JOURNAL OF BACTERIOLOGY, Jan., 1966
Vol. 91, No. 1

Fatty Acids Synthesized from Hexadecane by *Pseudomonas aeruginosa*

ETHEL M. ROMERO AND RODOLFO R. BRENNER

Instituto de Fisiología, Facultad de Ciencias Médicas, Universidad Nacional de la Plata, La Plata, Argentina

Received for publication 21 August 1965

ABSTRACT

ROMERO, ETHEL M. (Universidad Nacional de la Plata, La Plata, Argentina), AND RODOLFO M. BRENNER. Fatty acids synthesized from hexadecane by *Pseudomonas aeruginosa*. J. Bacteriol. 91:183–188. 1966.—The lipids extracted from *Pseudomonas aeruginosa* incubated with hexadecane in a mineral medium were separated into a nonpolar and three polar fractions by thin-layer chromatography. The fatty acid composition of the four cellular fractions and that of the lipids excreted into the medium was studied by gas-liquid chromatography. Saturated fatty acids with 14 to 22 carbons were recognized, together with monoenoic, dienoic, and hydroxylated acids. Hydroxylated fatty acids were principally found in two polar fractions containing rhamnose and glucose; the other polar fraction, containing serine, alanine, ethanolamine, and leucine, was richer in monoenoic fatty acids. Octadecadienoic acid was found in the neutral fraction.

From the beginning of this century, many investigators have shown that many bacteria, including *Pseudomonas aeruginosa*, are able to grow in a mineral medium containing hydrocarbons (32). From a petroleum-contaminated soil, Solari et al. (27) isolated a microorganism identified as *P. aeruginosa*. Linday and Donald (18), working with *Pseudomonas* and *Nocardia*, proved the synthesis of myristic, palmitic, and stearic acids and the excretion of waxes. All of these substances were derived from octadecane and docosane. The metabolism of hexadecane by the strain of *P. aeruginosa* isolated by Solari, and the fatty acid composition of the cellular lipids and fatty acids excreted into the medium, are reported in this work.

MATERIALS AND METHODS

Organisms. The strain of *P. aeruginosa* studied was isolated and kindly furnished by A. A. Solari (Facultad de Química y Farmacia, Universidad Nacional de la Plata). The *n*-hexadecane (provided by YPF, Florencio Varela, Argentina) was 99.9% pure, estimated by gas-liquid chromatography.

Preparation of the cells for lipid extraction. P. aeruginosa was grown on agar slants for 24 hr at 37 C; a suspension was then made in 10 ml of water and transferred to 250 ml of sterile mineral medium (*p*H 7.0) containing 1% *n*-hexadecane. The composition of the mineral medium was: K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $NH_4H_2PO_4$, 1 g; NaCl, 5 g; and distilled water, 1,000 ml. The mineral medium and the hexadecane were sterilized separately. The bacteria were incubated at 32 C in a rotary shaker (23) at 250 rev/min for 48 hr, and the growth was controlled by opacimetry (530 mμ), *p*H estimation, and microscopic observation. This growth was then inoculated into 2,750 ml of the same mineral medium with 1% *n*-hexadecane, and was incubated at *p*H 7.1 and 31 C, in a 5-liter New Brunswick (model F-05) fermentor, under a stream of air (2 liters/min) and with an agitation of 1,000 rev/min. The growth was followed, as before, by opacimetry, *p*H estimation, and microscopic observation (Fig. 1).

After 4 days, the cells were harvested by centrifugation at 4,000 rev/min for 45 min, and the supernatant liquid was stored in a freezer under nitrogen for further studies.

Cellular lipids. The lipids of the bacteria were extracted with chloroform-methanol (2:1) by the method of Folch et al. (10). Samples were transmethanolized with 3 N HCl-methanol and sublimated (29). Fatty acid esters were analyzed by gas-liquid chromatography in a Pye apparatus with an argon ionization detector. A column heated at 195 C and filled with 10% polyethylene glycol adipate on chromosorb W (100 to 120 mesh) was used.

Fractionation of the cellular lipids. The cellular lipids were applied to thin-layer chromatographic microplates (6 by 6 cm) of silica gel G (Merck), 250 μ thick. The chromatogram was developed in petroleum (60 to 80 carbons)-diethyl ether-acetic acid (90:10:1; v/v). Two spots, detected with iodine vapors, presented the same R_F values (0.47 and 0.20) as triglycerides and free fatty acids, respectively. A third spot, similarly detected, did not move from the origin, and represented most probably a polar lipid.

The polar lipids were easily separated by thin-layer

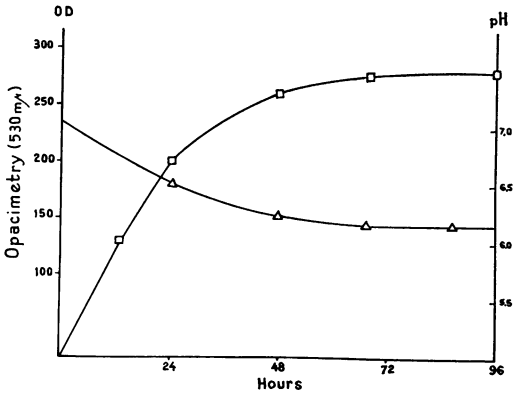

FIG. 1. *Growth curve of Pseudomonas aeruginosa determined by opacimetry (530 mμ) and pH variations.*

chromatography on the same microplates with chloroform-methanol-water (62:25:4, v/v) as the developing solvent. Three spots were stained by iodine vapor. The top one (F_3) was ninhydrin-positive, and had the same R_F (0.72) as phosphatidyl ethanolamine. The central spot (F_2) was ninhydrin- and choline-positive (26), and had the same R_F (0.52) as phosphatidyl choline; the lowest one (F_1) (R_F, 0.38) was also ninhydrin-positive.

From this first approach, it was decided that polar lipids would be separated on silica gel G macroplates (20 by 20 cm), 500 μ thick, by using chloroform-methanol-water (65:25:4, v/v) as in the first assay. The positions of the spots were recognized by staining only the edges of the plates with iodine and ninhydrin. The corresponding central parts of the plates were scraped off and extracted with methanol acidified with HCl. The three polar fractions, F_1, F_2, and F_3, and a nonpolar one that ran with the solvent front were collected. The fatty acid composition of the nonpolar lipids (triglycerides) was estimated by gas-liquid chromatography after direct transmethanolysis with HCl-methanol. Lipids of fractions F_1, F_2, and F_3 were saponified with 2.5 N KOH. The fatty acids were esterified with 3 N HCl-methanol. The water-soluble components that separated from the fatty acids were stored for subsequent analysis. The fatty acid composition of F_1, F_2, and F_3 was determined by gas-liquid chromatography (Table 1).

Analysis of water-soluble hydrolysate. The aqueous phases separated after saponification of F_1, F_2, and F_3 were hydrolyzed with HCl and investigated for the presence of primary amines, carbohydrates, and phosphorus.

Amino acids and primary amines from lipid hydrolysates were identified by bidimensional paper chromatography on Whatman no. 1 filter paper. A Shandon apparatus was used. The papers were developed in 1-butanol-acetic acid-water (4:1:5; v/v) and phenol-water (8:2; v/v). The spots were located by spraying with ninhydrin, and the amino bases were identified by comparing them with their corresponding standards.

Carbohydrates were identified by the method of

TABLE 1. *Fatty acid composition (g 100 g) of methyl esters of triglycerides and polar lipids fractionated by thin-layer chromotography from Pseudomonas aeruginosa*

Fatty acid*	Triglycerides	Polar fractions			Equivalent chain length
		F_1 (R_F = 0.38)	F_2 (R_F = 0.52)	F_3 R_F = 0.72)	
14:0	4.4	3.7	5.1	5.5	14
14:1	0.2	Traces	4.0	2.8	14.20
15:0	0.2	Traces	—	1.9	15
15:1	—	Traces	3.9	—	15.20
9:0h	1.4	—	—	—	15.60
16:0	22.3	23.0	11.3	28.3	16.0
16:1	6.7	2.1	6.9	3.6	16.20
10:0h	—	—	—	Traces	16.60
17:0	Traces	Traces	R	0.6	17.00
17:1	—	Traces	6.3	Traces	17.20
11:0h	2.7	—	—	—	17.60
18:0	3.4	18.7	11.8	21.1	18:0
18:1	19.7	15.0	23.2	13.7	18.20
12:0h + 18:2	—	5.0	5.4	0.5	18.60
18:2	35.2	—	—	—	18.80
19:0	—	Traces	—	3.7	19.0
19:1	2.2	—	2.1	—	19.20
13:0h	—	4.8	—	Traces	19.60
20:0	—	Traces	1.0	0.6	20.00
20:1	1.5	—	3.7	0.7	20.20
14:0h	—	23.9	0.7	13.4	20.60
21:0 + x_1	Traces	—	—	3.7	21.00
x_2	—	0.7	1.9	Traces	21.70
x_3	—	1.5	—	—	21.87
22:0	Traces	—	0.9	Traces	22.00
22:1	—	—	10.9	—	22.30

* First figure denotes number of carbon atoms; second figure, number of double bonds; h indicates hydroxylated; x = unidentified.

Stahl and Kaltenbach (28). Samples of F_1, F_2, and F_3 hydrolysates were applied to thin-layer chromatographic plates of kieselguhr G (Merck). The plates were developed with a mixture of 65 ml of ethyl acetate and 35 ml of isopropanol-water (2:1), and the carbohydrates were located by spraying with a fresh mixture of 9 ml of 95% ethyl alcohol, 0.5 ml of concentrated sulfuric acid, and 0.5 ml of anisaldehyde. The plates were heated at 90 to 100 C for 5 min. Only rhamnose (R_F, 0.91) and glucose (R_F, 0.84) were recognized (Table 2).

The phosphorus content of the different spots was estimated by means of the Bartlet (3) method, as modified by Doizaki and Zieve (8).

Lipids excreted into the medium. The cell-free medium obtained after centrifugation of the culture was acidified with HCl and heated; all of the lipids were repeatedly extracted with petroleum ether. The lipid extract was saponified with 4% alcoholic KOH

TABLE 2. *Water-soluble components hydrolyzed from polar fractions F_1, F_2, and F_3, separated by thin-layer chromatography*

Component	F_1	F_2	F_3
Serine	+	+	+
Alanine	−	+	−
Ethanolamine	−	+	+
Leucine	−	+	−
Unidentified			
Ninhydrin-positive spots	−	+	+
Rhamnose	+	+	−
Glucose	+	−	−
Phosphorus	+*	+*	+

* Weak.

for 2 hr, and the unsaponifiable fraction was extracted with ether. The fatty acids were recovered after acidification, esterified as previously with 3 N HCl-methanol, sublimated, and analyzed by gas-liquid chromatography.

RESULTS

Fatty acids were identified by gas-liquid chromatography in 10% polyethylene glycol adipate, and the curves were compared with the chromatograms obtained after hydrogenation of the samples (9). Standards of myristate, palmitate, stearate, arachidate, behenate, palmitoleate, oleate, 9-eicosenoate, and linoleate were used to compare the retention times. From the plot of the retention times relative to methyl stearate, versus chain length (22), the equivalent chain length of all the esters was calculated and tabulated (Tables 1 and 3). The composition was calculated by the method of peak triangulation. Quantitative comparison with National Heart Institute Fatty Acid Standard D (13) agreed with the stated composition data, with a relative error of less than 13% for major components (>10% of total mixture) and of less than 3% for minor components (<10% of total mixture). In triglycerides, octadecadienoic acid (18:2) was the major acid present, with palmitic (16:0) and octadecenoic (18:1) next in concentration. Other saturated and monounsaturated acids were also found (Table 1).

A big difference was found between the fatty acid composition of neutral and polar lipids (Table 1). An important difference in the polar lipids was shown by the presence of only traces of octadecadienoic acid and of a higher concentration of stearic acid. In addition, fraction 2 included 10.9% docosenoic acid (22:1); in fractions 1 and 3, high concentrations of hydroxylated fatty acids were calculated.

From the retention times of the methyl esters of F_1 and F_3, the existence of hydroxylated fatty acids was presumed. They were separated by thin-layer chromatography on silica gel G (30). The plates were developed with diethyl ether-hexane (30:70), and the spots were located by spraying the plates with alcoholic 2,7-dichlorofluorescein. The presence of monohydroxylated fatty acid methyl esters (R_F, 0.30) was easily recognized by comparison with a standard β-hydroxymyristate. They were clearly separated from normal fatty acid esters, which migrate more quickly, and dihydroxy fatty acids esters, which moved only slightly from the line of application. The zone of the monohydroxylated esters was scraped off; it was extracted with acidified methanol and analyzed by gas-liquid chromatography. An important peak with the same equivalent chain length (20.60) as β-hydroxymyristate synthesized in the laboratory (31) was recognized, together with smaller peaks. According to the work of Kaneshiro and Marr (15), polar columns of polyesters may be used to separate α-hydroxy from β-hydroxy acids. In consequence, the fatty acid ester of equivalent chain length (20.60) was considered to be β-hydroxymyristate. The other hydroxy acids found were considered to belong to the same β-hydroxy series because they fell together with β-hydroxymyristate in the same straight line when the logarithms of the retention times were plotted as a function of the number of carbon atoms (Fig. 2). Jarvis and Johnson (14) also found β-hydroxydecanoic acid in *P. aeruginosa* grown in glycerol, and Bergström et al. (4) recognized small amounts of β-hydroxydecanoic acid and β-hydroxyoctanoic acid in the same bacteria.

Hydroxy fatty acids were bound to the lipids of the polar fractions F_1 and F_3. These fractions were very rich in rhamnose. Both of them contained

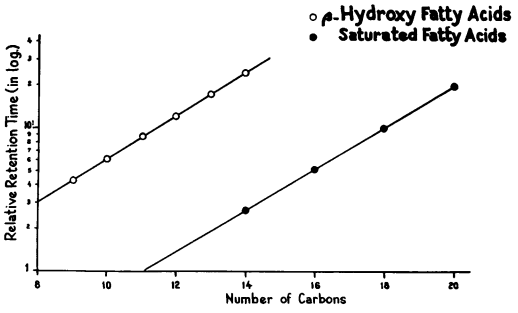

FIG. 2. *Relative retention times in gas-liquid chromatography of methyl esters of n-saturated and β-hydroxy monocarboxylic acids. The column was 10% polyethylene glycol adipate, in Chromosorb W (100 to 120 mesh), operated at 195 C.*

TABLE 3. *Composition (g/100 g) of fatty acids excreted into the medium of methyl esters*

Fatty acid*	Composition	Equivalent chain length
14	Traces	14
14:1	Traces	14.30
15:0	0.6	15.00
15:1	Traces	15.30
9:0	1.3	15.60
16:0	33.5	16.00
16:1	Traces	16.30
17:0	6.6	17.00
11':0h	Traces	17.60
18:0	43.0	18.00
18:1	8.6	18.30
18:2	1.1	18.80
13:0h	1.5	19.60
20:0	3.8	20.00
20:1	Traces	20.30
20:2	Traces	20.80
21:0	Traces	21.00
21:1	Traces	21.30
21:2	Traces	21.80
22:0	Traces	22.00

* First figure denotes number of carbon atoms; second figure, number of double bonds; h indicates hydroxylated.

glucose and serine, but fraction F_3, very rich in phosphorus, gave a positive test for ethanolamine and showed the same R_F of phosphatidylethanolamine by thin-layer chromatography. In consequence, both fractions were constituted by more than one lipid. The fatty acids of the polar fraction F_2 were bound to ethanolamine, serine, alanine, leucine, and choline. It presented the same R_F as phosphatidylcholine, and it also gave a weak positive test for rhamnose.

Saturated fatty acids were the major acid excreted into the medium (Table 3). Stearic (43.0%) was the principal acid, with palmitic next in concentration. Hydroxy fatty acids were practically absent.

DISCUSSION

Azoulay and Senez (2) proposed that the metabolism of *n*-alkanes by *P. aeruginosa* is accomplished in the following sequence of reactions:

Hydrocarbon → primary alcohol → aldehyde → fatty acid

Chouteau et al. (7) proved that in this reaction pioscianine and nicotinamide adenine dinucleotide behave as hydrogen acceptors. However, Ali Khan et al. (1) found that *Pseudomonas* grown in octane produced suberic as well as adipic acid. However, he could not separate these products in *Pseudomonas* cultured in hexane. At the same time, Kester and Foster (16) admitted that ω oxidation was the mechanism used by *Corynebacterium* and other bacteria for the oxidation of *n*-alkanes. However, the absence of dicarboxylic acids in our culture would agree with the mechanism of hexadecane oxidation proposed by Azoulay and Senez.

In this work, *P. aeruginosa* was shown to metabolize hexadecane and synthesize saturated, monounsaturated, octadecadienoic, and β-hydroxylated acids.

Block (5) reported that β-hydroxy acids are intermediates of monounsaturated acid synthesis from acetate in anaerobic, facultatively anaerobic, and even obligately aerobic bacteria such as *P. fluorescens* (6). This so-called (by Block) "anaerobic" route does not involve dehydrogenation of long-chain saturated fatty acids, but rather elongation of a carbon chain of an already β,γ-unsaturated acid produced from β-hydroxy acids of appropriate length (10 and 12 carbons). β-Hydroxy acids derive in the conventional way from acetyl and malonyl coenzyme A (19). The presence of the homologous series of β-hydroxy acids in our *P. aeruginosa* strain is, in consequence, due very probably to the same synthetic mechanism. At least they seem not to be direct products of hexadecane metabolism, since hydroxy acids were also found by Bergström et al. (4) and by Jarvis and Johnson (14) in *P. aeruginosa* cultured in hydrocarbon-free media. Jarvis and Johnson (14) also reported that β-hydroxydecanoic was the most abundant hydroxy acid found in their culture and that it was bound to a lipid. Hauser and Karnovsky (11, 12) proved that this product was excreted into the medium. We also found β-hydroxy acids bound to at least two kinds of polar rhamnolipids. However, β-hydroxymyristate was our most abundant hydroxy acid, and both rhamnolipids were found in the cells and not in the medium. β-Hydroxymyristic acid cannot be a precursor either for vaccenic or for oleic acid if it were desaturated and elongated following Block's "anaerobic" route. However, it has also been found in *Escherichia coli* by Marr and Ingraham (21); this bacterium is also classified by Block as following the anaerobic synthetic route to monounsaturated acids.

The presence of a very high concentration of octadecadienoic acid in the neutral lipid of our bacteria states a special problem, since, according to the opinion of Block (5) and others, this acid is synthesized in living beings through oxidative dehydrogenation of already formed monounsaturated acids, in a completely different path-

way (aerobic) that gives way to β-hydroxy acids and monounsaturated acids of the vaccenic type. In consequence, its presence may suggest that *P. aeruginosa* could follow both anaerobic and aerobic routes to synthesize unsaturated acids. Unfortunately, shortage of material prevented us from studying the position of the double bonds in monounsaturated and octadecanoic acid, which might furnish us with a key for solving this problem.

The fatty acids of the polar fraction F_2 were quite different from all of the other fractions. They were especially rich in monounsaturated acids of 14, 15, 16, 17, 18, 19, 20, and 22 carbons, and were practically free from hydroxy acids. These fatty acids were bound to a minor amount of phosphatidylcholine and to a special kind of lipid containing ethanolamine and the amino acids serine, alanine, and leucine. The structure of these lipids has not yet been determined, but in all probability they are lipoamino acids. The existence of lipoamino acids was practically unknown until MacFarlane (20) recognized phosphatidylglycerol alanine in *Clostridium welchii*. Sinha and Gaby (25) proved the existence of lipid-amino acids in *P. aeruginosa*. But these lipid-amino acids, separated by thin-layer chromatography in silicic acid, were associated with noncholine lipids composed primarily of ethanolamine and serine phosphatides. They were shown to contain several ninhydrin-positive compounds in addition to ethanolamine, serine, and alanine, and the structure proposed was based on three molecules of glycerol bound by phosphoric acid bridges with two amino bases esterified to the same acids. The amino acids would be esterified to the central glycerol, whereas four fatty acids would be bound to the outer glycerol. Such a structure would be very rich in phosphorus and rather basic in reaction; it does not seem to agree very well with our fraction F_2. Other kinds of lipid-amino acids, such as peptidolipine and fortuitine (17), which are also water-soluble, could also be found in fraction F_2. Work is in progress to try to elucidate these problems.

ACKNOWLEDGMENT

We are grateful to Arturo Solari for suggesting this work and for the *P. aeruginosa* strain used, to Rodolfo Ertola for lending the fermentor, and to YPF Laboratories of Florencio Varela for providing the hexadecane.

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