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Chlorinated Fatty Acid Distribution in *Mycobacterium convolutum* Phospholipids after Growth on 1-Chlorohexadecane†

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The composition of phospholipids from *Mycobacterium convolutum* R22 was determined after growth at two temperatures (20 and 30°C) with 1-chlorohexadecane as the substrate. Comparisons were made with the phospholipids of cells grown on *n*-hexadecane. Phosphatidylinositolmannosides and phosphatidylethanolamine (PE) were the major phospholipids in *n*-hexadecane-grown cells. In 1-chlorohexadecane-grown cells, phosphatidylinositolmannosides were approximately half of the total phospholipids, with lesser amounts of PE and cardiolipin (CL). The relative level of PE was greater at 20°C (versus that at 30°C) after growth on either substrate. A determination was made of structure and positional distribution of constituent fatty acid in both CL and PE. The relative amount of unsaturated fatty acid was higher at 20°C. There were two $C_{16:1}$ fatty acids ($C_{16:1\Delta9}$ and $C_{16:1\Delta11}$), and these had positional preferences in both CL and PE. The positional sites of chlorinated fatty acids differed in both CL and PE at the two temperatures. The results confirm that microorganisms can specifically distribute chlorinated fatty acids into cellular phospholipids.

The effect of various environmental factors on the lipid composition of microorganisms has been reviewed (4, 10, 12, 16, 24). Responses in the cell to temperature and other environmental manipulations include changes in the relative amounts of neutral lipid to phospholipid, levels of individual phospholipids, chain length, and the degree of unsaturation or branching in the acyl chain of the fatty acids.

We reported earlier that hydrocarbon-utilizing mycobacteria can oxidize terminally chlorinated *n*-alkanes at the methyl terminus and incorporate the resultant chlorinated fatty acid into cellular lipids (18, 19). These chlorinated fatty acids constitute a major portion of the cellular phospholipids (G. L. Murphy and J. J. Perry, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K220, p. 208). This study was initiated to determine whether there is a positional preference in the incorporation of the chlorinated fatty acids. Growth was at 20 and 30°C to determine whether temperature affected distribution of the chlorinated fatty acids in cellular lipids.

MATERIALS AND METHODS

Microorganism and growth conditions. *Mycobacterium convolutum* R22 (1) was grown on L-salts medium (29) with KH_2PO_4 and $Na_2HPO_4 \cdot 7H_2O$ (each at 4 g/liter) as the added phosphate. The pH of the medium was adjusted to 7 before autoclaving. Liquid hydrocarbon substrates were added at 0.2% (vol/vol). The organism was grown at 20 or 30°C on a rotary shaker and harvested during the logarithmic phase of growth. Hexadecane was obtained from Sigma Chemical Co. (St. Louis, Mo.), and 1-chlorohexadecane was from Fluka Chemical Corp. All data reported were substantiated by at least three separate experiments.

Extraction and separation of lipids. Lipids were extracted from wet or lyophilized cells by the method of Bligh and Dyer (2). Neutral lipids and polar lipids were separated on an activated silicic acid column (Unisil 100/200-mesh). The total lipid extract was placed on the column, and $CHCl_3$ was the eluant for neutral lipids followed by the addition of CH_3OH to remove the polar lipids.

Thin-layer chromatography. Phospholipids were identified chromatographically with 0.25-mm Silica Gel G (Fisher Scientific Co., Pittsburgh, Pa.) thin-layer plates activated at 110°C for 60 min. The solvent for a one-dimensional system was $CHCl_3$-CH_3OH-7 N NH_3 (65:30:4, vol/vol/vol). For two-dimensional chromatography the aforementioned was the first solvent followed by $CHCl_3$-CH_3OH-CH_3COOH-H_2O (65:25:25:4, vol/vol). Preparative separation of phospholipids was accomplished on 0.5-mm Silica Gel H (Analtech) thin-layer plates (activated as above) developed with the first solvent. The individual lipids were visualized with iodine vapors.

Identification of phospholipids. Phospholipids were detected with the phosphorus reagent of Dittmer and Lester (7). The phospholipids were identified by comparing mobilities in the solvent systems with standards and by specific spray reagents as described by Christie (3).

Phosphorus determination. The relative amount of an individual phospholipid was determined by thin-layer chromatography in the one-dimensional system. The phospholipid was scraped from the thin-layer chromatographic plates, and total phosphorus was determined (in the presence of Silica Gel H) by the method described by Christie (3). Appropriate amounts of KH_2PO_4 in the presence of Silica Gel H served as the standard.

Enzymatic hydrolysis of phospholipids. Purified cardiolipin (CL) and phosphatidylethanolamine (PE) were hydrolyzed with snake venom (*Trimeresurus flavoviridis*) in an ether-borate buffer system(3). The mixture was incubated for 24 h at room temperature followed by the addition of $CHCl_3$-CH_3OH (2:1, vol/vol) and then dried over anhydrous sodium sulfate. The reaction products were separated by thin-layer chromatography. Free fatty acids and lysophospholipid bands were scraped from plates and esterified directly on silica gel by heating with 2 ml of benzene and 2 ml of 10% BCl_3 in CH_3OH in sealed glass ampoules at 110°C for 30 to 60 min. Fatty acid methyl esters were extracted with diethyl ether and analyzed by gas-liquid chromatography as described previously (19). Whole-cell fatty acid methyl esters

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TABLE 1. Effect of temperature and growth substrate on the phospholipid composition of *M. convolutum* R22 when grown at 20 and 30°C

Temp (°C)	Substrate	mol% of total phospholipid[a]		
		PIM	PE	CL
30	C_{16}	36.7 ± 3.4	34.6 ± 3.0	28.6 ± 0.6
	ClC_{16}	49.3 ± 1.0	21.0 ± 1.7	30.2 ± 1.6
20	C_{16}	30.4 ± 4.0	44.3 ± 4.2	26.2 ± 2.6
	ClC_{16}	52.1 ± 5.3	30.5 ± 4.6	17.4 ± 5.3

[a] Moles percent was determined from total lipid phosphorus calculations and based on 2 mol of P per mol of CL and 1 mol of P per mol of PE or PIM. Numbers represent the average of three separate experiments ± standard deviation.

were prepared and analyzed by the method of Dunlap and Perry (8).

RESULTS

The relative amount of each phospholipid component in *M. convolutum* R22 after growth on *n*-hexadecane or 1-chlorohexadecane at 20 and 30°C was determined (Table 1). The major phospholipids present were phosphatidylinositol-mannosides (PIM), PE, and CL. In cells grown at 30°C with *n*-hexadecane as the substrate, PIM and PE were the major phospholipids and were present in nearly equal amounts. Cells grown with 1-chlorohexadecane as the substrate (30°C) contained PIM at a higher level, accounting for one-half of the total phospholipid. The relative amount of PE in these cells was significantly lower than was present in comparable cells grown on *n*-hexadecane.

Growth at 20°C on *n*-hexadecane yielded cells with PE as the major phospholipid (44%), and PIM and CL were present in equivalent amounts. In 1-chlorohexadecane-grown cells, PIM was about half the total phospholipid. The relative amount of CL in these cells was quite low (17%).

M. convolutum was grown at 20 and 30°C with *n*-hexadecane as the substrate. Intact cells and purified phospholipids were subjected to fatty acid analysis. The results of these analyses are presented in Tables 2 and 3. The major fatty acids in the organism after growth at these temperatures were C_{16}, C_{17br}, $C_{16:1\Delta9}$; and $C_{16:1\Delta11}$ (90% of the total). The fatty acid constituents in PIM (positions not determined) were equivalent qualitatively and quantitatively

TABLE 2. Fatty acid distribution in *M. convolutum* after growth on *n*-hexadecane at 20°C and the positional distribution of fatty acids in CL and PE[a]

Fatty acid	% Distribution[b] of fatty acid in:					
	Cell	CL		PE		PIM
		sn-1	*sn*-2	*sn*-1	*sn*-2	
C_{14}	6.2	5.1	4.5	Tr	3.6	2.0
C_{15}	Tr	Tr	1.3	Tr	1.1	Tr
C_{16}	48.6	17.7	26.4	20.5	26.5	52.1
C_{17br}[c]	3.8	24.5	6.7	54.2	40.5	30.4
$C_{16:1\Delta9}$	35.8	37.6	31.6	5.8	8.3	6.2
$C_{16:1\Delta11}$	3.1	4.1	24.6	2.0	14.5	5.8
C_{18}	ND	1.2	1.1	6.9	1.3	ND
C_{19br}	Tr	4.5	Tr	6.6	3.4	3.0
$C_{18:1}$	Tr	4.8	3.0	3.8	Tr	ND

[a] The organism was grown on a rotary shaker at 20°C. Substrates were added at 0.2% (vol/vol).

[b] Recorded as the percentage of the total fatty acids present. ND, None detected; Tr, trace.

[c] br, 10-methyl branched.

TABLE 3. Fatty acid distribution in *M. convolutum* after growth on *n*-hexadecane at 30°C and the positional distribution of fatty acids in CL and PE[a]

Fatty acid	% Distribution[b] of fatty acid in:					
	Cell	CL		PE		PIM
		sn-1	*sn*-2	*sn*-1	*sn*-2	
C_{14}	6.9	1.5	5.9	Tr	4.4	3.0
C_{15}	Tr	Tr	1.8	ND	1.1	Tr
C_{16}	51.1	10.0	42.7	25.9	33.6	55.5
C_{17br}	9.7	67.7	30.3	56.1	52.2	28.7
$C_{16:1\Delta9}$	26.1	3.1	1.7	ND	ND	6.6
$C_{16:1\Delta11}$	3.0	3.1	11.0	ND	1.1	1.6
C_{18}	Tr	Tr	1.7	9.7	1.8	ND
C_{19br}	1.3	12.9	4.6	8.3	5.9	4.0
$C_{18:1}$	Tr	Tr	Tr	ND	ND	ND

[a] Conditions and abbreviations are as in Table 2.

in cells grown at either temperature. There was a marked difference in the total unsaturated fatty acid in PE after growth at the two temperatures, with $C_{16:1\Delta9}$ and $C_{16:1\Delta11}$ constituting 23% of the total at *sn*-2 in PE of cells grown at 20°C and 1.1% of those grown at 30°C. At 30°C there was a corresponding increase in saturated and branched fatty acids. CL from these cells had a higher level of the unsaturated fatty acids at both the *sn*-1 and *sn*-2 positions when grown at 20°C as compared with cells grown at 30°C (6 versus 41% and 12 versus 55% at *sn*-1 and *sn*-2, respectively). There were more saturated and branched fatty acids in CL of cells grown at 30°C.

The fatty acids present in cells grown at 20 and 30°C with 1-chlorohexadecane as the substrate were determined (Table 4). There was a marked increase in 14-carbon fatty acid (ClC_{14}) in the organism after growth on the chlorinated substrate. Another difference in fatty acid composition was the somewhat higher levels of unsaturated fatty acids in cells grown at 20°C. Cells grown at 20°C had 18% chlorinated unsaturated fatty acids (14% $ClC_{16:1}$ plus 4% $ClC_{18:1}$) and 6%

TABLE 4. Distribution of fatty acids in cells and PIM of *M. convolutum* after growth on 1-chlorohexadecane at 20 and 30°C[a]

Fatty acid	Cellular distribution (% total)		PIM (% total)	
	20°C	30°C	20°C	30°C
C_{14}	ND	Tr	ND	2.7
C_{15}	ND	3.8	ND	2.1
C_{16}	3.9	5.2	10.6	16.0
C_{17br}	Tr	Tr	1.2	Tr
$C_{16:1\Delta9}$	1.1	Tr	Tr	6.1
$C_{16:1\Delta11}$	Tr	Tr	Tr	ND
C_{18}	Tr	Tr	ND	4.1
C_{19br}	Tr	1.3	1.0	Tr
$C_{18:1}$	Tr	Tr	Tr	4.1
ClC_{12}	1.6	1.1	Tr	ND
ClC_{13}	Tr	Tr	Tr	ND
ClC_{14}	26.2	25.6	19.7	16.1
ClC_{15}	4.2	6.7	8.0	5.7
$ClC_{15:1}$	Tr	Tr	ND	ND
ClC_{16}	38.1	22.1	29.7	19.2
ClC_{17br}	4.4	16.0	17.3	14.6
$ClC_{16:1}$	14.2	5.9	4.2	1.7
ClC_{18}	ND	1.7	ND	ND
ClC_{19br}	1.6	8.6	7.2	5.7
$ClC_{18:1}$	4.5	1.9	1.1	ND

[a] Conditions and abbreviations are as in Table 2 except that ClC_n is a fatty acid with Cl distal to — COOH.

total branched. At 30°C the total of $ClC_{16:1}$ plus $ClC_{18:1}$ was 8% with 24% branched chlorinated acids (16% ClC_{17br} plus 8.6% ClC_{19br}). The major difference in the composition of PIM was in the relative amount of chlorinated fatty acids (Table 4). At 30°C about 35% of the total fatty acid was without chlorination compared with 13% in PIM from cells grown at 20°C.

The fatty acid profile in CL obtained from cells grown on 1-chlorohexadecane is presented in Table 5. The relative level of unchlorinated fatty acids at the *sn*-2 position is approximately twice that at the *sn*-1 position (both temperatures). The increased level of unchlorinated fatty acids at the *sn*-2 position is accompanied by a corresponding increase in chlorinated fatty acids. Branched fatty acids constitute a higher percentage of the total in the CL fraction of cells grown at 30°C compared with that in cells grown at 20°C.

The fatty acid composition of PE in cells grown on the chlorinated hydrocarbon is depicted in Table 6. There is a shift in the total unchlorinated fatty acids with 40% of the total in the *sn*-1 position at 20°C and 14% in that position at 30°C. The *sn*-2 position has 21% at 20°C and 42% at 30°C.

DISCUSSION

This study was initiated to determine the effect of a terminal chlorine substituent on the degree of unsaturation, branching, and positional distribution of fatty acids in the polar lipids of a mycobacterium. The organism was grown in 1-chlorohexadecane at two temperatures (20 and 30°C) as this can effect alterations in lipid composition. Previous studies have clearly shown that chlorohexadecane (18) and *n*-hexadecane (8) are terminally oxidized and incorporated directly into cellular lipids. CL and PIM have been reported to be the major phospholipids of mycobacteria (11, 21). In this study, PE and PIM were the major phospholipids (based on moles percent) in *n*-hexadecane-grown *M. convolutum* R22. PIM was the major phospholipid at 30°C, whereas PE was the major one at 20°C. The increase in PE and decrease in PIM at lower growth temperatures has been observed by Taneja et al. (27) in *Mycobacterium smegmatis*. Dhariwal et al. (6), however, observed increased percentages of CL and decreased levels of PIM at lower temperatures in *Mycobacterium phlei*.

TABLE 5. Fatty acid positional distribution in CL of *M. convolutum* after growth on 1-chlorohexadecane at 20 and 30°C[a]

Fatty acid	% Positional distribution in CL at:			
	20°C		30°C	
	sn-1	*sn*-2	*sn*-1	*sn*-2
C_{14}	ND	1.1	Tr	1.4
C_{15}	ND	ND	ND	Tr
C_{16}	3.4	7.5	3.0	9.0
C_{17br}	1.2	Tr	1.2	Tr
$C_{16:1\Delta9}$	Tr	1.2	1.6	1.0
$C_{16:1\Delta11}$	Tr	1.2	Tr	Tr
C_{18}	1.0	2.7	1.0	4.0
C_{19br}	1.0	Tr	1.7	Tr
$C_{18:1}$	1.2	1.7	Tr	1.1
ClC_{12}	6.7	ND	3.4	ND
ClC_{13}	1.6	ND	Tr	Tr
ClC_{14}	35.6	30.6	17.7	31.0
ClC_{15}	3.8	2.8	2.6	2.8
$ClC_{15:1}$	ND	1.4	ND	1.8
ClC_{16}	23.9	18.4	25.0	16.3
ClC_{17br}	6.0	7.6	23.3	12.8
$ClC_{16:1}$	8.1	14.6	7.4	9.1
ClC_{18}	ND	ND	ND	Tr
ClC_{19br}	2.0	2.1	6.3	3.0
$ClC_{18:1}$	3.1	4.3	3.6	2.5

[a] Conditions and abbreviations are as in Tables 2 and 4.

TABLE 6. Fatty acid positional distribution in PE of *M. convolutum* after growth on l-chlorohexadecane at 20 and 30°C[a]

Fatty acid	% Positional distribution in PE at:			
	20°C		30°C	
	sn-1	*sn*-2	*sn*-1	*sn*-2
C_{14}	1.4	2.0	Tr	3.1
C_{15}	ND	ND	Tr	2.3
C_{16}	21.7	10.2	7.2	20.5
C_{17br}	4.0	2.2	1.2	1.2
$C_{16:1\Delta9}$	ND	Tr	2.5	2.1
$C_{16:1\Delta11}$	ND	Tr	ND	ND
C_{18}	10.2	2.8	2.0	9.8
C_{19br}	4.2	1.9	1.6	1.1
$C_{18:1}$	ND	1.3	ND	2.2
ClC_{12}	ND	ND	Tr	ND
ClC_{13}	ND	ND	Tr	ND
ClC_{14}	10.1	28.7	21.7	17.4
ClC_{15}	ND	3.0	4.8	2.2
$ClC_{15:1}$	ND	ND	ND	ND
ClC_{16}	12.8	15.6	21.4	11.4
ClC_{17br}	26.6	15.5	18.1	14.1
$ClC_{16:1}$	ND	5.4	6.4	1.9
ClC_{18}	ND	ND	ND	3.4
ClC_{19br}	8.1	7.2	8.7	6.4
$ClC_{18:1}$	ND	2.4	2.3	ND

[a] Conditions and abbreviations are as in Tables 2 and 4.

The moles percent of individual phospholipids in cells after growth on 1-chlorohexadecane differed from those in cells grown on *n*-hexadecane at both 20 and 30°C. In 1-chlorohexadecane-grown cells, the relative level of PIM was equivalent and at a higher level than in *n*-hexadecane-grown cells. At a growth temperature of 20°C the level of PE was higher with either of the two substrates. The effect of a decreased growth temperature on PE level varies among organisms, increasing in some (5, 9, 13, 25) and decreasing in others (12, 15, 17, 20, 22, 23). An increase in PE at 20°C has been attributed to a lower turnover rate as the growth temperature decreases (26).

Cells grown on *n*-hexadecane at 20°C had a higher percentage of unsaturated and lesser amounts of branched fatty acid than cells grown at 30°C (Tables 2 and 3). Taneja et al. (27) observed a decrease in branched-chain acids at lower growth temperatures in *M. smegmatis*. Similar results were observed by Kawaguchi and Seyama (14) in corynebacteria that possess 10-methyl branched fatty acids. *M. phlei* (6) and *M. smegmatis* (26) have increased levels of unsaturated fatty acids after growth at 27°C compared with those at 37°C. In *M. convolutum* the 16-carbon saturated fatty acid was selectively incorporated at the *sn*-2 in both CL and PE of cells grown at 20 or 30°C, similar to earlier reports (20, 28). There is evidence for the presence of two $C_{16:1}$ fatty acids in *M. convolutum* as revealed by mass spectrometry (data not reported). The $C_{16:1\Delta9}$ fatty acid occurs at higher levels in the *sn*-1 position in CL and in equal amounts at the *sn*-1 and *sn*-2 positions of PE. The $C_{16:1\Delta11}$ level was increased after growth at 20°C at the *sn*-2 position of CL and PE. Both of these $C_{16:1}$ fatty acids were present in greater amounts in CL than in PE. Similar results were obtained with *Mycobacterium butyricum* (20), *Mycobacterium bovis* (28), and *Mycobacterium tuberculosis* (20). The distribution of fatty acids in PIM (Table 4) did not differ significantly after growth on

1-chlorohexadecane at either temperature. However, the organism did have considerably more chlorinated fatty acids at 20°C (87.2% of the total) than at 30°C (63%).

The growth temperature for strain R22 had a marked effect on the positional distribution of fatty acids when 1-chlorohexadecane was the substrate. At 30°C chlorinated fatty acids were a large percentage of the total in both CL and PE. They were present, for the most part, in higher percentages at the *sn*-1 position of both CL and PE, except ClC_{14} in CL. The high level of ClC_{14} may result from the large Cl^- increasing the bulk of the fatty acid to near that of the C_{16} normally present. The positional distribution at 20°C differed from that at 30°C. In CL, the ClC_{14} fatty acid was present at a higher level in the *sn*-1 position. $ClC_{16:1}$ and $ClC_{18:1}$ appear in higher percentages at the *sn*-2 position, similar to the pattern in 30°C-grown cells.

In PE, the percentage of chlorinated fatty acids was again less than that found in CL. Moreover, the positional preferences of unchlorinated fatty acids at 20°C were reversed from those found at 30°C. In contrast to CL, the amount of branched-chain chlorinated fatty acids was not lower in PE at the lower growth temperature.

The presence of a chlorine substituent causes alterations in phospholipid composition and in the nature of their constituent fatty acids. The effect of changes in growth temperature on the lipid composition differed with the two substrates. CL from *n*-hexadecane-grown cells contained a higher level of unsaturated fatty acids and a lesser relative amount of branched fatty acids at the lower growth temperature. The major change in the CL wiht 1-chlorohexadecane as the substrate (at 20°C) was a relative decrease in branched chlorinated fatty acids. Fatty acid changes in PE at 20°C were equivalent to those in CL with *n*-hexadecane as the substrate. A major response in 1-chlorohexadecane-grown cells was in positional distribution of both chlorinated and other fatty acids.

This is the first clear evidence that chlorinated fatty acids can become an integral part of a microbial cell. The presence of chlorine does result in alterations in phospholipids and their constituent fatty acids. How membrane fluidity might be affected by chlorination is unknown at this time. We also need to know whether chlorinated fatty acids in microbial lipids can be passed on to the lipids of predators that ingest them. Both of these questions are under investigation.

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