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Phospholipid Derived from Hydrocarbons by Fungi

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Strain no. 250-2, a hydrocarbon-utilizing filamentous fungi isolated from oil-polluted soils, was identified as an *Aspergillus* sp. This organism grew well on both glucose and *n*-alkane (C11～16) as sole carbon and energy sources. The most suitable substrate for growth was *n*-C15, and for phospholipid formation, *n*-C16.

The major fatty acid in cellular lipids was C18: 2 acid (46～60%) in all cases. The proportion of odd-chain fatty acids was relatively high in the cells grown on odd-chain *n*-alkanes. Phospholipids in cellular lipid were phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and cardiolipin (CL). CL especially was the major phospholipid in *n*-C11-grown cells. The changes of phospholipids depended on the growth phases and substrates in all cases. Although the major fatty acids in all phospholipids except CL were C16: 0 (1～58%), C18: 0 (6～66%), and C18: 2 (9～53%) acids, those of CL were C16: 0 (27～36%) and C18: 2 (46～54%) as were those of cellular lipids. Fatty acid compositions of phospholipid also depended on the growth substrates in all cases.

Many reports have described hydrocarbon utilization by bacteria and yeasts,[1] but there is very little on the utilization of hydrocarbons[2-6] or on the effects of hydrocarbons on the composition of phospholipids in fungi.[7,8] The quality and quantity of cellular fatty acids and phospholipids formed from hydrocarbons are greatly influenced by the type of organism and the hydrocarbons used as substrates.

To define the lipid biosynthesis of hydrocarbon-utilizing filamentous fungi, we examined the assimilation of hydrocarbons and the nature and diversity of the phospholipids of a newly isolated strain of filamentous fungi, no. 250-2, growing at the expense of a homologous series of odd and even *n*-alkanes as sole carbon and energy sources.

Materials and Methods

Microorganisms used We isolated 49 strains of hydrocarbon-utilizing filamentous fungi from oil-polluted soils in 1979. One of these strains, no. 250-2, grew more rapidly and formed more lipid than any of the others on kerosene culture. Because of this, strain no. 250-2 was the most suitable for our purpose, and was identified as a species of *Aspergillus* in accordance with the methods described in Illustrated Genera of Imperfect Fungi 3rd Ed. (1972)[9] and *Kin-rui Zukan* (1978).[10]

Cultivation Strain no. 250-2 was preincubated on potato-dextrose-agar slants for 7 days at 28°C. About 10^7 spores were inoculated into 60 ml of sterile mineral medium (pH 6.0) containing 1% of either *n*-alkane or glucose. The composition of the mineral medium was: $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and yeast extract, 5 g, in 1 *l* of distilled water. The *n*-alkanes used were undecane (*n*-C11), dodecane (*n*-C12), tetradecane (*n*-C14), pentadecane (*n*-C15), and hexadecane (*n*-C16), all of purity over 99%. They were used without further purification.

The cultures were grown statically in 300-ml Erlenmeyer flasks at 28°C. The cells of each culture were harvested by filtration, then washed successively with distilled water and *n*-hexane. The washed cell was lyophilized and weighed. The cells in the three growth phases (lag or early log, log, and stationary) of strain no. 250-2 were used for further analysis.

Analysis of fatty acids Cellular lipid was extracted with chloroform-methanol (1: 2, v/v) by the method of Allen *et al.*[11] The extracted lipid was

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methylated using 5% methanolic HCl.[12] Fatty acid methyl esters obtained were diluted in acetone, and their compositions were analyzed by gas liquid chromatography (GLC) using a Shimadzu gas chromatograph GC-6AMPTF. Each peak detected was identified by comparing of its retention time to those of the standard mixture of fatty acid methyl esters.

The column was glass tubing (3 mm × 3 m) packed with 10% diethyleneglycol succinate on 60/80 mesh Chromosorb W (acid washed and silanized), with the oven at 200°C, the injection port and detector, 280°C; and the nitrogen flow rate, 30 ml/min. The proportions of fatty acids were calculated by the usual method using a Shimadzu integrator C-R1A.

Analysis of phospholipids The extracted cellular lipid was separated into lipid classes by thin-layer chromatography (TLC) for the characterization of phospholipids (PL).

The extracted lipid was diluted in chloroform-methanol (3: 1, v/v), used in TLC, and developed by the method of Touchstone *et al.*[13] The TLC plate was a Silica Gel 60 (E. Merck, Darmstadt, W. Germany), and the solvent system was chloroform-ethanol-triethylamine-water (30: 34: 35: 8 by vol.). The chromatograms obtained were exposed to iodine vapor for about 5 min, and also detected by spraying with molybdenum blue reagent by the method of Dittmer and Lester.[14]

For the quantitative analysis, PL in the area corresponding to each authentic PL was transferred with the silica gel on the plate and re-extracted with chloroform-methanol (3: 1, v/v). After the evaporation of solvent under N_2 gas, phospholipids were estimated by assaying the phosphorus content of an aliquot of lipid extract using a modification of the method of Bartlett.[15] The phosphorus contents were multiplied by 25 to give the total PL content in dry weight of cells. Furthermore, the fatty acid composition of each phospholipid was analyzed as described above.

Results and Discussion

Growth and cellular fatty acids The growth curves of strain no. 250-2 on glucose and on various *n*-alkanes are shown in Fig. 1. The growth on each *n*-alkane indicates that this organism can assimilate hydrocarbons as well as glucose. *n*-C15 was an especially suitable growth substrate for this organism.

On the other hands, the previously reported fungi, *Hormodendrum hordei*,[3] *Cladosporium* sp.,[3] *Penicillium lilacinum* M-56-8,[4] *Aspergillus versicolor* M-15-6,[4] and *Cladosporium resinae*[6,7] grew better on glucose than on hydrocarbons, so *Aspergillus* sp. no. 250-2 is unusual among fungi in its hydrocarbon assimilation.

The compositions of cellular fatty acids are summarized in Table 1. In even-chain *n*-alkane-grown cells, the dominant feature is that all fatty acids detected and identified are even-chain fatty acids. In general, the major acids in these cells were C18:2 (48–59%), C16:0 (10–30%), and C18:1 (4–14%) acids. *n*-C16-grown cells contained a slight higher concentration (19–30%) of C16:0 acid than others.

In odd-chain *n*-alkane-grown cells, the proportions of odd-chain fatty acids (C15:0 and C17:0 acids) were only slightly increased (almost 10% in total).

These results suggest that cellular fatty acids are mainly formed by *de novo* synthesis. In *n*-C15- and *n*-C16-grown cells, fatty acids may also be formed by terminal oxidation and incorporated into the cellular lipid as such or after elongation by C2 units.

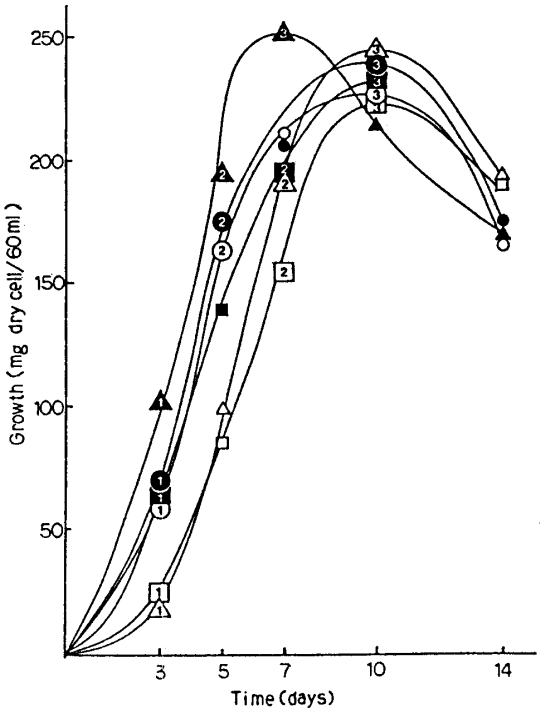

Fig. 1. Growth on glucose and on various hydrocarbons. Cells in three phases (1~3) of growth (1: lag or early log, 2: log, 3: stationary) were harvested.
Symbols: *n*-C11 (○); *n*-C12 (●); *n*-C14 (△); *n*-C15 (▲); *n*-C16 (□); glucose (■).

Table 1. Fatty acid composition of cellular lipids.

Carbon source	Phase	Fatty acid* (%)								
		14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3
n-C11	1	0.4	0.5	17.3	1.0	2.1	3.5	6.0	57.6	5.4
	2	0.3	0.5	17.7	0.7	2.6	3.9	4.4	59.4	4.6
	3	0.4	0.8	23.0	0.1	3.4	5.6	6.5	50.2	2.4
n-C12	1	0.5	0.3	16.2	1.0	1.2	3.6	5.2	56.8	5.4
	2	0.3	0.4	20.0	0.4	1.2	5.5	4.8	54.7	2.9
	3	0.3	0.6	19.8	0.5	1.2	5.8	5.4	54.5	2.5
n-C14	1	0.8	0.2	17.0	1.3	0.9	3.4	6.8	48.2	9.1
	2	2.0	t	17.4	0.1	0.3	3.4	5.3	59.0	3.1
	3	1.8	0.1	18.5		0.3	3.1	5.3	59.5	2.4
n-C15	1	0.1	3.7	11.9	3.5	4.2	2.6	7.8	47.6	4.5
	2		5.2	10.2	1.4	6.1	2.6	8.3	52.5	3.9
	3		5.3	11.6	2.1	8.5	3.3	8.6	45.5	2.5
n-C16	1	0.3	t	19.4	0.5	0.6	3.5	8.6	48.5	8.5
	2	0.4	0.8	29.5			0.6	6.3	53.1	4.0
	3	2.6	0.5	24.0		t	1.4	6.8	53.3	1.6
Glucose	1	0.6	0.4	16.3	1.4	0.4	5.8	13.3	48.1	7.2
	2	0.4	0.6	16.2	0.9	0.4	5.5	14.4	49.8	5.7
	3	0.5	0.7	16.7	0.8	0.5	4.9	13.9	51.6	4.7

t: less than 0.1%
*Recorded as percent of the total fatty acids present (including unidentified acids).

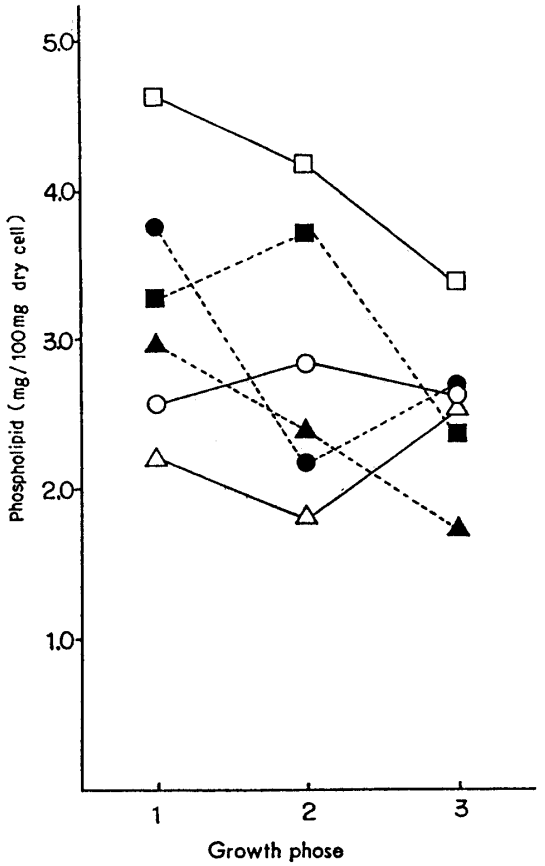

Fig. 2. Contents of cellular phospholipid.
Symbols: *n*-C11 (○); *n*-C12 (●); *n*-C14 (△); *n*-C15 (▲); *n*-C16 (□); glucose (■).

Cellular PL formation PL contents are shown in Fig. 2. PL contents (mg/100 mg dry cell) obtained from glucose-grown cells and from *n*-alkanes-grown cells were: 2.6–2.8 on *n*-C11; 2.2–3.8 on *n*-C12; 1.8–2.5 on *n*-C14; 1.7–3.0 on *n*-C15; 3.4–4.7 on *n*-C16; and 2.4–3.7 on glucose. The PL content of *n*-C16-grown cells was significantly higher than those grown on the other substrates. Its content reached the maximum (4.7 mg/100 mg dry cells) in early log phase of growth, and then decreased gradually with the passage of cultivation. And it was two or three times higher than that of *Cladosporium resinae*.[7]

These results suggest that *n*-C16 is the most suitable substrate for PL formation, and the decrease of PL derived from *n*-alkanes will probably be utilized for the biosynthesis of the other cellular components.

Components of cellular PL As shown in Table 2, cellular PL was separated into five components as follows: PC, PS, PI, PE, and CL. The five components were generally formed in cells grown on any substrate, and no other components were detected in this experiment.

Table 2. Thin layer chromatogram of phospholipids obtained from cellular lipids.

Phospholipid	$R_f \times 100$						
	authentic	*n*-C11	*n*-C12	*n*-C14	*n*-C15	*n*-C16	glucose
Phosphatidyl-choline	10	10	9	10	13	11	11
Phosphatidyl-serine	21	21	18	22	23	21	20
Phosphatidyl-inositol	26	25	24	27	27	25	25
Phosphatidyl-ethanolamine	29	29	29	31	31	29	29
Cardiolipin	44	44	45	47	47	43	43

Solvent system; chloroform : ethanol : triethylamine : water = 30 : 34 : 35 : 8 (by vol.)

The composition of PL is summarized in Table 3. In *n*-C11-grown cells, the major component was CL (28–55%), and PE (21–35%) followed. Moreover, in *n*-C12-grown cells, the major components were PE (23–32%) and CL (19–31%), and PI (18–22%) followed. The variations of PL compositions depended greatly on growth phases and on substrates in any case. However, CL was a minor component in the cells of the fungi described previously. Therefore, the high content of CL in *n*-C11- and *n*-C12-grown cells was characteristic of the phospholipids in strain no. 250-2.

Fatty acid composition of the isolated PL The fatty acid profiles of PL resulting from the growth at the expense of *n*-alkanes are summarized in Table 4. The data of PL were obtained from cells in the stationary phase of growth, and CL of *n*-C15- and *n*-C16-grown cells were in the log phase of growth.

The dominant feature is that all fatty acids

Table 3. Composition of cellular PL.*

Carbon source	Phase	PL (%)**				
		PC	PS	PI	PE	CL
n-C11	1	1.3	6.5	3.1	34.9	54.3
	2	5.9	6.9	6.4	26.0	54.8
	3	14.2	16.3	20.6	21.0	28.0
n-C12	1	9.1	21.0	18.4	32.1	19.4
	2	7.2	16.0	22.1	23.3	31.2
	3	17.6	11.1	19.7	32.3	19.2
n-C14	1	25.1	18.2	14.8	28.8	13.1
	2	21.6	26.1	18.8	24.7	8.9
	3	30.3	5.0	19.7	12.4	32.6
n-C15	1	ND	33.5	2.9	63.5	ND
	2	46.1	3.7	ND	28.4	21.8
	3	8.8	63.7	17.1	10.4	ND
n-C16	1	26.5	ND	ND	49.8	23.8
	2	29.7	4.6	17.1	29.1	19.4
	3	17.1	71.9	0.5	10.5	ND
Glucose	1	ND	35.6	4.9	37.7	21.9
	2	3.0	4.2	41.6	41.3	9.9
	3	27.0	31.8	3.7	21.2	16.4

* Recorded as percent of total PL present.

** Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CL, cardiolipin. ND, not detected.

Table 4. Fatty acid composition of cellular PL.

PL	Carbon source	Fatty acid* (%)								
		14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3
PC	*n*-C11			t		4.8	15.7		52.7	3.3
	C12	4.7	1.9	32.6		2.9	16.4		9.6	
	C14	1.2	0.9	4.5		0.7	21.3		40.1	
	C15	3.6	18.8	15.8		3.3	21.3	8.1	16.5	2.3
	C16	0.9	1.5	21.3		1.1	28.3		26.0	
	glucose			28.6			33.0		9.4	
PS	*n*-C11	0.9	0.7	31.5		3.3	6.7		41.6	1.0
	C12	2.8	1.3	30.2		1.1	9.8	4.3	30.2	
	C14	5.4	5.2	46.0		1.7	10.3	6.1	11.8	
	C15	3.6	4.5	16.2		4.6	18.7	6.6	22.5	
	C16	2.2	0.2	28.5		0.2	12.8	18.0	24.1	2.7
	glucose			66.1		2.4	15.7		6.7	1.9
PI	*n*-C11	4.9	2.0	27.2		2.1	5.9	3.9	24.7	
	C12	12.2	5.9	57.8		6.9	22.7		t	
	C14	5.4	2.2	18.9	0.3	1.1	26.2	7.9	22.6	
	C15	3.8	4.5	20.3		4.1	18.6	7.8	19.4	0.6
	C16	4.5	0.3	29.7		t	15.9	9.2	9.4	
	glucose		1.1	49.1		1.7	15.7		16.1	
PE	*n*-C11	5.4	0.5	28.6		0.7	6.7	13.3	18.6	
	C12	2.7	0.7	27.9		0.8	53.4		t	
	C14		2.7	0.9		1.9	41.5	3.9	34.3	
	C15	1.4	6.7	8.8		4.8	31.1	8.6	25.9	
	C16			t			66.2		25.3	
	glucose	8.2	t	26.3		t	22.4		t	
CL	*n*-C11			26.2		1.1	7.7	4.7	52.0	2.1
	C12		t	35.5			9.1	4.0	45.5	1.6
	C14			27.0		1.7	6.7	1.3	54.4	
	C15**		1.2	4.0		5.1	2.9	5.1	73.5	6.4
	C16**			48.6		3.6	3.9	4.8	34.6	1.2
	glucose		0.3	35.8			5.9	5.4	45.6	2.6

t: less than 0.1%.

* Recorded as percent of total fatty acids (including unidentified acids) present in stationary phase.

** Data of *n*-C15 and *n*-C16 obtained were at log phase.

of PL detected and identified were even-chain fatty acids. The proportion of C18:0 acid was higher (6–66%) in all the PL except CL (7–9%), as compared with cellular lipids (less than 6%). Moreover, the proportion of C16:0 acid was much higher (1–58%) including CL (27–36%) as high as that of cellular lipid (10–30%). The proportion of C18:2 acid was also high (9–53%) especially in CL (46–54%) as high as that of cellular lipid (48–60%).

It is very interesting that the major proportions of fatty acids in CL were different from those of the other PL.

These results suggest that fatty acid incorporated preferentially into PL from *n*-alkane may be served by *de novo* fatty acid biosynthesis in *Aspergillus* sp. no. 250-2.

We found none of the marked differences in the effects of fungi as compared with those of other microorganisms, yeasts[16,17] and bacteria.[18]

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