experiments. Cells from each medium contained more extractable (free) lipids than non-

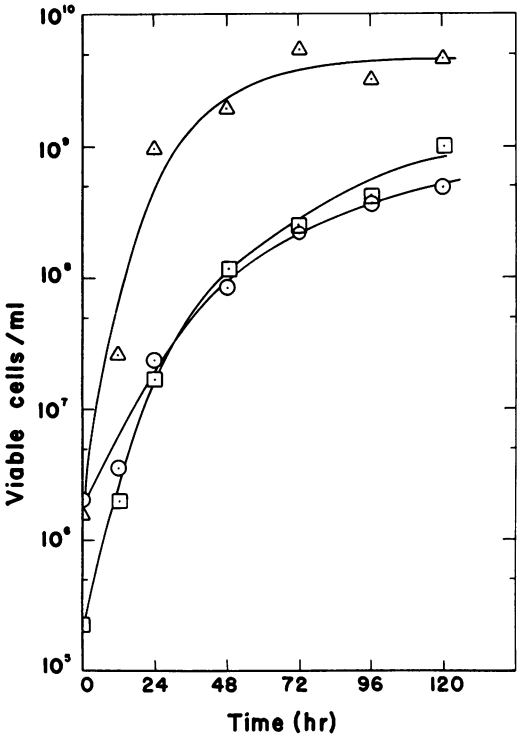

FIG. 1. *Growth of P. aeruginosa in a mineral salts solution with JP-4 jet fuel* (⊙), *in a mineral salts solution with tridecane* (⊡), *and in TSB* (△).

TABLE 1. *Lipid composition of P. aeruginosa cells grown in TSB or in hydrocarbon media*

Medium	Percentage of cellular dry weight (mean ± SD)[a]		
	Free lipids	Bound lipids	Total lipids
JP-4	6.04 ± 2.41	1.91 ± 0.60	7.95 ± 2.35
Tridecane	5.01 ± 1.36	2.07 ± 0.56	7.08 ± 0.82
TSB	6.42 ± 3.22	1.80 ± 0.65	8.22 ± 2.03

[a] Data indicate average values from five experiments.

extractable (bound) lipids. Free lipids represented 75.5, 70.7, and 78.1% of total lipid content of cells from JP-4, tridecane, and TSB, respectively. Only free lipids were analyzed further.

TLC of free lipid extracts resolved four fractions (Table 2). Based on comparisons with reference compounds, the fractions were termed "polar fraction," "acid fraction," "neutral fraction," and "hydrocarbon fraction," respectively. The polar fraction contained ninhydrin-positive components. Extracts of cells from each medium contained all four fractions. Based on densitometric tracings, the neutral fraction was the largest component in each case, followed by, in decreasing order, hydrocarbon, acid, and polar fractions. Further characterization of the various fractions was not undertaken.

Fatty acid profiles of free lipids from cells grown on three media are shown in Fig. 2 to 4. The C_{12} methyl ester was added to esters from each extract as an internal standard. Each extract contained a spectrum of C_{14} to C_{20} methyl esters and two unidentified components (A and B). Tridecane-grown cells yielded a trace of C_{13} methyl ester. Temperature-programmed determinations and a number of isothermal determinations were made, but esters shorter than C_{13} or longer than C_{20} were not detected. Methyl esters were not observed when JP-4 fuel was chromatographed. Both saturated and unsaturated components were present in all cells. The presence of unsaturated methyl esters was confirmed by peak reduction in chromatograms of brominated materials. A chromatogram of brominated esters from JP-4-grown cells is shown in Fig. 5. This chromatogram also shows the presence of several small peaks that were absent in Fig. 2 to 4. These peaks represent components that were present in minute quantities and were visible only when large samples were analyzed. Peaks representing the unidentified components (A and B) were not present in chromatograms of brominated materials.

Only straight-chain saturated and unsaturated

TABLE 2. *Chromatographic separation of free lipids from cells of P. aeruginosa*

TLC fraction	R_F from cells grown in			Reference compound	R_F of reference compound	Designation of fraction
	JP-4	Tridecane	TSB			
1	0.0 -0.15	0.0 -0.15	0.0 -0.15	DL-α-Lecithin	0.0	Polar fraction
2	0.21-0.47	0.20-0.47	0.21-0.47	α-Hydroxystearic acid	0.25	Acid fraction
3	0.84	0.84	0.79	Tripalmitin	0.80	Neutral fraction
4	1.00	1.00	1.00	Octadecane	1.00	Hydrocarbon fraction

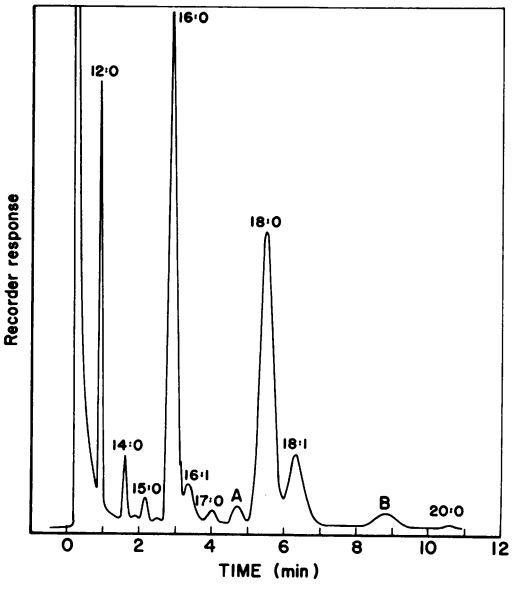

FIG. 2. *Fatty acid profile of free lipids from JP-4-grown cells of P. aeruginosa. Numbers refer to number of carbon atoms in acid: number of double bonds. The 12:0 peak represents an internal standard. Chromatographed on 10% DEGS.*

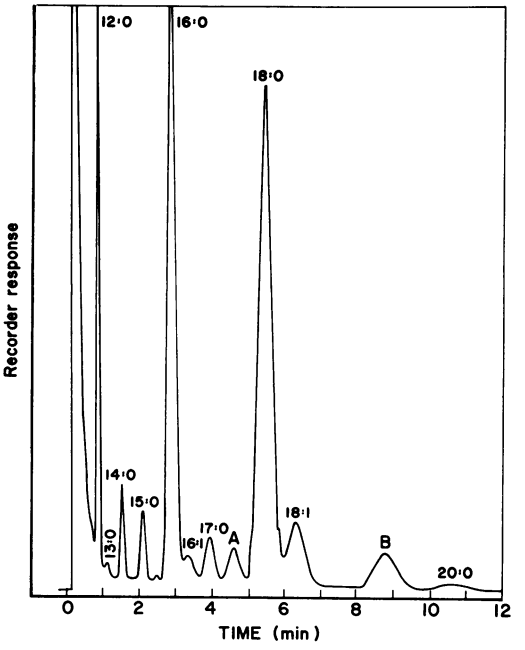

FIG. 3. *Fatty acid profile of free lipids from tridecane-grown cells of P. aeruginosa. Numbers refer to number of carbon atoms in acid: number of double bonds. The 12:0 peak represents an internal standard. Chromatographed on 10% DEGS.*

methyl esters were detected. Unsaturated methyl esters from cells grown on each medium were $C_{16:1}$, $C_{18:1}$, and components A and B.

The fatty acid composition of cells is shown in Table 3. Saturated C_{16} and C_{18} were the major components in cells from each medium. The predominant unsaturated acid present in JP-4- and tridecane-grown cells was $C_{18:1}$, but TSB-grown cells contained both $C_{18:1}$ and component B as major unsaturated acids. Tridecane-grown cells contained more C_{15} and C_{17} esters than cells grown on the other two media. Cells grown in TSB, JP-4, and tridecane contained 24.64, 20.02, and 16.02% unsaturated acids, respectively.

DISCUSSION

JP-4 fuel may contain 5,000 to 10,000 different hydrocarbons, but paraffins and cycloparaffins comprise 75 to 90% of the fuel. In general, microorganisms attack long-chain normal alkanes more readily than other hydrocarbons (25).

TSB supported more rapid growth and higher cell yield than either hydrocarbon medium. Cultures in TSB were in the maximal stationary phase after 72 hr, whereas viable counts were still increasing in hydrocarbon cultures between 96 and 120 hr. The *p*H of all cultures gradually increased, to 7.1 (JP-4), 7.2 (tridecane), and 7.9 (TSB), which indicated that acids did not accumulate in the systems or were masked by other products. These findings are not unusual, because *P. aeruginosa* is primarily an oxidative organism. The slightly alkaline *p*H in each cul-

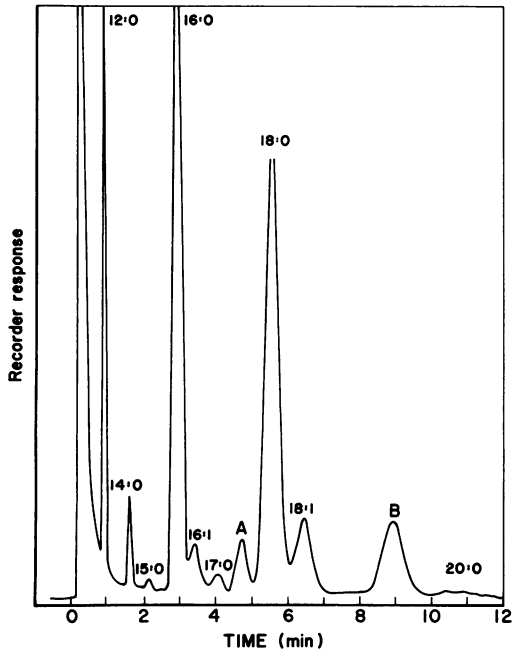

FIG. 4. *Fatty acid profile of free lipids in TSB-grown cells of P. aeruginosa. Numbers refer to number of carbon atoms in acid: number of double bonds. The 12:0 peak represents an internal standard. Chromatographed on 10% DEGS.*

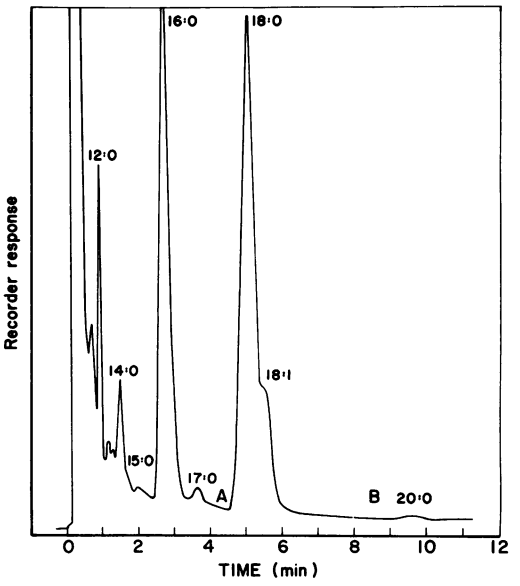

FIG. 5. *Fatty acid profile of free lipids in JP-4-grown cells of P. aeruginosa after bromination. Numbers refer to number of carbon atoms in acid: number of double bonds. The 12:0 peak represents an internal standard. Chromatographed on 10% DEGS.*

ture at the time of harvest suggests that basic products were formed during growth. Cell-free filtrates were not examined for lipid materials. Romero and Brenner (24) grew *P. aeruginosa* on hexadecane and found significant quantities of saturated fatty acids and large quantities of cellular lipids in the cell-free filtrates.

JP-4-, tridecane-, and TSB-grown cells contained total lipid materials that averaged 7.9, 7.1, and 8.2% of cellular dry weight, respectively. Inspection of the data from five experiments (Table 1) indicated that these differences were not statistically significant. Most bacteria contain between 1.0 and 10% lipid material on a dry weight basis. However, values higher than 20% of cellular dry weight have been found in both gram-positive and gram-negative species (1). In addition, age, *p*H, and culture medium may greatly influence the quantitative and qualitative composition of bacterial lipids (1). In the present work, growth on hydrocarbons did not increase the lipid content of *P. aeruginosa* over that of cells grown on TSB. Cells grown on each medium contained more free than bound lipids.

Chromatographic fractionation (Table 2) and estimation of the quantity of each fraction suggested that the free lipids were similar in cells from all three media. However, the techniques used were semiquantitative and would not reveal differences within fractions.

Fatty acids with carbon chains shorter than

TABLE 3. *Fatty acid composition of free lipids of P. aeruginosa cells grown in TSB or in hydrocarbon media*[a]

Fatty acid	Medium		
	JP-4	Tridecane	TSB
13:0[b]		Trace	
14:0	2.64	2.56	2.25
15:0	1.13	2.37	0.32
16:0	38.39	32.62	36.39
16:1	3.75	1.52	3.65
17:0	0.49	2.34	0.46
A	1.90	2.32	3.14
18:0	37.29	44.04	35.89
18:1	11.19	7.37	8.00
B	3.18	4.81	9.85
20:0	Trace	Trace	Trace

[a] Calculated as percentage of total peak area obtained from a diethylene glycol succinate column.

[b] Numbers represent number of carbon atoms in acid: number of double bonds.

C_{13} or longer than C_{20} were not detected in the free lipids, and all cells contained both saturated and unsaturated acids. No attempt was made to identify minor peaks, which may represent hydroxy- or branched-chain fatty acids. Saturated C_{16} and C_{18} acids were the major components present in cells grown on each medium. Unsaturated $C_{16:1}$ and $C_{18:1}$ and unsaturated components A and B were present in all cells. Romero and Brenner (24) found large quantities of C_{16} and C_{18} saturated and unsaturated acids in cells of *P. aeruginosa* grown on hexadecane. Although qualitatively the fatty acid composition of cells from the three media was the same, quantitative differences were found (Table 3). Quantitative comparison with National Heart Institute Fatty Acid Standard D (9) agreed with the stated composition data, with a relative error of less than 11% for major components (> 10% of total mixture) and less than 9% for minor components (< 10% of total mixture).

Certain *Candida*, *Nocardia*, and *Mycobacterium* species oxidize hydrocarbons by monoterminal or diterminal mechanisms with no change in the carbon skeleton (3, 4, 7, 14, 15). The predominant fatty acids and alcohols produced from such oxidations have chain lengths analogous to the chain length of the hydrocarbon in the medium. In contrast, *P. aeruginosa* oxidizes hydrocarbons nonspecifically, yielding products of mixed types (7, 8). *Micrococcus cerificans* shows a correlation between chain length of even-carbon alkane substrates and chain length of even-carbon fatty acids. When grown on odd-chain alkanes (C_{11} to C_{17}), cells contained both odd- and even-chained fatty acids (17). A *Mycobacterium* species contained significant amounts of C_{15} acid when grown on odd-chain alkanes but not when grown on even-chain alkanes (4). This correlation was not observed when C_{11} and C_{12} alkanes were the substrates (5). In the same study, a different *Mycobacterium* species and a *Brevibacterium* species yielded high levels of C_{13} and C_{17} fatty acids when the corresponding alkanes were the substrates. Therefore, whether an organism oxidizes an alkane directly or degrades it is species-dependent and may also be influenced by the chain length of the alkane.

Although odd-chain fatty acids increased in cells from media containing odd-chain hydrocarbons (JP-4 and tridecane), these acids were not obtained in large quantities, even from the homologous substrate (tridecane). C_{16} and C_{18} acids were the predominant acids in tridecane-grown cells; only a trace of C_{13} acid was found. Therefore, these data suggest that when grown on tridecane this strain of *P. aeruginosa* oxidized the hydrocarbon principally by nonspecific mechanisms and that the organism's fatty acids were derived chiefly from de novo synthesis rather than from direct incorporation of a hydrocarbon substrate.

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