Journal of Industrial Microbiology, 2 (1988) 337–348
Elsevier

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Biosynthesis of acyclic methyl branched polyunsaturated hydrocarbons in *Pseudomonas maltophilia*

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Received 3 April 1987
Revised 24 September 1987
Accepted 25 September 1987

Key words: Biosynthesis; Fatty acid; Hydrocarbon; Lipid; *Pseudomonas*

SUMMARY

The hydrocarbon composition of *Pseudomonas maltophilia* was determined by gas chromatography-mass spectrometry. Mono-, di- and tri-unsaturated alkenes were identified with a predominance of polyunsaturated components. The carbon chains of the alkenes contained single methyl branches in *iso* and *anteiso* position and double methyl branches in the *iso-iso* and *anteiso-anteiso* configurations. The composition of the hydrocarbons from cells grown in synthetic media enriched with amino acids or volatile fatty acids demonstrated that the probable precursors incorporated into individual hydrocarbons were branched and normal fatty acid chains in the range from C_3 to C_{16}. The probable fatty acid precursors which were connected together to form the major triunsaturated hydrocarbon chains were two monounsaturated chains, whereas the major diunsaturated chains resulted from condensation of saturated and monounsaturated chains. The probable precursors for the major monounsaturated hydrocarbons were C_{14} (C_{15}) and C_{16} (C_{15}) fatty acids. The accumulation of hydrocarbons was not detected until the cells were in the late exponential phase of growth; the maximal levels were reached at the mid-stationary phase of growth.

INTRODUCTION

Triterpenoids, isoprenoid and nonisoprenoid hydrocarbons are among the oldest known and most ubiquitous chemicals on earth, having been found in fossil remains and a variety of geological environments and sediments [15,16,29,32,33,35,38]. Apparently the biosynthetic pathways for synthesizing the isoprenoid-derived compounds existed before the evolution of chlorophyll biosynthesis. Unlike the isoprenoid-derived compounds, the biosynthesis of saturated and unsaturated acyclic hydrocarbons is not ubiquitous to living systems and, accordingly, its occurrence in nature is supported by both biogenic and abiogenic theories. Evidence against the biogenic theory is that no organism has been found that synthesizes the types of alkanes commonly found in sediments and ancient deposits. Alkanes and alkenes are synthesized by plants, yeast and algae but in relatively small quantities

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[26,39]; the hydrocarbons are odd-numbered carbon chains that apparently resulted from the decarboxylation of fatty acids [6,8,9,10,14,16, 27,30,34]. Two bacteria, species of *Micrococcus* [24,31,39,42] and *Pseudomonas maltophilia* [45], synthesize acyclic aliphatic hydrocarbons in quantities that range from 0.25 to 2% of the cellular dry weight. Other bacteria synthesize either trace quantities or have no detectable levels of such hydrocarbons [39]. The best-studied of the hydrocarbon-producing bacteria are those in the taxonomic family Micrococcaceae. *Micrococcus* species synthesize symmetrically- and asymmetrically branched monounsaturated alkenes in the range from C_{22} to C_{32} [1,24,41–43] in concentrations that range from 0.25 to 1.5% of the cellular dry weight. Discrete alkene patterns produced by different *Micrococcus* species were useful in developing the taxonomy of the Micrococcaceae, illustrating the discrete hydrocarbon biosynthetic pathways [24,31,43]. The alkenes are apparently derived from head-to-head (carboxyl end to carboxyl end) condensation with a reduction of one carboxyl group and a decarboxylation of the other. The unsaturated position occurs between carbons 1 and 2 on the fatty acid that undergoes reduction. Thus, the double bond occurs next to the site of condensation of the two acids [1–3,42,44]. The other bacterium which synthesizes appreciable quantities of aliphatic acyclic hydrocarbons is *P. maltophilia* [45]. The yield of such hydrocarbons was reported as 1.4% of the cell dry weight. They range from C_{22} to C_{32} and were tentatively identified as a mixture of saturated and unsaturated branched and normal chains [45]. *Micrococcus* species [24,46] and *P. maltophilia* [18,21,46] are free-living, ubiquitous microbes that have been isolated from a variety of sources including soil, water, foods, and clinical specimens. Furthermore, *P. maltophilia* strains have been isolated from soils in petroleum zones and from bacterial enrichment cultures of aromatic and hydrocarbon compounds, althought they did not dissimilate the enrichment substrates [4,11,12,19,20,23].

The interest in microbial aliphatic hydrocarbon biosynthesis has existed as long as the curiosity as to how fossil fuel was derived. While the actual answer may never be obtained, curiosity about the mechanistic basis for the biosynthesis of hydrocarbons in selected microorganisms persists. Interest in the potential production of microbial hydrocarbons has been spurred by the importance of hydrocarbons as petrochemical substitutes and additives and by their geological and evolutionary importance. The purpose of this report is to present our findings on the identification of the aliphatic acyclic hydrocarbons synthesized by *P. maltophilia* and the metabolic regulation of hydrocarbon biosynthesis.

MATERIALS AND METHODS

Organisms

Sixteen strains of *P. maltophilia* were evaluated for aliphatic acyclic hydrocarbon biosynthesis. They were ATCC strains, 13637, 17444, 17445, 17666, 17668, 17673, 17674, 17679, 17677, and laboratory isolates RH 447, RH 609, RH 460, RH 139, RH 430, RH 1168, RH 1157. The laboratory strains were obtained from the culture collection of W. Kloos, North Carolina State University, Raleigh. Strain ATCC 17674 was selected for detailed studies on the chemical structure and biosynthesis of aliphatic hydrocarbons.

Culture conditions

P. maltophilia strains were grown in trypticase soy broth (TSB), brain heart infusion (BHI), or a synthetic medium consisting of a mineral medium containing ten salts [17], 3% glucose and 0.1% amino acids (1 × concentration) at pH 7.5. The 18 amino acids were all the L-form and were present in the proportions reported for α-casein [41]. Synthetic medium was also used with differing ratios of amino acids and branched amino acids or fatty acid supplements as follows: synthetic medium with the balanced amino acid mixture (SM(1 × AA)); synthetic medium with one-tenth the concentration of the balanced amino acid mixture (1/10 × AA) fortified with 270 mg/l each of leucine, isoleucine and valine; synthetic medium with one-tenth of the balanced amino acid mixture with 800 mg of leucine, isoleucine or valine; synthetic medium with the bal-

anced amino acid mixture with 800 mg of propionic, butyric, caproic, caprylic, myristic or palmitic acid.

Cells were cultivated at 30°C on an orbital shaker operating at 150 rpm. Unless otherwise indicated, cell preparations were harvested during the early stationary phase of growth by centrifugation at 5000 × *g* at 20°C for 15 min. The cells were washed twice with physiological saline containing a mixture of salts (9 g NaCl, 0.25 g $MgSO_4$, 1.8 mg $MnCl_2$, 0.22 mg $ZnSO_4$, 0.08 mg $CuSO_4$ and 0.05 mg $FeCl_3$ per liter) and each time sedimented by centrifugation as above. The fresh cell preparations were immediately lyophilized, weighed and then suspended in saline solution and extracted as described below.

Extraction and fractionation of lipids

Total lipids were extracted by the modified method of Bligh and Dyer [7] as described by Tornabene [40]. The lipid extracts were concentrated under a stream of nitrogen gas or by flash evaporation, then dried under vacuum, and their weights were determined gravimetrically.

Total lipid extracts were fractionated on a heat-activated silicic acid column (Unisil, activated silicic acid 200–325 mesh, Clarkson Chemical Company, Williamsport, PA) with redistilled hexane, benzene, chloroform, acetone and then methanol as described elsewhere [40]. The fractions were reduced in volume by flash evaporation, then taken to dryness under a stream of nitrogen gas and further dried under vacuum over KOH.

Thin-layer chromatography

Total and column-fractionated lipids, purified lipids, lipid derivatives and hydrolyzed lipids were characterized by thin-layer chromatography (TLC) on 20 × 20 cm glass plates coated (0.6–1 mm layers) with Stahl silica gel G, or Supelco precoated hard-layered TLC silica gel plates. Chromatography was carried out in lined jars by the ascending method using solvent mixtures: (a) diethyl ether/benzene/ethanol/acetic acid (40:50:2:0.2, by volume) as first solvent and hexane/diethyl ether (96:4, by volume) as second solvent for separating nonpolar lipids [40]; (b) chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by volume) [40]; or (c) two-dimensional polar lipid solvent: chloroform/methanol/acetone/diethylamine/water (120:34:37.5:6:4.5, by volume) in the first dimension and chloroform/methanol/ammonium hydroxide (65:25:5) in the second dimension for separation of polar lipids [13]. Spots were visualized by exposure to iodine vapors, acid charring and ninhydrin for amino acids, molybdate for phosphate [47], Draggendorf stain for quarternary amines [5] and α-naphthol solution for glycolipids [37]. Preparative TLC was utilized to isolate individual components according to procedures described elsewhere [40].

Analytical methods

Organic dry weight and ash content were determined in prefired Coors crucibles at 400°C for 12 h. Protein was assayed as decribed by Lowry et al. [28]. Total carbohydrates were analyzed by the phenol-sulfuric acid method following acid hydrolysis in 2 N HCl for 1 h at 100°C [25]. The preparation of trimethylsilyl ether derivatives of hydroxylated hydrocarbons was obtained by OsO_4 and bis-trimethylsilyl acetamide treatments as previously described [42]. Fatty acid methyl esters were prepared by esterification with methanolic HCl [22]. Lipids were hydrogenated catalytically (Pt) in an atmosphere of H_2 to reduce the unsaturated components.

Aliphatic hydrocarbons and fatty acids were analyzed by gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC-MS). A Varian 3700 Gas Chromatograph equipped with dual flame ionization detectors and a Varian CDS 402 data system was used with the following columns: (a) 99 m × 0.75 mm stainless steel capillary column coated with 3% OV-17 operated at 8 psi of He from 150°C to 250°C at 4°C/min and then held isothermally; (b) 30 m × 0.2 mm OV-351 fused silica capillary column with 0.25 μm film thickness (J. & W. Scientific, Inc., Rancho Cordova, CA) operated at 8 psi of He and 125°C to 220°C at 4°C/min and then held isothermally; (c) 30 m × 0.2 mm DB-5 fused silica capillary column, with a film thickness of 0.25 μm (J. & W. Scientific, Inc.) operated at 8 psi of He from 125°C to 230°C at

4°C/min and then held isothermally. The GC-MS analyses were performed using a Hewlett Packard 5890A Gas Chromatograph-5970 Series Mass Selective Detector equipped with a Hewlett Packard 9133 data system. The column used was an HP-5 (Crosslinked 5% Phenyl Methyl Siloxane) 25 m × 0.2 mm with 0.33 μm film thickness operated at 7 psi of He from 60°C to 300°C at 4°C/min and then held isothermally.

RESULTS

Test organism

Sixteen different strains of *P. maltophilia* were evaluated for total lipid production and hydrocarbon biosynthesis. The growth responses of the strains in TSB were similar, but the total lipids varied from 3.9 to 9.8% with the percentages of hydrocarbons ranging from 3.9 to 11.1 (Table 1).

Table 1

Total lipid production of *P. maltophilia* strains[a]

Strains	Total lipid[b]	Hydrocarbons[c]
ATCC 17673	5.8	4.5
ATCC 17677	6.1	5.6
ATCC 17668	3.9	3.9
ATCC 17679	9.5	10.1
ATCC 17674	9.0	11.1
ATCC 17445	9.6	8.8
ATCC 17444	6.8	10.8
ATCC 17666	9.6	8.9
RF 460	6.4	6.6
RH 609	8.2	6.9
RH 447	8.3	6.2
RH 611	8.8	9.6
RH 229	8.2	7.8
RH 139	8.0	6.7
RH 1168	8.8	7.1
RH 1157	9.8	4.0

[a] Cells were cultivated in TSB at 30°C and harvested at the early stationary phase of growth.

[b] Values are the percentages based on the total organic dry weight.

[c] Values are the percentages of the total lipids as recovered in the hexane and benzene eluates from silicic acid columns.

Comparative analyses of the hydrocarbon compositions of the 16 strains demonstrated that the hydrocarbon compositions were virtually identical with the exception of minor variation in the relative intensities of individual components. ATCC strain 17674 consistently produced the largest amount of hydrocarbons, and it exhibited the best growth response in the synthetic medium of the strains tested. Thus, strain ATCC 17674 was selected for further studies.

Composition of lipids and cellular components

When cultured to stationary phase in trypticase soy broth, *P. maltophilia* ATCC 17674 contained 11.8% carbohydrates, 76.3% proteins and 9.0% lipid components of ash-free dry weight. The different classes of lipids fractionated on silicic acid using organic solvents of differing polarities were hydrocarbons in the hexane (9.3%) and benzene (1.7%) eluants, non-glyceride neutral lipids in the benzene eluant (5.7%), a mixture of nonpolar lipids in the chloroform eluant (17.9%), pigments, glycolipids and phospholipids in the acetone eluant (37.4%), and phospholipids, aminolipids and glycolipids in the methanol eluant (29.7%). This distribution of lipids was similar to those reported elsewhere for *P. maltophilia* [45].

The production of hydrocarbon by *Pseudomonas* was not detected until the cells reached late exponential growth phase; the hydrocarbon production approached the maximum level in the mid-stationary growth phase. Accordingly, the approximate 7–11% hydrocarbon levels of the inoculum dropped to concentration levels below 1% after 4 h incubation. The rapid drop in hydrocarbon levels was attributed to the rate of cell division exceeding hydrocarbon synthesis. Some degradation of hydrocarbons might have occurred during the exponential phase, but there was no evidence to support such a proposal.

Identification of aliphatic hydrocarbons

The hydrocarbons detected by GC-MS were homologues of branched unsaturated chains that ranged from C_{26} to C_{32} (Fig. 1). To determine the branching points, the hydrocarbons were hydro-

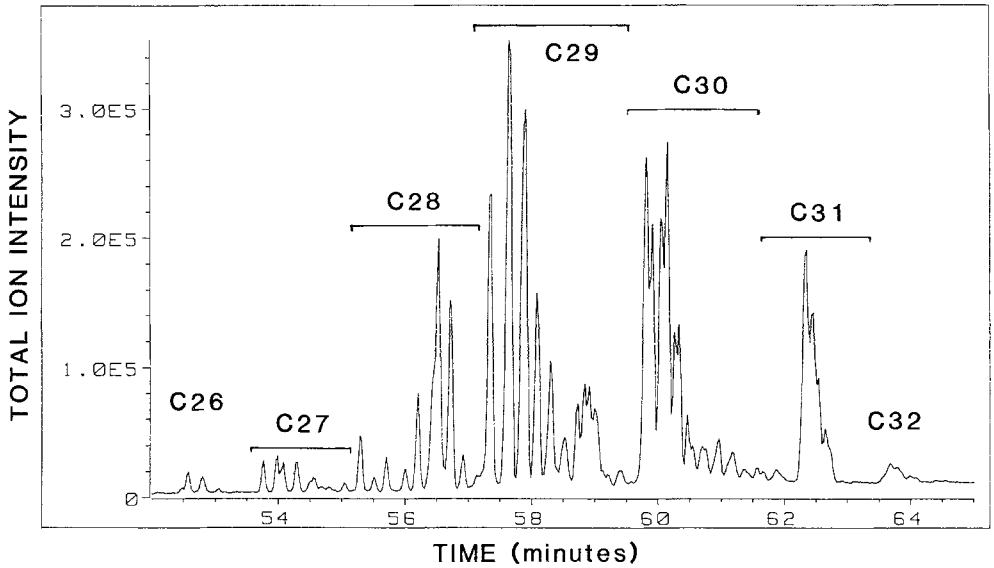

Fig. 1. Total ion chromatogram of hydrocarbons of *P. maltophilia*. Cells were cultivated in synthetic medium with the balanced amino acid mixture (1 × AA). The sample was analyzed on a fused silica HP-5 capillary column at 7 psi of He, from 60°C to 300°C at 4°C/min and held isothermally with a mass selective detector (HP-5970)/gas chromatogram (HP-5980A) operated at 70 eV.

genated and further analyzed by GC-MS. The total ion chromatogram (Fig. 2) contained a pattern of odd-numbered alkanes with distinct tetraplet arrangements of components comprising the C_{27}, C_{29}, C_{31} fractions with terminal branches at one or both ends of the carbon chains. The branching configurations were identified as *iso, anteiso, iso-iso,* and *anteiso-anteiso*. Relatively small quantities of normal alkanes were also identified. The C_{28} and C_{30} even-numbered alkanes, however, did not contain dual terminal branched components; they were comprised of mixtures of normal chains and single branched components of the *iso* or *anteiso* form. Only one C_{32} component was detected which had the *iso* configuration.

The alkenes were converted into oxygenated derivatives and the corresponding trimethylsilyl (TMS) ethers and analyzed by GC-MS to determine the location of the double bonds. The C_{29} alkenes had three double bonds between C-8/C-9, C-14/C-15, and C-21/C-22. Positional isomers of $C_{29:2}$ alkenes with different retention times were also identified, both with double bonds between C-8/C-9 and C-14/C-15. They were assumed to be hydrocarbons with different branching configurations. Also identified was a $C_{29:1}$ alkene with an internal double bond at C-14/C-15. The $C_{30:3}$ alkenes had double bonds between C-8/C-9, C-14/C-15, and C-21/C-22. The $C_{30:2}$ alkenes had double bonds between C-9/C-10 and C-16/C-17. The double bond positions of the relatively minor components were not determined. The relative position of the double bonds of the major C_{29} and C_{30} components are depicted in Table 2 demonstrating the uniformity of the double bond position(s) in each of the odd- and even-numbered carbon chains. The branched structure of the unsaturated carbon chains could not be determined from the mass spectra of the TMS derivatives because the fragmentation pattern was dominated by fission products of the vicinal ether groups.

The hydrocarbons recovered in the benzene eluate were predominantly the same di- and triunsaturated species that were also identified in the hexane eluate. The fatty acids which could serve as likely precursors for these hydrocarbons are discussed below.

Biosynthesis of aliphatic hydrocarbons

A series of nutritional studies was conducted in

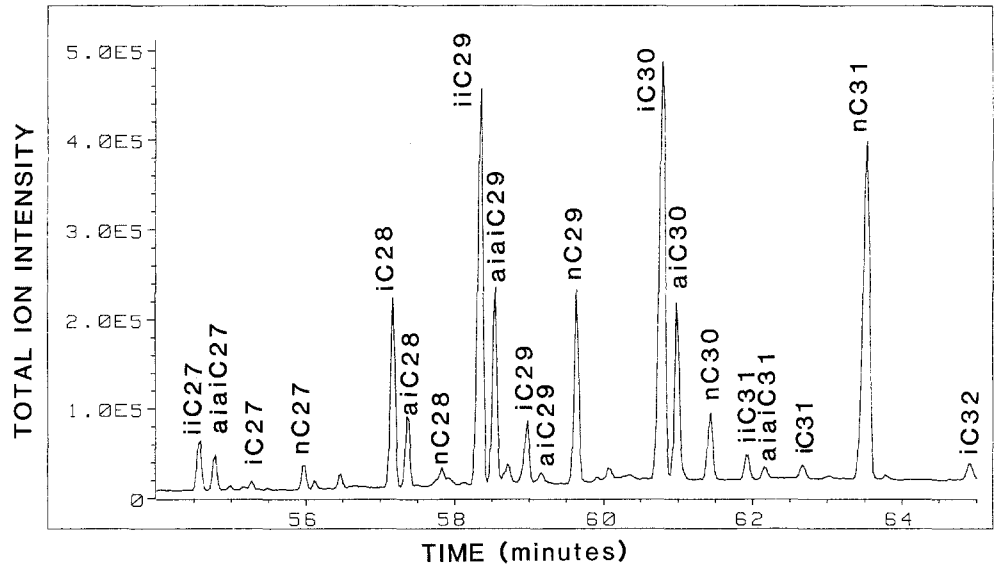

Fig. 2. Total ion chromatogram of hydrogenated hydrocarbons of *P. maltophilia* cultivated in TSB. See Fig. 1 legend for analytical conditions. i = *iso* methyl branch; ii = *iso* methyl branches on both ends of the carbon chain; ai = *anteiso* methyl branch; aiai = *anteiso* methyl branches on both ends of the carbon chain; n = normal.

order to explore the mechanism of hydrocarbon biosynthesis and to identify the precursors of hydrocarbon biosynthesis. Organisms grown on the TSB or BHI gave higher cell yields than those activated on SM(1 × AA), but the percentage of the total production of hydrocarbons of ash-free dry weight cells (AFDW) was about the same (Table 3). In one of the nutritional studies, the level of the balanced amino acids of the synthetic medium (SM(1 × AA)) was reduced ten-fold and excess branched amino acids were added (SM(1/10 × AA) + Leu, Ile, Val). This resulted in a ten-fold decrease in cell yield, 0.2 to 0.02 mg/l, accompanied by an almost two-fold increase in the hydrocarbon concentration per unit of cell weight (Table 3). An increase in the lipid content was noted as well, i.e., from 8.3% to 15.2%. Doubling the concentration of amino acids (SM(2 × AA)) provided a moderately better cell yield but lower lipid production.

The relative distribution of the various hydrocarbons from cells cultivated in different formulations of the synthetic media with enrichments of individual amino acids are summarized in Table 4. The synthetic medium enriched with valine promoted the formation of single *iso*-branched odd-numbered carbon hydrocarbons and *n*-C_{28}, *n*-C_{29},

Table 2

Relative position of the double bonds in the C_{29} and C_{30} hydrocarbons

The identities were determined by GC-MS of TMS-derivatives of the oxygenated alkenes.

Configuration	Identity
$C_{13}H_{27}$ - CH=CH - $C_{14}H_{29}$	$C_{29:1(\Delta 14)}$
C_7H_{15} - CH=CH - C_4H_8 - CH=CH - $C_{14}H_{29}$	$C_{29:2(\Delta 8,14)}$
C_7H_{15} - CH=CH - C_4H_8 - CH=CH - C_5H_{10} - CH=CH - C_7H_{15}	$C_{29:3(\Delta 8,14,21)}$
$C_{13}H_{27}$ - CH=CH - C_5H_{10} - CH=CH - C_8H_{17}	$C_{30:2(\Delta 9,16)}$
C_7H_{15} - CH=CH - C_4H_8 - CH=CH - C_5H_{10} - CH=CH - C_8H_{17}	$C_{30:3(\Delta 8,14,21)}$

Table 3

Hydrocarbon production by *P. maltophilia* grown in different growth media

Growth media	Cell yield (mg/l)	Lipids[d]	Hydrocarbons[e]
Trypticase soy broth	0.6	9.5	7.6
Brain heart infusion	1.2	8.7	10.8
SM(2 × AA)	0.4	7.5	8.0
SM(1 × AA)[a]	0.2	8.3	9.5
SM(1/3 × AA) + 200 mg Leu, Ile, Val[b]	0.2	8.9	12.6
SM(1/10 × AA) + Leu, Ile, Val[c]	0.02	15.2	18.7

[a] Synthetic medium (SM) consisted of mineral salts, glucose and the indicated level of a balanced mixture of 18 amino acids (AA).
[b] The branched amino acids were each present at 200 mg/l.
[c] The branched amino acids were each present at 270 mg/l.
[d] The values are percentages based on the ash-free dry weight.
[e] Hydrocarbons were separated by column fractionation of lipid extracts on silicic acid with hexane. The values are percentages based on the weight of eluted lipid.

n-C_{30} and *n*-C_{31} hydrocarbons as major components. Interestingly, valine did not stimulate the formation of the dual *iso-iso* branched hydrocarbon nor the major even-numbered carbon *iso*-branched chains (Table 4). The formation of the *iso*-branched even-numbered carbon and odd- or even-numbered *iso-iso* branched hydrocarbons was stimulated by leucine supplementation. Correspondingly, the isoleucine supplement stimulated the formation of the *anteiso*-branched hydrocarbons (Table 4). The *anteiso-anteiso* branch occurred only in relatively small quantities and was restricted to cells cultivated in the balanced synthetic medium used as a control. Only odd-numbered members of the *anteiso-anteiso* branched configuration were detected.

The mechanism by which these unsaturated hydrocarbons are formed was further investigated in order to determine whether or not the double bonds mark the sites of condensation of two short chain fatty acids such as C_3, C_4, C_6, C_7 or C_8 to form the C_{14}–C_{18} fatty acids. Fatty acids containing three, six and eight carbons were chosen on the basis of the carbon length between double bonds as shown in Table 2. The C_7 and C_9 fatty acids were not available for testing. The acids were added as supplements to synthetic medium with the balanced mixture of amino acids (SM(1 × AA) + C_3, C_6 or C_8). Alkenes produced by cells cultivated in synthetic medium supplemented with propionic acid were predominantly C_{29} and C_{30} with two and three double bonds (Table 5). The percentages of alkenes listed in Table 5 are reported as the sum of all positional isomers of the same carbon number and do not distinguish between branched and normal structures. A more accurate depiction of the results is shown in Fig. 3. The distribution pattern is significantly different from the typical pattern shown in Fig. 1. The significant alteration of the distribution pattern points to the possibility that propionic acid is not one of the principal metabolic intermediates in the hydrocarbon biosynthetic pathway. The addition of this short-chain acid to the growth medium selectively altered the hydrocarbon production pathway. The results demonstrated, however, that the organism was able to convert this metabolic intermediate into alkenes. Caproic acid and caprylic acids, in turn, enhanced the synthesis of certain odd-numbered carbon chains. Most notable were the marked increases in the C_{27} alkenes with one or two double bonds and C_{31} alkenes with two or three double bonds. The C_{28} and C_{30} alkenes were correspondingly reduced in quantity as well as the C_{29} alkene with one double bond. The addition of C_4 fatty acid or longer-chain fatty acids as supplements resulted in no significant alteration in the hydrocarbon pattern.

Table 6 contains a list of the total free fatty acids. The fatty acids were mostly saturated with

Table 4

Composition of hydrocarbons of *P. maltophilia* grown in synthetic media supplemented with branched amino acids

Hydrogenated hydrocarbon[a]	Media composition[b]			
	SM(1 × AA)	SM(1/10 × AA) + Val	SM(1/10 × AA) + Leu	SM(1/10 × AA) + Ile
i,i-C_{27}	1.9	–[c]	2.5	–
ai,ai-C_{27}	1.3	–	–	–
i-C_{27}	–	1.8	–	–
ai-C_{27}	–	–	–	1.3
n-C_{27}	1.0	–	0.8	1.3
i,i-C_{28}	–	–	0.5	–
i-C_{28}	7.2	3.4	9.7	–
ai-C_{28}	2.6	–	–	11.8
n-C_{28}	–	3.1	–	1.8
i,i-C_{29}	17.3	2.2	41.1	–
ai,ai-C_{29}	7.3	–	–	4.2
i,ai.C_{29}	–	–	–	8.8
i-C_{29}	2.5	6.2	2.2	–
ai-C_{29}	–	–	–	3.4
n-C_{29}	7.5	17.7	3.5	12.0
i,i-C_{30}	–	–	6.1	–
i-C_{30}	21.4	12.2	27.4	3.2
ai-C_{30}	7.3	–	–	24.8
n-C_{30}	2.9	10.5	0.7	5.2
i,i-C_{31}	1.1	–	4.6	–
i-C_{31}	–	3.1	0.4	–
ai-C_{31}	–	–	–	1.1
n-C_{31}	18.8	39.8	6.2	21.7
i-C_{32}	–	–	1.3	–

[a] i = *iso* methyl branch; i,i = *iso* methyl branches on both ends of the carbon chain; ai = *anteiso* methyl branch; ai,ai = *anteiso* methyl branches on both ends of the carbon chain; n = normal.

[b] The values are the percentages based on the total peak area of hydrocarbons. When added, the branched amino acids were present at 800 mg/l.

[c] – = not detected.

straight chains or branched with *iso* or *anteiso* configurations. The chain lengths ranged from 14 to 20 carbon atoms. Two cyclopropane fatty acids were detected as well.

DISCUSSION

The only occurrence of polyunsaturated acyclic non-isoprenoid lipids reported in procaryotes are the polyunsaturated fatty acids of the blue-green bacteria that contain a photosynthetic apparatus like those of eucaryotes [40]. The presence of the polyunsaturated methyl branched acyclic hydrocarbons in *P. maltophilia* are unique, since such compounds have not previously been found [39]. These hydrocarbons in *P. maltophilia* clearly distinguish it from all other pseudomonads studied, which have no detectable levels of hydrocarbons [39,45]. This contraposition is consistent with the findings of other studies that demonstrated several compositional and metabolic characteristics that differentiated *P. maltophilia* from the other pseudomonads [37]. It has been proposed [30] that bacterial hydrocarbons are end-products that result from the metabolic regulation of the fatty acid pathway.

Table 5

Composition of alkenes of *P. maltophilia* grown in synthetic media supplemented with short-chain fatty acids

Hydrocarbon	Media composition[a]			
	SM(1 × AA)	SM(1 × AA) + propionic acid	SM(1 × AA) + caproic acid	SM(1 × AA) + caprylic acid
$C_{26:2}$	0.4	–	0.3	–
$C_{26:1}$	–	–	0.5	0.6
$C_{27:3}$	–	–	0.5	0.7
$C_{27:2}$	2.1	2.9	7.2	6.2
$C_{27:1}$	1.1	1.4	4.5	5.5
$C_{28:3}$	1.7	2.1	0.6	0.9
$C_{28:2}$	7.7	4.7	3.4	4.5
$C_{28:1}$	4.7	2.7	2.2	2.8
$C_{29:3}$	8.0	18.4	8.8	4.8
$C_{29:2}$	19.6	31.8	21.6	23.0
$C_{29:1}$	15.7	8.8	1.6	2.6
$C_{30:3}$	13.7	9.5	6.6	6.5
$C_{30:2}$	15.7	13.9	5.8	8.2
$C_{30:1}$	2.4	1.6	–	–
$C_{31:3}$	6.5	2.3	30.6	26.9
$C_{31:2}$	0.8	–	5.7	6.9

[a] The values are the percentages based on the total hydrocarbon weight. The dashed line indicates that none was detected. When used, the short-chain fatty acids were present at 800 mg/l.

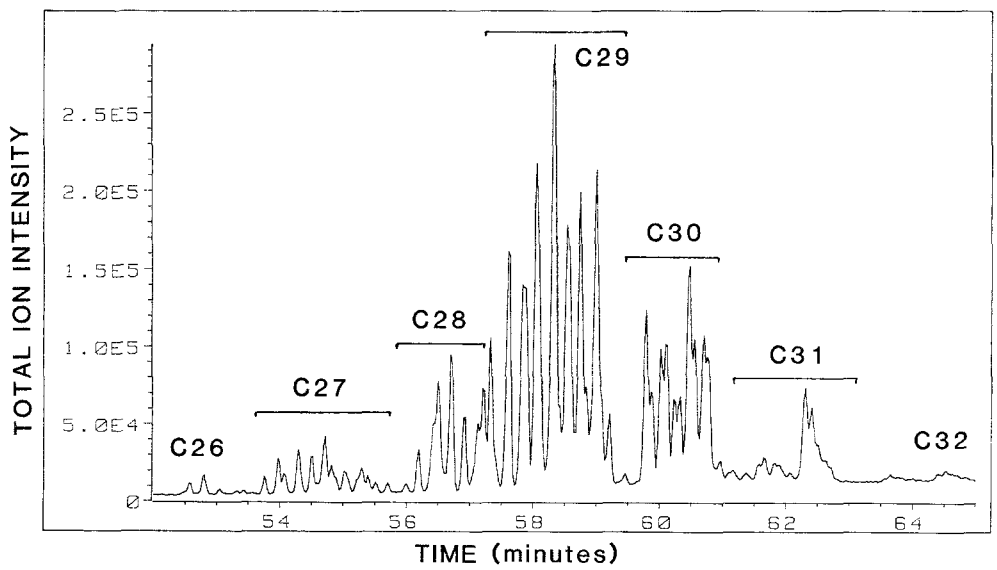

Fig. 3. Total ion chromatogram of hydrocarbons of *P. maltophilia* grown in synthetic medium with excessive supply of propionic acids (C_3 fatty acids). See Fig. 1 legend for analytical conditions.

Table 6

Fatty acids of *P. maltophilia*[a]

Fatty acid identity[b]	Composition[c]
i-$C_{14:0}$	0.7
n-$C_{14:0}$	1.0
i-$C_{15:0}$	28.0
ai-$C_{15:0}$	13.2
n-$C_{15:0}$	1.3
i-$C_{16:0}$	7.3
n-$C_{16:0}$	22.8
i-$C_{17:0}$	9.3
ai-$C_{17:0}$	2.0
n-$C_{17:0}$	1.0
cyc-$C_{17:0}$	4.3
i-$C_{18:0}$	1.3
n-$C_{18:0}$	3.5
n-$C_{18:1}$	0.6
i-$C_{19:0}$	0.4
ai-$C_{19:0}$	1.3
cyc-$C_{19:0}$	0.3
n-$C_{20:0}$	0.1

[a] Cells were cultivated in TSB at 30°C.

[b] i = *iso* methyl branch; ai = *anteiso* methyl branch; n = normal; cyc = cyclopropane.

[c] The values are the percentages based on the total peak area of fatty acids.

The biosynthesis of predominantly branched polyunsaturated hydrocarbons in *P. maltophilia* appears to be similar to that of the *Micrococcus* species. The carbon skeletons of the family of hydrocarbon isomers found in the well-studied *Micrococcus* species are predominantly monounsaturated alkenes with repeating distribution patterns for compounds of different chain length [1,41,42,44]. The alkenes of *Micrococcus* species result from head-to-head (carboxyl end to carboxyl end) condensation of two fatty acids with decarboxylation of one of the fatty acids [3,42,44]. It has been proposed that this head-to-head route is the terminal step in the elongation process, with the position of the double bond residing at carbons 1 and 2 of the fatty acid that was not decarboxylated [1,3,42,44]. The *iso* and *anteiso* configurations were derived from leucine and isoleucine, respectively. The data in Table 4 support a proposal that the *iso* and *anteiso* configurations of the hydrocarbons of *P. maltophilia* were also derived from leucine and isoleucine, respectively. Leucine supplements significantly enhanced the biosynthesis of *iso-iso* branched hydrocarbons and *iso* branched configuration of the even-numbered carbon chains. Valine was not a preferred precursor of the *iso* configuration, resulting in a reduction of the relative amount of *iso*-C_{28} and all *iso-iso* configurations. Valine supplements, however, caused an enhancement in the relative amounts of *iso* branched odd-numbered carbon chains and non-branched chains. For valine to be incorporated in *iso* branched odd-numbered chains, assuming the amino acid followed the standard catabolic route of deamination and decarboxylation, the even-numbered carbon precursor would have to be condensed with selected fatty acids. The incorporation of valine into non-branched chains suggests that the valine was metabolically degraded into key intermediates that were either incorporated directly or indirectly by inducing the formation of precursors that supported *n*-chain biosynthesis. The precursor fatty acids condensed into the hydrocarbons are not known. If, in the case of *P. maltophilia*, short-chain fatty acids are incorporated by condensation mechanism (head-to-tail) into longer fatty acids with the double bond resulting at the site of condensation, the length of the possible precursor fatty acid units would be C_3 to C_9 (Table 2). The predominant fatty acid incorporated would then be predicted to be C_8 (Table 2), followed by C_7, C_9 and C_6. Supporting evidence for this proposal was obtained from the nutritional studies employing short-chain fatty acids (Table 5). When the minimal medium was enriched with caproic (C_6) and caprylic (C_8) acids there was a two- to five-fold increased production of di- and triunsaturated alkenes with odd-numbered carbons (Table 5). The C_7 and C_9 fatty acids were not available for incorporation studies. Enrichment with propionic acid almost exclusively stimulated the di- and triunsaturated C_{29} alkenes; however, this precursor created a significant increase in the total number of C_{29} components detected. The additional components were identified as a mixture of di- and triunsaturated positional isomers. The position of the double

bonds in all of the isomers was not determined. Since this distribution pattern was not observed in any of the other studies, it was concluded that propionic acid is not normally a major intermediate in the metabolic pool committed to alkene biosynthesis. The feeding of acetic and butyric acids had no specific effects on the distribution of biosynthesized alkenes (data not shown). These even-numbered fatty acids are apparently primary metabolites in the cellular pool and, thus, not pivotal metabolic control points. The biosynthesis of hydrocarbons late in the growth cycle suggests that secondary metabolites are involved.

The mechanism by which the hydrocarbons are synthesized remains unknown. The evidence suggests that the condensation of *iso* and *anteiso* branched precursors with acetate and other volatile fatty acids, possibly by the standard head-to-tail route, results in saturated and unsaturated fatty acids of the C_{14}–C_{18} range. These acids are then condensed into alkenes, perhaps by the head-to-head (carboxyl end to carboxyl end) mechanism. Before this proposal for biosynthesis of hydrocarbons can be tested, however, improved techniques for determining both branching and double bond positions of mixed branch polyunsaturated hydrocarbon isomers will have to be developed. Moreover, additional experiments will have to be conducted to delineate the exact metabolic interaction between the nature and composition of lipids and hydrocarbons.

ACKNOWLEDGEMENT

This work was supported in part by subcontract XK-4-04143 from the Solar Energy Research Institute to T.G.T.

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