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Assimilation of Chlorinated Alkanes by Hydrocarbon-Utilizing Fungi†

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The fatty acid compositions of two filamentous fungi (*Cunninghamella elegans* and *Penicillium zonatum*) and a yeast (*Candida lipolytica*) were determined after the organisms were grown on 1-chlorohexadecane or 1-chlorooctadecane. These organisms utilized the chlorinated alkanes as sole sources of carbon and energy. Analyses of the fatty acids present after growth on the chlorinated alkanes indicated that 60 to 70% of the total fatty acids in *C. elegans* were chlorinated. Approximately 50% of the fatty acids in *C. lipolytica* were also chlorinated. *P. zonatum* contained 20% 1-chlorohexadecanoic acid after growth on either substrate but did not incorporate C_{18} chlorinated fatty acids.

A number of reports indicate that selected *n*-alkanes can be incorporated into the cellular lipids of filamentous fungi after terminal oxidation of the substrate (4, 8, 11, 12). Equivalent incorporation of substrate also occurs in yeasts (13, 14, 18, 20). This direct incorporation of substrate into cellular fatty acids has been thoroughly examined in bacteria (5, 6, 15–17). In addition, bacteria will incorporate various other substrate molecules (after terminal oxidation) into cellular fatty acids, including 1-alkenes (6, 17), cyclohexylalkanes (2), and chlorinated alkanes (19). The major requirement for this incorporation is the presence, in the molecule, of an alkyl chain of appropriate length. Long-chain *n*-alkanes of >30 carbons are apparently cleaved in the central portion of the molecule, and the resultant fatty acids of moderate length (C_{14} to C_{18}) can be directly incorporated (9).

The similarity of the terminal oxidation pathways in various microbes and the previous report that substrate alkanes and 1-alkenes can be incorporated into the cellular fatty acids of fungi suggest that these eucaryotes might also incorporate chlorinated alkane substrates. This investigation examined whether substrate chlorinated alkanes can be assimilated into the cellular structures of yeasts and fungi. Such incorporation would indicate another route whereby chlorinated compounds enter the biosphere.

Microorganisms. The organisms used in this study were the filamentous fungi *Cunninghamella elegans* (3) and *Penicillium zonatum* (10) and the yeast *Candida lipolytica* (obtained from C. E. Cerniglia, National Center for Toxicological Research, Jefferson, Ark.). Stock cultures were maintained on a mineral salts medium (21) with added *n*-hexadecane (0.2%, vol/vol) or on Sabouraud-glucose agar (Difco Laboratories, Detroit, Mich.).

Media and growth conditions. The fungi were cultured in liquid medium with hydrocarbons as the substrate as previously described (4). The fungal inocula consisted of spores and mycelia eluted from a petri dish culture with 20 ml of the sterile mineral salts medium. After growth, the mycelial mat was recovered and rinsed gently with petroleum ether to remove excess substrate. The yeast cells were grown on a rotary shaker to the late-log phase, harvested by centrifugation, and rinsed with petroleum ether. The chlorinated alkanes 1-chlorohexadecane and 1-chlorooctadecane were obtained from Fluka Chemical Corp., Hauppauge, N.Y.

Fatty acid analysis. Fatty acid methyl esters (FAME) were prepared from intact cells as previously described (5). Residual substrate was removed as described previously (19) except that Silica Gel G (Analtech) thin-layer plates were used. Bands migrating as FAME were eluted with diethyl ether and, after concentration under a stream of N_2, were analyzed by gas-liquid chromatography (GLC).

The FAME were analyzed with a Hewlett-Packard 5880A GLC equipped with a flame ionization detector. Separation was attained on a 15-m SP-2330 fused silica capillary column (Supelco, Inc., Bellefonte, Pa.) with helium as the carrier gas at an inlet pressure of 50 kPa. Column oven temperature was held isothermal at 150°C for 3 min, programmed to 175°C at 5°C/min, programmed to 200°C at 2°C/min, and held at 200°C for 9 min. For isothermal runs, the oven temperature was held at 170°C. Injector and detector temperatures were 250 and 300°C, respectively.

FAME were identified as previously described (5, 6) and by comparison of retention times with those of standards. Chlorinated fatty acids (Cl-fatty acids) were identified by comparison of retention times to Cl-fatty acids from *Mycobacterium convolutum* R22. After growth on 1-chlorooctadecane, *M. convolutum* Cl-fatty acids were positively identified by electron capture GLC and by GLC-mass spectrometric analysis in the electron impact and methane chemical ionization modes (19).

The filamentous fungi *C. elegans* and *P. zonatum* and the yeast *C. lipolytica* were grown on *n*-hexadecane, *n*-octadecane, 1-chlorohexadecane, and 1-chlorooctadecane. The cellular fatty acids were determined by GLC and examined for the presence of Cl-fatty acids. *M. convolutum* R22 contains significant levels of Cl-fatty acids when grown on chlorinated alkanes (19). The profile of Cl-fatty acids recovered from *M. convolutum* R22 after growth on 1-chlorooctadecane (Fig. 1) served as a standard for identification.

GLC profiles of each organism after growth on 1-chlorohexadecane are presented in Fig. 2. The retention times under the conditions used indicate that Cl-fatty acids were produced by each of these organisms.

Results are presented in Tables 1, 2, and 3 indicating the fatty acid composition of *C. elegans*, *P. zonatum*, and *C.*

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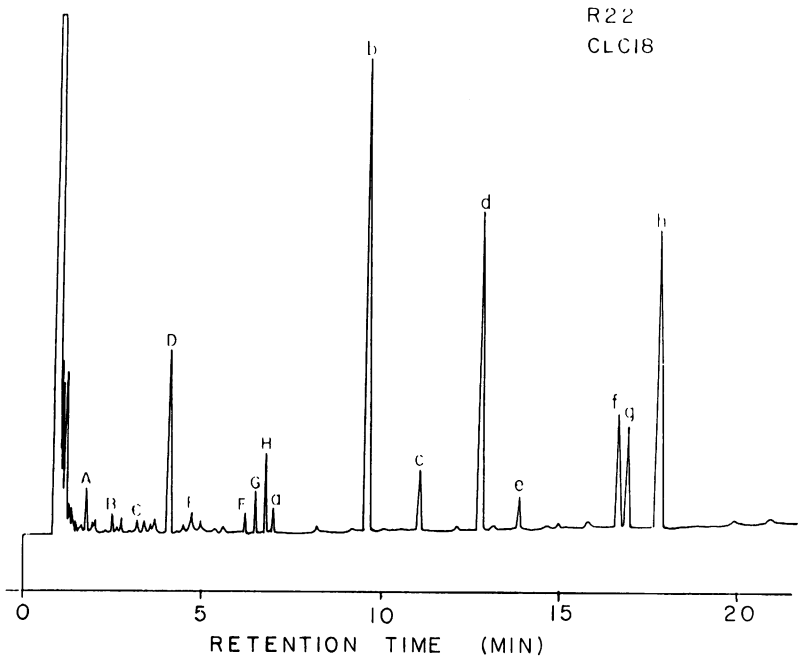

FIG. 1. GLC profile of the FAME obtained from *M. convolutum* R22 after growth on 1-chlorooctadecane. A, C_{12}; a, ClC_{12}; B, C_{14}; b, ClC_{14}; C, C_{15}; c, ClC_{15}; D, C_{16}; d, ClC_{16}; E, $C_{16:1}$; e, $ClC_{16:1}$; F, C_{18}; f, ClC_{18}; G, branched C_{19}; g, branched ClC_{19}; H, $C_{18:1}$; h, $ClC_{18:1}$.

lipolytica after growth on either *n*-alkanes or 1-chlorinated alkanes.

Growth of *C. elegans* on *n*-hexadecane resulted in a cellular composition predominantly of C_{16} and C_{18} fatty acids (Table 1). Growth on *n*-octadecane resulted in somewhat lower levels of C_{16} fatty acids and relatively higher amounts of C_{18} fatty acids. Growth on chlorinated alkanes resulted in the presence of 60 to 70% Cl-fatty acids in *C. elegans*. The major fatty acids in 1-chlorohexadecane-grown cells were ClC_{16} and $ClC_{16:1}$ with lesser amounts of $ClC_{18:1}$.

TABLE 1. Major fatty acids of *C. elegans* after growth on *n*-alkanes and 1-chlorinated alkanes[a]

Fatty acid	% of total fatty acids on the following growth substrate:			
	C_{16}	ClC_{16}	C_{18}	ClC_{18}
$C_{13:1}$	ND[b]	1.7	ND	1.0
C_{14}	1.0	ND	Tr	ND
C_{16}	32.9	6.3	16.8	5.2
$C_{16:1}$	5.7	1.1	Tr	1.0
$C_{17:1}$	ND	ND	ND	1.9
C_{18}	1.4	Tr	12.0	1.2
$C_{18:1}$	24.7	12.8	36.9	10.4
$C_{18:2}$	20.2	8.9	17.1	3.9
$C_{18:3}$	14.6	7.3	15.5	2.2
ClC_{14}[c]		3.6		4.6
ClC_{15}		Tr		Tr
ClC_{16}		30.1		14.4
$ClC_{16:1}$		13.3		5.0
$ClC_{17:1}$		2.6		Tr
ClC_{18}		ND		2.5
$ClC_{18:1}$		11.9		41.5
Unidentified	ND	ND	ND	2.0

[a] Organisms were grown in stationary culture at 28°C for 10 days. Substrates were added at 0.2% (vol/vol).
[b] ND, None detected.
[c] ClC_n, Fatty acid with Cl distal to –COOH.

However, cells grown on 1-chlorooctadecane contained $ClC_{18:1}$ as the major fatty acid. Odd-carbon-length Cl-fatty acids were present after growth on both 1-chlorohexadecane and 1-chlorooctadecane.

P. zonatum had $C_{18:2}$ as the major cellular fatty acid after growth on *n*-hexadecane and *n*-octadecane (Table 2). The amount of C_{16} fatty acid was somewhat higher in *n*-hexadecane-grown cells than in cells grown on *n*-octadecane. The diunsaturated fatty acid $C_{18:2}$ was predominant in 1-chlorohexadecane- and 1-chlorooctadecane-grown cells. The sole Cl-fatty acids present in *P. zonatum* after growth on 1-chlorohexadecane were ClC_{16} and $ClC_{16:1}$. *P. zonatum* grown on 1-chlorooctadecane contained ClC_{16} as the major Cl-fatty acid with trace amounts of odd- and even-chain-length

TABLE 2. Major fatty acids of *P. zonatum* after growth on *n*-alkanes and 1-chlorinated alkanes[a]

Fatty acid	% of total fatty acids on the following growth substrate:			
	C_{16}	ClC_{16}	C_{18}	ClC_{18}
$C_{13:1}$	ND[b]	2.6	ND	1.0
C_{14}	ND	1.3	ND	Tr
C_{16}	24.8	18.1	17.4	23.8
C_{17}	Tr	1.0	1.0	2.4
brC_{18}[c]	ND	ND	1.2	ND
C_{18}	2.5	2.4	5.4	4.4
$C_{18:1}$	8.0	8.4	8.2	5.2
$C_{18:2}$	52.6	44.7	62.4	41.7
$C_{18:3}$	7.7	Tr	2.6	Tr
$C_{20:2}$	1.8	ND	Tr	ND
ClC_{16}[d]		19.7		19.0

[a] Organisms were grown in stationary culture at 28°C for 10 days. Substrates were added at 0.2% (vol/vol).
[b] ND, None detected.
[c] br, Branched.
[d] ClC_n, Fatty acid with Cl distal to –COOH.

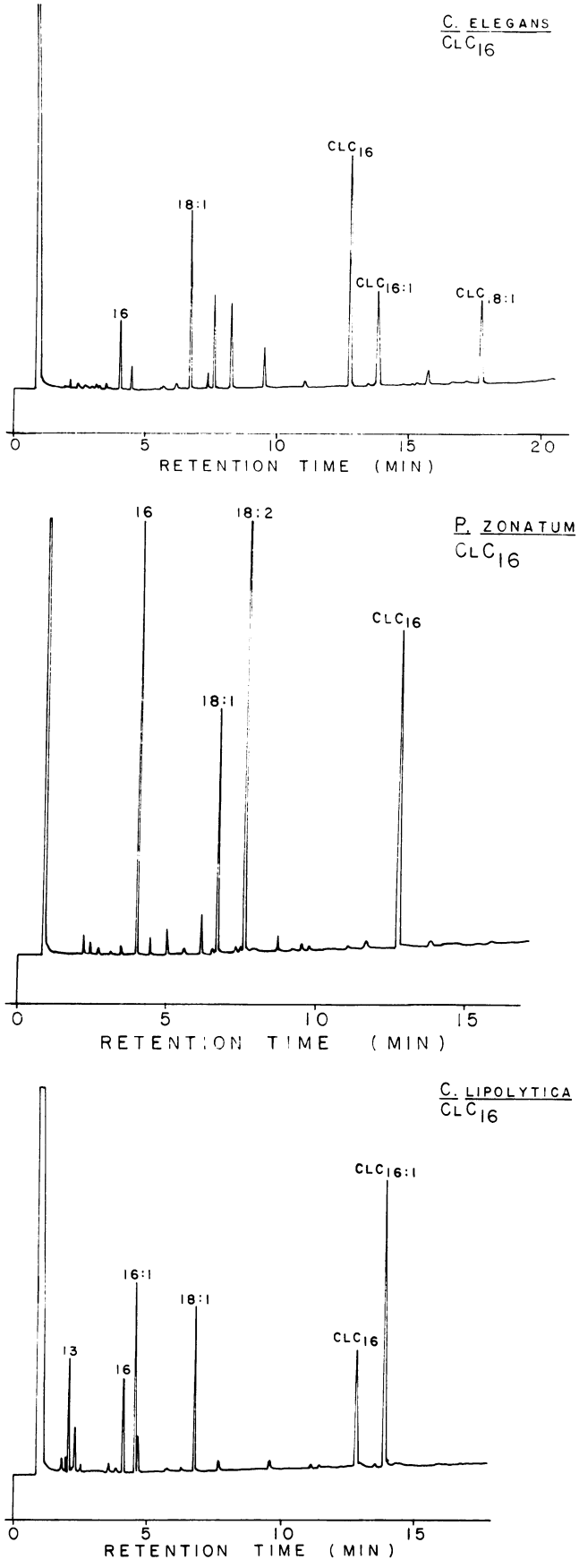

FIG. 2. GLC profiles of the FAME obtained from *C. elegans*, *P. zonatum*, and *C. lipolytica* after growth on 1-chlorohexadecane.

TABLE 3. Major fatty acids of *C. lipolytica* after growth on *n*-alkanes and 1-chlorinated alkanes[a]

Fatty acid	% of total fatty acids on the following growth substrate:			
	C_{16}	ClC_{16}	C_{18}	ClC_{18}
C_{13}	ND[b]	5.9	ND	ND
$C_{13:1}$	ND	2.2	ND	5.0
$C_{14:1}$	Tr	ND	2.2	ND
C_{15}	Tr	ND	1.0	ND
C_{16}	29.3	7.2	15.4	11.6
$C_{16:1}$	30.3	17.2	6.7	4.3
$C_{17:1}$	1.0	ND	1.9	3.5
C_{18}	Tr	Tr	7.9	2.5
$C_{18:1}$	23.2	13.8	44.7	18.3
$C_{18:2}$	13.2	Tr	20.9	3.6
ClC_{14}[c]		Tr		Tr
ClC_{15}		Tr		2.3
ClC_{16}		14.3		24.7
$ClC_{16:1}$		39.4		18.4
$ClC_{17:1}$		ND		4.0
ClC_{18}		ND		1.7

[a] Organisms were grown on a rotary shaker at 28°C and harvested during late log phase of growth. Substrates were added at 0.2% (vol/vol).
[b] ND, None detected.
[c] ClC_n, Fatty acid with Cl distal to –COOH.

Cl-fatty acids. Growth on either of the chlorinated alkanes resulted in a sum total of about 20% Cl-fatty acids.

C. lipolytica contained mostly C_{16} fatty acids when grown on *n*-hexadecane and predominantly C_{18} fatty acids when grown on *n*-octadecane (Table 3). Cells grown on 1-chlorohexadecane had $ClC_{16:1}$ as the major fatty acid. There were no Cl-fatty acids with a chain length longer than the substrate. Cells grown on 1-chlorooctadecane had low levels of C_{18} Cl-fatty acid (1.7%). The major fatty acids in these cells were ClC_{16} and $ClC_{16:1}$. Traces of odd-carbon-chain-length Cl-fatty acids were also present after growth on either of the chlorinated alkane substrates.

Results presented in Fig. 2 and Tables 1, 2, and 3 indicate that filamentous fungi and yeasts incorporate chlorinated alkanes into cellular lipids. Terminal oxidation at the methyl end of the substrate followed by direct incorporation into cellular fatty acids appears to be the pathway of chlorinated alkane assimilation in these organisms.

Major amounts of terminally chlorinated fatty acids were present in the filamentous fungus *C. elegans* (60 to 70%) and in the yeast *C. lipolytica* (ca. 50%) after growth on 1-chlorohexadecane and 1-chlorooctadecane. These levels are comparable to those reported earlier for bacteria (19). The filamentous fungus *P. zonatum* incorporated markedly less Cl-fatty acid (ca. 20%) when grown on these chlorinated substrates.

The occurrence of ClC_{14} and ClC_{16} fatty acids in these eucaryotic organisms after growth on 1-chlorooctadecane suggests that β-oxidation of the substrate does occur. Also, the presence of odd-chain-length Cl-fatty acids in each of the test organisms indicates the presence of an α-oxidation pathway, probably operating at a low level. An α-oxidation pathway that produces small amounts of odd-chain-length fatty acids from even-carbon-chain-length substrates has been suggested from previous studies with both bacteria (2, 6) and yeasts (13, 14, 18). Chain lengthening was apparent

only in *C. elegans*, as indicated by the presence of $ClC_{17:1}$ (2.6%) and $ClC_{18:1}$ (11.9%) in cells grown on 1-chlorohexadecane. Moreover, the unidentified fatty acid (2.0%) in 1-chlorooctadecane-grown *C. elegans* is apparently ClC_{19}, suggesting that chain lengthening of this substrate might occur. Neither *P. zonatum* nor *C. lipolytica* contained any Cl-fatty acids longer than the original substrate.

C. elegans was the only one of the organisms tested that contained $ClC_{18:1}$ in excess of trace amounts (11.9 and 41.5% after growth on 1-chlorohexadecane and 1-chlorooctadecane, respectively). There was no evidence that the chlorinated C_{18} fatty acid was desaturated to more than one double bond. All attempts to demonstrate the presence of chlorinated diunsaturated or polyunsaturated fatty acids were negative. If the desaturation of $C_{18:1}$ and other monoenoic acids actually occurs at the phospholipid level as suggested by others (1, 7), the presence of the terminal chlorine atom might well block this conversion.

The profile of fatty acids in *P. zonatum* indicates that there are lesser amounts of polyunsaturated fatty acids present in cells after growth on chlorinated alkanes than are present in *n*-alkane-grown cells. This decrease in polyunsaturated acids is accompanied by an almost equal increase in Cl-fatty acids. However, the level of $C_{18:2}$ remains quite substantial, suggesting that in this organism Cl-fatty acids are unable to displace membrane polyunsaturated fatty acids. Both *C. elegans* and *C. lipolytica* grown on chlorinated alkanes had a more significant decrease in total polyunsaturated fatty acids when compared with cells grown on *n*-alkanes. The similarities between *C. elegans* and *C. lipolytica* (as opposed to *P. zonatum*) suggest a commonality in lipid synthesis in these organisms.

Chlorinated alkane assimilation by filamentous fungi and yeasts occurs in a manner similar to that described in previous reports for *n*-alkanes and 1-alkenes. This investigation, along with the previous study (19), provides further evidence that chlorinated compounds can be incorporated into the cellular structures of both procaryotic and eucaryotic organisms. The ability of various organisms to incorporate such compounds might be a pathway for substantial amounts of chlorinated compounds to enter the biosphere.

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