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Phospholipid Metabolism During Penicillinase Production in *Bacillus licheniformis*

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During membrane-bound penicillinase production, *Bacillus licheniformis* forms vesicles and tubules that do not appear in the absence of penicillinase production. The major lipids of *B. licheniformis* were shown to be phospholipids. The proportions, metabolism, and the total phospholipid per cell were shown to be essentially the same in the uninduced control, induced and constitutive penicillinase forming cells during both the exponential and stationary growth phases. Membrane phospholipids were not secreted into the medium during penicillinase formation. In the shift from the exponential to the stationary growth phase, there was an accumulation of phosphatidyl glycerol and a marked decrease in cardiolipin. These two lipids had the most active turnover of their phospholipid phosphate of all the lipids studied.

A significant proportion of the inducible exoenzyme penicillinase in *Bacillus licheniformis* is membrane-bound. This bound penicillinase is accessible to exopenicillinase antibodies in whole cells and can be liberated by proteolytic enzymes from cells in hypotonic medium (12) or from membrane fragments, in which it comprises 6 to 8% of the total membrane mass (13). Detailed studies have shown that penicillinase secretion is associated with the development of mesosome-like vesicles (pits in negatively stained preparations) and microtubules in the periplasmic space (9). The periplasmic tubules contain 60% of the bound penicillinase activity. These tubules can be released after protoplasting the cells (26). These isolated microtubules contain a specific activity of penicillinase that is six times greater than in washed membrane preparations (26). The process of penicillinase secretion can be partially separated from its synthesis. Synthesis stops either when the inducer is destroyed by penicillinase activity (21) or when protein synthesis is inhibited by chloramphenicol (14). The maximal rate of release in the absence of synthesis is about 8.7% of the bound penicillinase released per bacterial doubling time (14). Release of bound penicillinase from the vesicles is both temperature and *p*H dependent (25). This release is inhibited by tertiary amines and detergents, suggesting that an enzyme may be involved (25). The penicillinase secreted by the cells is identical to the penicillinase released from membranes by trypsin except for a terminal lysine (1, 22). The membrane-bound penicillinase apparently exists in a polymerized form of the exoenzyme that is in a different conformational state as reflected by its ability to bind deoxycholate (28). The transformation from a lipophilic polymer to lipophobic exoenzyme might be expected for an enzyme that must pass through the membrane (28). The kinetics of penicillinase secretion suggest a membrane-bound intermediate with a long residence time (6), although the residence time is shorter during periods of rapid synthesis (27). The extensive membrane tubular vesicles and pits that appear when penicillinase secretion is induced, and the fact that most penicillinase secretion involves a membrane-bound intermediate that is converted from a lipophilic to lipophobic protein, suggested that *B. licheniformis* would be an ideal system in which to test the involvement of the membrane lipids in the secretory process.

MATERIALS AND METHODS

Growth of B. licheniformis. *B. licheniformis* strain 749 and 749C were supplied by B. K. Ghosh and J. O. Lampen. The cells were maintained on potato agar slants (B. K. Ghosh, *personal communication*). Inocula were prepared by growth on nutrient agar petri plates for 18 hr at 35 C. Approximately 10^8 cells were collected from the petri plates and inoculated into 1,000 ml of the casein hydrolysate plus salts medium (23) in low-form Erlenmeyer flasks and grown with shaking at 35 C. Cells were harvested by centrifugation at 16,000 × *g* for 10 min, the medium was decanted, and the cells were suspended in 50 mM phosphate buffer, *p*H 7.6. Turnover experi-

ments involved growth with ^{32}P, centrifugation, washing at room temperature, and inoculation into warm nonradioactive medium (37). There was no lag period after the exponentially growing cells were resuspended in the nonradioactive medium. Bacterial density was measured by the absorbance at 750 nm in 13-mm diameter round tubes. The absorbance was linearly related to the dry weight of both strains (36). An absorbance of 1.0 was equivalent to 0.3 mg (dry weight) per ml.

Penicillinase assay. Penicillinase was assayed by iodometric titration (20) with penicillin G as standard. The penicillinase was assayed both in the supernatant medium and after release from the resuspended bacterial cells by trypsin (12, 13). Penicillinase secretion was induced in the presence of 2.4 μM cephalosporin C (9). Cephalosporin C (kindly supplied by R. S. Griffin, Lilly Laboratories, Indianapolis, Ind.) is slowly hydrolyzed by penicillinase (V_{max} = 0.5% of benzylpenicillin) and induces continuous production of the enzyme (21).

Extraction and analysis of the lipids. The lipids were extracted from the intact cells by the Bligh and Dyer method (4). Fatty acids were released from the extracted lipids and cells by saponification (35) and were assayed colorimetrically (15) with palmitic acid used as the standard. Neutral lipids and phospholipids were separated chromatographically with a silicic acid column (35). No lipid phosphate was recovered in the neutral lipid fraction, and the recovery of fatty acids was quantitative. The individual phospholipids were separated by two-dimensional chromatography on silica gel-impregnated paper (Whatman SG-81; reference 39). Lipids were quantitatively recovered from the papers (36) after location by autoradiography (34) and were deacylated by mild alkaline methanolysis (34). Mild alkaline methanolysis quantitatively deacylates the lipids into fatty acid methyl esters and glycerol phosphate esters (36). The glycerol phosphate esters were separated by two-dimensional chromatography on aminocellulose paper (Whatman AE-81; reference 38) or on cellulose thin-layer plates (Eastman 6064) by using the solvents described previously (30). Authentic standards were utilized from other bacteria (34, 36). For lipid analysis, the lipids from cells grown in the presence of ^{32}P were separated by chromatography on SG-81 paper, located by autoradiography and the paper corresponding to the spots cut out. The radioactivity in the lipid was determined by putting the paper into the toluene scintillation fluid and counting in the scintillation spectrometer (34). The paper was then removed (no ^{32}P remained in the scintillation fluid), rinsed twice in fresh toulene, cut into small pieces, and digested in perchloric acid; the phosphate was determined colorimetrically (36). Carrier-free $H_3{}^{32}PO_4$ in 0.02 N HCl (Tracer Lab, Waltham, Mass.) was used.

RESULTS

Extraction of the lipids. Exponentially growing *B. licheniformis* 749C was extracted with the Bligh and Dyer procedure. The fatty acids recovered in the extract, the fatty acids remaining in the residue, and the fatty acids in the unextracted cells were compared (Table 1). The Bligh and Dyer procedure extracted 96% of the total fatty acids. Of the lipids extracted by this procedure, the neutral lipids accounted for 20% of the total fatty acids. The total phospholipid content, 80 μmoles of lipid P per g (dry weight), is typical of other bacteria (34).

Separation and identification of the phospholipids. *B. licheniformis* was grown for two doublings (3 hr) in the presence of 800 μc of $H_3{}^{32}PO_4$ per 1,000 ml. The lipids were separated by chromatography in two dimensions on silica gel-impregnated paper (Fig. 1). Four chromatograms similar to that illustrated in Fig. 1 were prepared, the phospholipids were located by autoradiography, and the lipid corresponding to each spot was eluted from the paper. The lipids were then individually deacylated by mild alkaline methanolysis, and the water-soluble glycerol phosphate ester derived from each lipid was identified by its chromatographic mobility in aminocellulose paper chromatography and Eastman cellulose thin-layer chromatography. Each ester was examined in two solvents on both solid phases. Phosphatidic acid (PA) gave rise to glycerol phosphate (GP). The phosphatidyl-ethanolamine (PE) gave rise to glycerol phosphoryl-ethanolamine (GPE). The phosphatidyl glycerol (PG) gave rise to glycerol phosphoryl glycerol (GPG). The cardiolipin (CL) gave rise to bis glycerol phosphoryl glycerol (GPGPG). A trace lipid phosphatidyl "X" (PX) gave rise to a glycerol phosphoryl ester with a unique chromatographic mobility. The lysyl phosphatidyl glycerol (LPG) gave rise to GPG. The spot nearest the origin after deacylation contained GPE, GPG, and GPGPG. The mobility of the lipid suggested the spot contained a mixture of lysophospholipids.

Penicillinase production. *B. licheniformis* 749 in the presence of 2.4 μM cephalosporin C, and the magnoconstitutive strain 749C produced 20 to 100 times the total penicillinase found in the uninduced 749 cells throughout the growth cycle (between 0.1 and 0.6 mg, dry weight, per ml).

The total penicillinase represented the penicillinase recovered in the supernatant fluid after centrifugation of the cells plus the penicillinase released from the cells after treatment with trypsin (12). Approximately 50% of the penicillinase was found to be membrane-bound in the uninduced, induced, and constitutive cells throughout the growth cycle.

Proportions of the phospholipids in B. licheniformis during penicillinase production in the exponential growth phase. A comparison of the proportions of the phospholipids extracted from

TABLE 1. *Fatty acid distribution in Bacillus licheniformis 749C*

Determination	Fatty acids (μmoles per g dry wt)
Total[a]	193
Bligh and Dyer extract	180
Neutral lipid	38
Phospholipid	150
Residue	8

[a] Exponentially growing cells (10.5 mg dry wt) were suspended in 1 ml of 50 mM phosphate buffer, *p*H 7.6. A 0.5-ml amount was saponified directly, and the fatty acids were determined colorimetrically (15); 0.5 ml was extracted by the Bligh and Dyer procedure (4). A portion of the extract was saponified and analyzed; the remainder was fractionated on a silicic acid column into neutral lipids (<0.01 μmole P) and phospholipids (0.42 μmoles P or 81 μmoles P per g dry weight) and saponified, and the fatty acids were analyzed. Recovery from the column was 104%. The residue of the Bligh and Dyer extract was saponified and the fatty acids were analyzed. The extract plus the residue represented 98% of the fatty acids found in the saponified bacterial suspension.

the 749 strain in the presence and absence of cephalosporin and from the 749C strain indicated essentially no difference in total phospholipid per gram, dry weight (Fig. 2). The proportions of the lipids remained about 34% PG, 24% Cl, 20% PE, 6% LPG, 6% PA, 6% lysophospholipids, and 4% PX throughout the exponential growth phase.

Metabolism of the phospholipid phosphate during penicillinase production. Exponentially growing *B. licheniformis* 749 was first grown in medium containing $H_3{}^{32}PO_4$, harvested, and then transferred to non-radioactive medium. The total ^{32}P in the LPG, PA, PX, PE, and the lysophospholipids did not change in 50 min (0.57 doublings; Fig. 3). The PG lost half its ^{32}P in 24 min (0.27 doublings), and the CL lost half its ^{32}P in 30 min (0.3 doublings). There were essentially no differences in phospholipid metabolism among induced, uninduced, and constitutive penicillinase-producing cells.

Excretion of phospholipid with penicillinase production. *B. licheniformis* was grown with 500 μc of ^{32}P per 1,000 ml for 10 hr during the exponential growth phase (0.07 to 0.19 mg, dry weight, per ml). Samples were removed, the cells were harvested by centrifugation, and the ^{32}P in the lipids from the cells and the surrounding medium were determined. The cell residue showed a logarithmic increase in ^{32}P that paralleled the density of the culture. The lipid ^{32}P in the supernatant medium represented less than 1% of the total ^{32}P added to the culture and did not change during the growth period. No phospholipid was released into the medium during penicillinase secretion in the exponential growth phase.

Phospholipid composition and metabolism during penicillinase production in the stationary growth phase. Recent studies (8) indicated that the greatest morphological changes which occur with penicillinase secretion occur in the stationary growth phase, about 10 hr after inoculation. Accordingly, the phospholipid composition and metabolism in the late exponential and early stationary growth phases were examined. There were no differences in either the proportions or metabolism of the phosphate of the phospholipids between the control strain, the control strain plus the inducer, or the constitutive strain in the growth period (Fig. 4 and 5).

There were differences in the phospholipid composition between exponential and stationary phase cells. The stationary phase cells contain twice the proportion of PG of exponentially

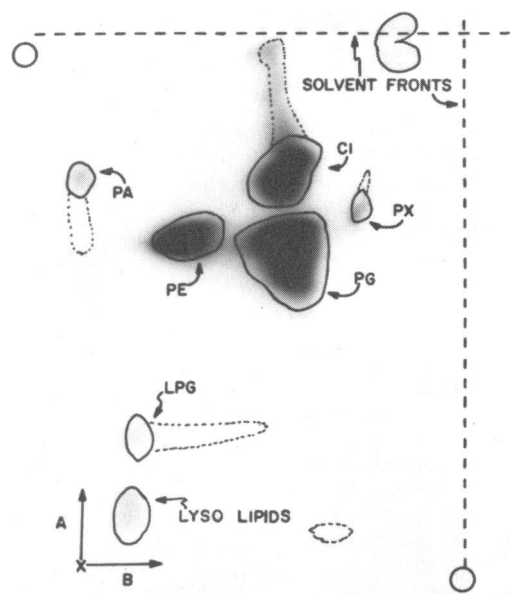

FIG. 1. *Autoradiogram of phospholipids from B. licheniformis 749 grown with ^{32}P after chromatography on silica gel-impregnated paper (SG-81). A total of 0.25 μmole of phospholipid (1.7 × 10^7 counts/min of ^{32}P) was spotted at the origin and chromatographed in solvent A [(first dimension) chloroform-methanol-diisobutylketone-acetic acid-water (12:5:23:13:2, v/v)] and then in solvent B [(second dimension) chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium acetate buffer, pH 10.4 (15:9:13:18:3, v/v)]. The autoradiogram was made by exposing the chromatogram to Kodak no-screen X-ray film for 24 hr and placing the developed film over a diagram of the chromatogram.*

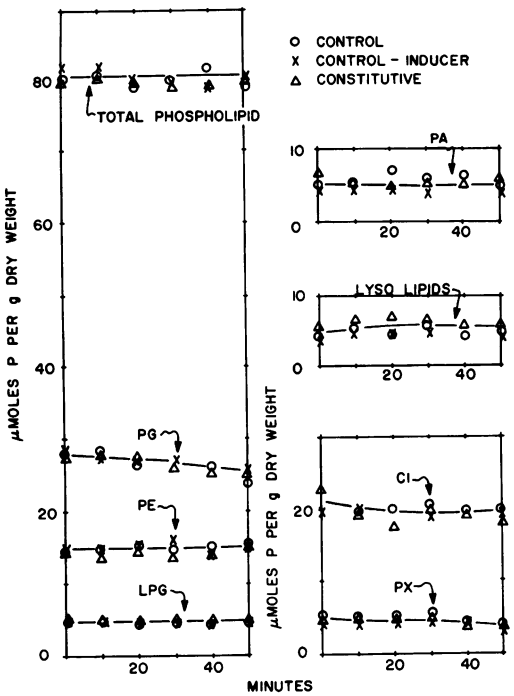

FIG. 2. *Proportions of the phospholipids of B. licheniformis during penicillinase secretion in the exponential growth phase. Strain 749 and the magnoconstitutive 749C strain were grown in 1,800 ml of casein hydrolysate plus salts medium (23) until the density reached 0.03 mg (dry weight) per ml when 2.4 μM cephalosporin C was added to one of the flasks containing strain 749. After the cells reached a density of about 0.07 mg (dry weight) per ml, 600 μc of $H_3{}^{32}PO_4$ was added, and the cells were allowed to grow to a density of about 0.1 mg (dry weight) per ml. The cultures were then centrifuged, washed in 50 mM phosphate buffer (pH 7.6), suspended in nonradioactive buffer at 35 C, and added to 1,800 ml of nonradioactive medium prewarmed at 35 C. At 10-min intervals, 250-ml samples were withdrawn (containing at least 2 μmoles of phospholipid), poured onto an equal volume of ice, centrifuged at 4 C, extracted, and chromatographed as in Fig. 1. During the period of growth in nonradioactive medium, the bacterial density increased from 0.1 mg (dry weight) per ml to 0.15 mg (dry weight) per ml with a doubling time of 85 to 90 min for all cultures. The proportion of phosphate in each lipid was determined after a total of 0.8 μmole of phospholipid was separated on three silica gel-impregnated papers, and spots corresponding to each lipid were pooled and analyzed (at least 0.04 μmole of PX). Symbols: O, strain 749 grown without inducer; X, strain 749 grown with 2.4 μM cephalosporin C during the labeling and the growth in nonradioactive medium; Δ, magnoconstitutive strain 749C. All ordinate scales are linear.*

growing cells but smaller proportions of the other lipids. As the growth rate decreased in the stationary growth phase, the proportion of CL decreased rapidly (Fig. 4). In the late exponential growth phase, the generation time increased twofold, but the time necessary for half the ^{32}P to disappear from the PG and CL increased threefold over the exponentially growing cells (Fig. 5). As the cells entered the stationary phase, the metabolism of the phospholipid phosphate essentially stopped. The proportion of ^{32}P in the lysolipids in the stationary phase was considerably higher than in the exponentially growing cells. In this growth period, the development of periplasmic vesicles and pits associated with penicillinase secretion was not reflected in the total lipid phosphate per cell (Fig. 5).

DISCUSSION

In cells actively producing penicillinase, the cell-bound form can represent up to 8% of the membrane (13). Although penicillinase secretion or storage is associated with the appearance of structures like the periplasmic tubules and pits in the membrane that contain the cell-bound enzyme (8, 9, 25, 26), recent evidence suggests that at least a portion of the penicillinase can be liberated without forming a membrane-bound intermediate (5, 27). With all of this supposed membrane-related activity, there was no detectable difference in the proportions or metabo-

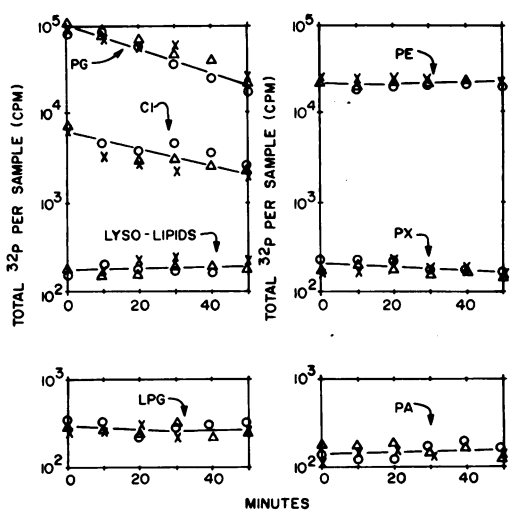

FIG. 3. *Turnover of ^{32}P in the phospholipids of B. licheniformis during penicillinase secretion. The total ^{32}P in each lipid during the growth in nonradioactive medium from the experiment described in Fig. 2 is plotted in Fig. 3; the same symbols are used.*

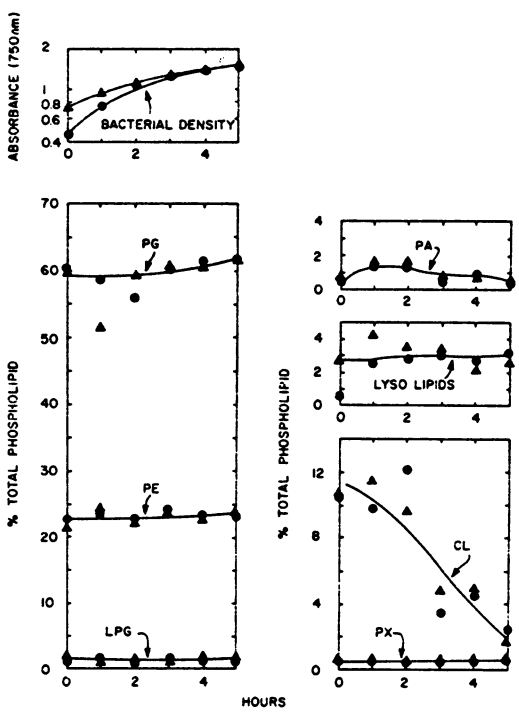

FIG. 4. *Phospholipid composition of B. licheniformis in the stationary growth phase. Symbols: △, lipids from strain 749C constitutive for penicillinase secretion; ○, strain 749, the control. Upper curve shows the increase in bacterial density of cells which had grown for 6 hr before the first sample, measured as the absorbance at 750 nm in 13-mm round cuvettes (7). Lipids were analyzed as in Fig. 2. Absorbancies at 750 nm between 0.05 and 0.6 correspond linearly to dry weights between 0.026 and 0.31 mg per ml.*

lism of the phospholipid phosphate in either the exponential or stationary growth phase, whether the cells were rapidly forming penicillinase or not (Fig. 2–5). The phospholipids form the most significant portion of the complex lipids in the membrane (Table 1), so that secretion or storage of cell-bound penicillinase apparently does not involve phospholipid metabolism. The great changes in morphology associated with penicillinase production are not reflected in the proportions, metabolism, or total lipid phosphate per cell (Fig. 2–5). Either these structures do not contain lipids or their formation results from rearrangements of existing lipids without metabolism of the phospholipid phosphate.

This study does indicate that *B. licheniformis* 749 contains phospholipids similar to those of *B. subtilis* and to those of another strain of *B. licheniformis* (3, 19, 24). The composition of the phospholipids shifts in the transition from exponential to stationary phase. There is a rapid loss of CL (6.3 μmoles) accompanied by an increase in PG (about 13 μmoles; Fig. 4). Perhaps a cardiolipin-specific phospholipase D like that found in *Haemophilus parainfluenzae* (17) may be active in *B. licheniformis*. The most rapid turnover was observed in PG and CL, which lost half their ^{32}P in about a third of a doubling time in the exponential growth phase (Fig. 3). As the cells enter the stationary growth phase, the loss of ^{32}P from these lipids decreases (Fig. 5). The relatively slow turnover of ^{32}P in phosphatidic acid in the exponential phase (Fig. 3) may indicate that the metabolically active pool of PA decayed so

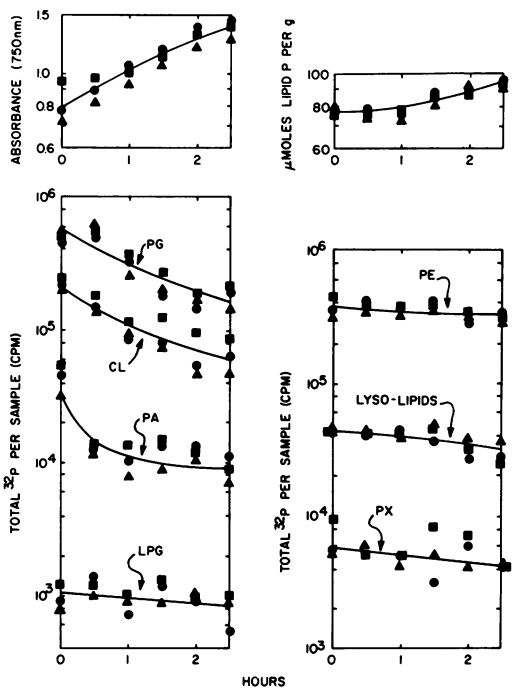

FIG. 5. *Turnover of ^{32}P in the phospholipids of B. licheniformis during the stationary growth phase. Strains 749 and 749C were grown in 1-liter flasks to a density of 0.03 mg (dry weight) per ml when 2.4 μM cephalosporin was added to one of the flasks containing strain 749. When the bacterial density reached 0.20 mg (dry weight) per ml, 900 μc of $H_3{}^{32}PO_4$ was added, and the cells were allowed to grow to a density of 0.4 mg (dry weight) per ml. At this point, the cultures were centrifuged, suspended in 50 mM phosphate buffer, and added to 1 liter of nonradioactive medium at 35 C as in Fig. 3. Samples of 150 ml were withdrawn, and the lipids were analyzed as in Fig. 2. Lipid analysis began 10 hr after the cultures were inoculated. Symbols are the same as in Fig. 2. Dry weight was determined from the absorbance at 750 nm.*

rapidly that it was not seen in these experiments. The turnover of ^{32}P in PA in the stationary phase actually looks biphasic, with a rapid initial loss of ^{32}P (Fig. 5).

The involvement of phospholipids in membrane functions has proved confusing. The metabolism of phospholipids is not affected by changing the extracellular *p*H in *Ferrobacillus ferrooxidans* (30), although aminoesters of PG accumulate during growth at lowered *p*H in other bacterial species (10, 11, 18, 19). Changes in phospholipid metabolism occur during the stimulation of phagocytosis in leukocytes (2, 29) but not in amoeba (33). Phospholipid metabolism is modified during changes in the bacterial electron transport system (7, 38) but not during the transition from rough to smooth endoplasmic reticulum in rat liver, or in the formation of chloroplast lamellae in *Chlamydomonas* (16, 31). In the present study, secretion of penicillinase, half of which was bound to the outside of the membrane and which occurs with marked changes in membrane morphology, has been shown to have no detectable effect on phospholipid metabolism. Perhaps these confusing data can be resolved if the membrane is fractionated into functional subunits before the involvement of the lipids is analyzed. Mosaicism in phospholipid composition has been demonstrated in the membrane of one bacteria (32), and the isolation of tesserae of specific functions could hold the promise of finally beginning the understanding of the role of phospholipids in membrane functions.

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