Phospholipids of *Nocardi a coeliaca*

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The lipids of *Nocardi a coeliaca* were separated into at least 10 components by the use of thin-layer chromatography. Phosphatidylcholine was the most abundant phospholipid in this organism, accounting for 25 to 40% of the total phospholipids. The major fatty acid components of the phosphatidylcholine were 14-methylpentadecanoic acid (41%), the other C₁₅ and C₁₇ iso- and anteiso-fatty acids (29%), and palmitic acid (13.5%). The next most abundant phospholipid was phosphatidylethanolamine (25 to 30%), followed by phosphatidylinositol (11 to 14%) and cardiolipin (7 to 15%). Phosphatidylethanolamine and phosphatidylinositol were very similar to the phosphatidylcholine in fatty acid composition, whereas cardiolipin was characterized by a higher content of palmitic acid (30%). In all of the phospholipids examined, only trace amounts of monounsaturated fatty acids were present. When washed cells of *N. coeliaca* were incubated with methionine-methyl-¹⁴C for 1 to 3 hr, the radioactivity was mainly incorporated into the choline moiety of the phosphatidylcholine. In contrast, acetate-¹⁴C or glycerol-¹³C was incorporated much more slowly into the phosphatidylcholine than into the other phospholipids and neutral lipids. No phosphatidylcholine was detected in 10 other species of *Nocardia* examined.

There have been several investigations on the lipids of mycobacteria (2, 3). In contrast, relatively little information is available concerning the lipids of *Nocardia*, the bacterium most closely related to the mycobacteria (5, 20). In a previous paper (32), we reported that *N. polychromogenes* contained four major phospholipids (cardiolipin, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol monomannoside) having 10-methylstearic acid residue as the major fatty acid component. However, later studies revealed that not all the species of *Nocardia* possess identical lipid and fatty acid compositions. In this paper, we show that *N. coeliaca* contains phosphatidylcholine as the most abundant phospholipid. To our knowledge, this report is the first to demonstrate the existence of phosphatidylcholine in the *Actinomycetales*.

**MATERIALS AND METHODS**

**Growth of organism.** A strain of *N. coeliaca* kindly supplied by M. Mayama, Shionogi Research Laboratories, Osaka, Japan, was used. *N. polychromogenes, N. asteroides, N. erythropolis, N. leishmanii, N. corallina, N. rubra, N. transvalensis, N. flav a,* and *N. madurae* were also kindly donated by M. Mayama, and *N. lutea*, by S. Fukui, Faculty of Engineering, Kyoto University, Kyoto, Japan. The medium used contained 1% polypeptone (Daigo-eiyo Chemical Co., Osaka, Japan), 0.5% yeast extract (Difco), and 1% glucose; the pH was adjusted to 7.0. The cells were inoculated from a 3-day subculture of the same medium, and were incubated at 30 C for 25 to 100 hr.

**Separation of cellular lipids.** Lipids were extracted with 10 volumes of chloroform-methanol (2:1, v/v) and washed by the method of Folch et al. (7); they were then concentrated with a rotary evaporator below 50 C, and finally were chromatographed on thin-layer plates (0.5 mm thick) of Silica Gel H (Merck Co., Darmstadt, Germany), in chloroform-methanol-acetic acid-water (85:15:10:4, v/v). Total lipids were demonstrated by charring the plates at 250 C for 15 min, after spraying them with 18 % H₂SO₄. Phosphorus, amino groups, reducing sugars, and choline were detected with Dittmer's reagent (6), ninhydrin, anthrone, and Dragendorff's reagent (30), respectively. Each phospholipid band was detected with 2',7'-dichlorofluorescein, scraped off with a razor blade, and then recovered from the plates with chloroform-methanol (1:2, v/v).

**Analysis.** Lipid phosphorus was determined by the method of Bartlett et al. (4). The lipids were hydrolyzed in 3 % HCl overnight, and the resulting water-soluble base was separated by paper chromatography with the solvent of n-butyl alcohol-phenol-80% formic acid-water saturated with KCl (50:50:3:10, v/v).

Gas-liquid chromatographic analysis of the methyl esters of fatty acids was carried out by use of a Packard instrument with argon ionization detector.
The column packed with 15% ethyleneglycol adipate polyester on Chromosorb W was operated at 185°C with a flow rate of 50 ml/min. The methyl esters of fatty acids were obtained by transmethylation of the lipids with 5% HCl-methanol for 3 hr, and were identified by comparison with the retention time of authentic standards.

**Incubation studies.** Flasks containing 2 μC of 14C-labeled precursor (methionine-methyl-14C, acetate-1-14C, or glycero-1-14C), 1 mmole of phosphate buffer (pH 7.0), and the washed-cell suspension of *N. coeliaca* (114 mg, dry weight) in a final volume of 5 ml were incubated with vigorous shaking at 30°C for 1 to 3 hr. After the reaction was stopped by the addition of 10 volumes of chloroform-methanol (2:1, v/v), the lipids were extracted, washed thoroughly with water to remove any free labeled precursor, and chromatographed on a thin-layer plate; then the radioactivity in the lipids was measured with a thin-layer chromatogram scanner (Nippon-musen Co., Tokyo, Japan). The per cent distribution of radioactivity in each lipid component was calculated from the peak area of radioactivity on the thin-layer chromatogram.

Infrared spectrum analysis of phospholipids was performed with a Hitachi ETI-G-type infrared spectrophotometer (Hitachi Co., Tokyo, Japan) as a thin film on KBr discs.

**Materials.** All chemicals used were of the highest purity commercially available. Organic solvents were redistilled before use. The lipids and fatty acids for use as reference standards were purchased from Applied Science Laboratories, State College, Pa. 13-Methyltetradecanoic acid (tuberculostearic acid) was obtained through the courtesy of J. Cason, University of California. Radioactive compounds were obtained from Dai-ichi Pure Chemical Co., Tokyo, Japan.

**RESULTS**

Chromatographic separation of the lipids of *N. coeliaca* on a thin-layer plate. When the total lipids of *N. coeliaca* were chromatographed on a thin-layer plate of silica gel, at least 10 different compounds were observed (Fig. 1). Individual lipid components were tentatively identified on the basis of the specific color reaction and the *Rf* values of water-soluble deacylation products on paper chromatography (Table 1).

**Spot a: neutral lipids.** This spot did not react with any reagent for detection of phosphorus, sugars, and free amino groups. It was shown to contain glycerides and free fatty acids with trace amounts of pigments, by the use of thin-layer chromatography with the solvent of hexane-ether-acetic acid (90:10:1, v/v).

**Spot b: glycolipid.** This spot reacted with anthrone reagent, and its *Rf* was the same as that of monogalactolipid isolated from plant tissues (24).

**Spot c: cardiolipin.** This spot contained phosphorus. The *Rf* value of the water-soluble product obtained after mild alkaline hydrolysis was the same as that of the sample obtained by hydrolysis of *Mycobacterium tuberculosis* polyglycerophosphate.

**Spot d: phosphatidylethanolamine.** This spot gave a positive ninhydrin reaction and contained phosphorus. Mild alkaline hydrolysis yielded a product with the same *Rf* value as that of egg yolk glycerylphosphorylcholine on paper chromatography.

**Spot e: unidentified phospholipid.** The *Rf* of the
unidentified component was very close to that of phosphatidylglycerol.

**Spot f**: phosphatidylcholine. As indicated in Fig. 1 and 2, the $R_F$ of this spot coincided with that of egg yolk phosphatidylcholine. The occurrence of phosphatidylcholine in *Actinomycetales* has not been demonstrated previously (13, 19). To determine whether the presence of phosphatidylcholine is common to genus Nocardia, 10 other strains (*N. polychromogenes*, *N. asteroides*, *N. erythropolis*, *N. leishmania*, *N. corallina*, *N. rubra*, *N. transvalensis*, *N. flava*, *N. madurae*, and *N. lutea*) were also examined. However, phosphatidylcholine was not detected in any of these species. A thin-layer chromatogram of the lipids of *N. polychromogenes* (32) is shown in Fig. 1, in comparison with that of the lipids of *N. coeliaca*. Spot f contained phosphorus and reacted with Dragendorf reagent. The water-soluble deacylation product of this spot gave the same $R_F$ as that of egg yolk phosphatidylcholine on paper chromatography. Acid hydrolysis of this spot yielded choline. The infrared spectrum of spot f showed good agreement with that of egg yolk phosphatidylcholine, in absorption at 2,920, 2,840, 1,030, and 970 cm$^{-1}$; a slight shift of ester absorption at 1,740 cm$^{-1}$ presumably suggests the occurrence of iso- and anteiso-fatty acid esters in the molecule (27).

**Spot g**: glycolipid and phosphatidylserine. Further identification of these components is needed. A compound similar to glycolipid was found to be abundant in *N. polychromogenes*, and its characterization will be described in a subsequent paper.

**Spot h**: phosphatidylinositol. The chromatographic behavior of this spot was the same as that of phosphatidylinositol from *N. polychromogenes* (32).

### Table 1. Characterization and tentative identification of the lipids of *N. coeliaca*

<table>
<thead>
<tr>
<th>Spot</th>
<th>P</th>
<th>NH$_3$</th>
<th>Choline</th>
<th>Sugar</th>
<th>Mild alkaline hydrolysate*</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Neutral lipid</td>
</tr>
<tr>
<td>b</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>Unknown glycolipid</td>
</tr>
<tr>
<td>c</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>GPGPG</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>d</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>GPE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>e</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>GPG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>f</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>GPC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>g</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>GPI</td>
<td>Phosphatidyserine</td>
</tr>
<tr>
<td>h</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Phosphatidylinositol</td>
<td>Phosphatidylinositol mannoside and peptidolipid</td>
</tr>
<tr>
<td>i</td>
<td>±</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Phosphatidylinositol</td>
<td>Unknown</td>
</tr>
<tr>
<td>j</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Phosphatidylinositol</td>
<td>Origin</td>
</tr>
<tr>
<td>k</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Phosphatidylinositol</td>
<td></td>
</tr>
</tbody>
</table>

*GPGPG, di(glycerolphosphoryl)glycerol; GPE, glycerolphosphorylethanolamine; GPG, glycerolphosphorylglycerol; GPC, glycerolphosphorylcholine; GPI, glycerolphosphorylinositol.*

**Spot i**: phosphatidylinositol monomannoside and peptidolipid. This spot gave positive reactions for anthrone and ninhydrin reagents, with faint coloration for phosphorus, possibly suggesting the overlapping of phosphatidylinositol monomannoside and lipids which contain amino acids. On hydrolysis, this spot gave mannose and amino acids including leucine and isoleucine.

The relative proportion of the individual phospholipids was as follows: cardiolipin, 7 to 15%; phosphatidylethanolamine, 25 to 30%; phosphatidylcholine, 25 to 40%; phosphatidylinositol, 11 to 14%; phosphatidylerine, 2 to 6%; phosphatidylinositol monomannoside, 2 to 4%. In conclusion, phosphatidylcholine was proved to be the most abundant phospholipid in *N. coeliaca*, although the amounts of the individual phospholipids varied considerably at different stages and under different growth conditions.

**Fatty acid composition of the individual lipids.** The fatty acid composition in *N. coeliaca* was determined by use of gas-liquid chromatography. The logarithms of the retention times of known mixtures of saturated, monoenoic, iso, anteiso, and tuberculostearic acid-type branched and cyclopropanoic fatty acid methyl esters were plotted against the number of carbon atoms, and the points were connected by straight lines. The unknown bacterial fatty acids were tentatively identified by their position on these graphs. From these relationships, it was revealed that at least four series of fatty acid homologues, saturated, iso, anteiso, and tuberculostearic acid-type fatty acids, occurred in *N. coeliaca* lipids. Table 2 lists the distribution of fatty acids in the individual lipids. The major fatty acids were normal saturated ($C_{14}$ and $C_{16}$) fatty acids and iso and anteiso-branched-chain ($C_{14}$ to $C_{17}$) fatty acids. Tuberculostearic acid-type fatty acids ($C_{17}$
and C₁₆ were found as the minor fatty acids in phospholipids. Only trace amounts of monoenoic fatty acids were detected by thin-layer chromatography on AgNO₃-impregnated silica gel (14). However, no polyunsaturated fatty acids were found.

These results preclude the possibility that the culture of *N. coeliaca* might have been contaminated with fungus-like organisms containing phosphatidylethanolamine, because fungi, like animals and plants, contain polyunsaturated fatty acids. Further, it is noted that, in *N. coeliaca*, phosphatidylethanolamine, phosphatidylethanolamine, and phosphatidylinositol were very similar in fatty acid composition whereas cardiolipin was characterized by much higher contents of palmitic acid (Table 2).

**Incorporation of the radioactive precursors into the individual lipids.** To demonstrate the biosynthetic activity of phosphatidylcholine in *N. coeliaca*, incubation studies with radioactive precursors were carried out. After the resting cells were incubated with methionine-methyl-¹⁴C, the lipids were separated on a thin-layer plate, followed by the measurement of radioactivity. Figure 3 shows that the radioactivity of methionine-methyl-¹⁴C was mainly incorporated into phosphatidylcholine and to a lesser extent into other phospholipids. As illustrated in Fig. 4, the radioactivity in phosphatidylcholine increased continuously during 3 hr of incubation. After acid hydrolysis of the labeled lipids, the radioactivity was exclusively recovered in the water-soluble phase. A paper radiochromatogram of the water-soluble product thus obtained indicated that the major radioactive peak was associated with the spot of authentic choline. These results indicate that *N. coeliaca* is able to synthesize phosphatidylcholine from methionine. For purposes of comparison, acetate-¹⁴C or glycerol-¹⁴C was used as the common precursor of lipid biosynthesis. When the washed cells were incubated with these precursors, higher levels of radioactivity were found in phosphatidylethanolamine, cardiolipin, and neutral lipids; incorporation into phosphatidylcholine and phosphatidylinositol occurred to a much lesser extent, in contrast to the pattern of incorporation of methionine-methyl-¹⁴C. At the initial stage of the incubation, the proportion of incorporation was higher

**TABLE 2. Fatty acid composition of the individual lipid classes from *N. coeliaca***

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Neutral lipid</th>
<th>Cardiolipin</th>
<th>Phosphatidylethanolamine</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidylinositol</th>
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</thead>
<tbody>
<tr>
<td>i 12-14:0</td>
<td>10.9</td>
<td>1.2</td>
<td>t</td>
<td>t</td>
<td>1.7</td>
</tr>
<tr>
<td>n 14:0</td>
<td>2.6</td>
<td>1.0</td>
<td>t</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>i 15:0</td>
<td>10.8</td>
<td>8.9</td>
<td>15.9</td>
<td>9.5</td>
<td>11.3</td>
</tr>
<tr>
<td>a 15:0</td>
<td>2.2</td>
<td>1.1</td>
<td>t</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>n 15:0</td>
<td>1.9</td>
<td>1.5</td>
<td>1.0</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>i 16:0</td>
<td>30.2</td>
<td>31.2</td>
<td>34.7</td>
<td>41.0</td>
<td>40.3</td>
</tr>
<tr>
<td>n 16:0</td>
<td>21.3</td>
<td>30.1</td>
<td>8.9</td>
<td>13.5</td>
<td>10.7</td>
</tr>
<tr>
<td>t 17:0</td>
<td>t</td>
<td>3.0</td>
<td>4.4</td>
<td>7.0</td>
<td>4.4</td>
</tr>
<tr>
<td>t 17:0</td>
<td>t</td>
<td>7.1</td>
<td>2.1</td>
<td>8.1</td>
<td>6.3</td>
</tr>
<tr>
<td>a 17:0</td>
<td>8.6</td>
<td>6.3</td>
<td>11.0</td>
<td>12.6</td>
<td>10.1</td>
</tr>
<tr>
<td>n 17:0</td>
<td>t</td>
<td>1.5</td>
<td>t</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>t 18:0</td>
<td>t</td>
<td>4.6</td>
<td>4.8</td>
<td>5.9</td>
<td>8.8</td>
</tr>
<tr>
<td>n 19:0</td>
<td>2.6</td>
<td>3.6</td>
<td>2.6</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
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<td>t</td>
<td>4.4</td>
<td>t</td>
<td>1.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Unknown</td>
<td>t</td>
<td>t</td>
<td>8.9</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>Unsat.</td>
<td>t</td>
<td>t</td>
<td>t</td>
<td>t</td>
<td>t</td>
</tr>
</tbody>
</table>

* Abbreviations: i, iso; a, anteiso; n, normal; t, tuberculostearic acid-type; unsat., unsaturated fatty acids.
in neutral lipids than in phospholipids. As the incubation time became longer, the radioactivity in phosphatidylethanolamine and cardiolipin increased markedly (Fig. 5). After hydrolysis, the radioactivity incorporated from acetate-1-14C or glycerol-1-14C was recovered in the ether- and water-soluble phases, respectively.

DISCUSSION

Lecithin (phosphatidylcholine) is known to be the most abundant phospholipid in animals and plants. By contrast, this phospholipid appears to occur in only relatively limited groups of bacteria (8, 9, 17, 21, 26). There have been no reports supporting the occurrence of lecithin or its related biosynthetic intermediates in the Actinomycetales. Lanelle et al. (20) reported that the phospholipids separated from Nocardia did not contain choline. Kataoka and Nojima (18) found that the phospholipids of Streptomyces griseus, N. polychromogenes, and Microbispora, as well as those of Mycobacterium, consisted of cardiolipin, phosphatidylethanolamine, and phosphatidylinositol mannioside, but not lecithin. We also found that 10 different species of Nocardia had no lecithin, unlike N. coeliaca. At present, we have no satisfactory explanation for such distinctive features of the lipid composition of N. coeliaca, although this organism, in the seventh edition of Bergey's Manual published in 1957, was described to differ from the other species of Nocardia in the absence of chromogenesis. Recently, Ikawa (13), reviewing bacterial phospholipids, observed that lecithin is present in bacteria requiring highly efficient electron transport, and he also speculated that lecithin-containing bacteria might be the more advanced form in the evolution. Hagen et al. (11) suggested the correlation between bacterial lecithin and intracytoplasmic membrane structure, and demonstrated lecithin to be present in Hyphomicrobium and Nitrocystis oceanus, organisms which have such complex structures (9, 11). Therefore, it may be of great interest to determine whether there are significant differences between N. coeliaca and other species of Nocardia in electron-transport system or intracellular structure.

Furthermore, the fatty acid composition of N. coeliaca is very characteristic. The most commonly occurring iso- and anteiso-fatty acids in gram-positive bacteria are 12- and 13-methyltetradecanoic acids (15, 23, 28, 29), whereas that in N. coeliaca is 14-methylpentadecanoic acid. In the lecithin of N. coeliaca, 14-methylpentadecanoic acid accounted for 41% of the total fatty

![Fig. 3. Radioactive scan of the lipid from N. coeliaca after incubation with methionine-methyl-14C for 3 hr at 30 C. Experimental details are described in Materials and Methods. Thin-layer plate, Silica Gel H (0.5 mm); solvent, chloroform-methanol-acetic acid-water (85:15:10.4, v/v). (A) Neutral lipids; (B) unidentified; (C) cardiolipin; (D) phosphatidylethanolamine; (E) unidentified; (F) phosphatidylcholine; (G) phosphatidylinositol; (H) origin.](http://jb.asm.org/)

![Fig. 4. Distribution of radioactivity among the individual lipid classes during incubation with methionine-methyl-14C. Experimental details are described in Materials and Methods. Symbols: O, phosphatidylcholine; △, phosphatidylethanolamine; ●, neutral lipids; ■, cardiolipin; Δ, phosphatidylinositol; □, other lipids.](http://jb.asm.org/)
These amount lecithin of the presented 87 other acids, but 13-methyltetradecanoic and 15- and 14-methylhexadecanoic acids comprised only 9.5, 6.5, and 12.6%, respectively (Table 2). The lecithin in animals (1), plants (10), algae (25), and yeast (I. Yano, Y. Furukawa, and M. Kusunose, unpublished data) has been recognized to contain generally highly unsaturated fatty acids as the major components. Further, the lecithin of Agrobacterium (12) and photosynthetic bacteria (31) was reported to contain a large amount of 18-carbon monoenoic acid. However, in the lecithin of N. coeliaca, only trace amounts of unsaturated fatty acids were detected; on the other hand, branch-chain fatty acids represented 87% of the total fatty acids (Table 2). These findings lead us to suggest that the lecithin in N. coeliaca might be substantially different from its counterparts in other organisms, in chemical or physical properties.

The results obtained from the incorporation of methionine-methyl-\(^{14}\)C into lecithin suggest that N. coeliaca possesses a pathway for the biosynthesis of lecithin by stepwise methylation (16, 22). It should be noted that the incorporation of acetate-\(^{1-14}\)C or glycerol-\(^{1-14}\)C was much slower in lecithin than in other lipids. It has been reported that in animals and plants lecithin had a very high turnover rate among various phospholipids (1, 10, 25). Therefore, it may be suggested that the lecithin in N. coeliaca plays a more important role with regard to the structure of the cells. Similar results were found by Lascelles and Szilagyi (21) in studies of Rhodopseudomonas, in which phosphatidylglycerol, rather than lecithin, was the most metabolically active phospholipid.

**ACKNOWLEDGMENTS**

We express our gratitude to M. Mayama, Shionogi Research Laboratories, Shionogi Co., Osaka, Japan, for his valuable suggestions and his generous gifts of many strains of Nocardia, and also to Y. Noda, Laboratory of Chemistry, Osaka City University Medical School, Osaka, Japan, for his valuable performance in infrared spectra.

**LITERATURE CITED**


Fig. 5. Distribution of radioactivity among the individual lipid classes during incubation with acetate-\(^{1-14}\)C. Experimental details and symbols are the same as those described in Fig. 4.


