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Incorporation and Modification of Exogenous Phosphatidylcholines by Mycoplasmas

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The uptake and modification of exogenous phosphatidylcholine (PC) by several *Mycoplasma* and *Spiroplasma* species was investigated. While in most *Mycoplasma* species and in all *Spiroplasma* species tested the PC appears to be incorporated unchanged from the growth medium, the PC of *M. gallisepticum*, *M. pulmonis*, and *M. pneumoniae* was disaturated PC, apparently formed by modification of 1-saturated-2-unsaturated PC from the growth medium. The modification of the exogenous PC by *M. gallisepticum* was inhibited by chloramphenicol under conditions that did not affect de novo synthesis of phosphatidylglycerol. A low activity of an endogenous phospholipase A was detected in native *M. gallisepticum* membranes. The activity was markedly stimulated by treating the membranes with low concentrations of the nonionic detergents. The PC modification was affected by the fatty acid composition of the exogenous PC species. Diunsaturated, 1-saturated-2-unsaturated, and 1-unsaturated-2-saturated PCs were modified to various extents, whereas the disaturated dipalmitoyl PC (DPPC) was not. Both modified and unmodified PCs were incorporated by the cells, but the unmodified DPPC was incorporated at a lower rate and to a lesser extent. The possibility that the incorporation of DPPC into *M. gallisepticum* cells is associated with the formation of intracytoplasmic membranes is discussed.

In the absence of a rigid cell wall (19), the cell membrane of mycoplasmas interacts directly with components of the growth medium. Most mycoplasmas are fastidious organisms requiring serum for growth (19, 30). Direct interaction of *Mycoplasma* or *Spiroplasma* species with the serum lipoprotein particles or other exogenous lipid donors results in the incorporation of exogenous lipid from the growth medium (6, 18, 20, 25). Since the major phospholipids in serum-containing growth medium are phosphatidylcholine (PC) and sphingomyelin (SPM), these phospholipids are the major phospholipids incorporated (20). Some *Mycoplasma* species incorporate more PC than SPM (20), whereas others, as well as all the *Spiroplasma* species tested, incorporate more SPM (6, 20). The incorporated lipids are intercalated into the bulk lipid bilayer (27). In *M. capricolum* the incorporation of exogenous PC was found to alter the de novo-synthesized phospholipids as well as the physical properties of the membrane (11), and more recently, the ability of mycoplasmas to incorporate exogenous phospholipids was correlated with their ability to take up cholesterol (8). In *M. gallisepticum*, cell growth was found to be greatly stimulated by the incorporation of exogenous SPM (22). The incorporation of exogenous phospholipids was first investigated by us in *M. gallisepticum* (25) in which it was found that the SPM was incorporated unchanged whereas the PC was modified by the cells. The modification involves the replacement of the unsaturated fatty acid that occupies position 2 of the serum PC with a saturated fatty acid giving rise to a disaturated PC.

In this report we show that the modification of exogenous PC is characteristic of several but not all mycoplasmas, and we attempted to determine whether incorporation and modification are tightly coupled.

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MATERIALS AND METHODS

Organism and growth conditions. *M. gallisepticum* (A5969), *M. pulmonis*, *M. pneumoniae*, *M. capricolum* (kid), *M. mycoides* subsp. *mycoides*, *M. arginini*, and *M. hominis* were taken from our collection. The *Spiroplasma* sp. strains BNR1, BC3, PBS1, MQ1, and B31 were kindly provided by R. F. Whitcomb (Beltsville, Md.). The *Mycoplasma* spp. were grown in 100- to 500-ml volumes of a modified Edward medium (21), and the *Spiroplasma* strains were grown in the medium of Saglio et al. (29). The medium was supplemented with either 4 to 10% horse serum or 0.5% delipidated bovine serum albumin and a mixture of lipids. To delipidate bovine serum albumin, 50 g of bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) was extracted three times with 200 ml of chloroform-methanol (1:1, vol/vol) at 4°C for 1 h with constant stirring. The extracted material was collected and dried overnight at room temperature. In most experiments the lipid mixture contained palmitic and oleic acids (10 μg of each per ml) and cholesterol (20 μg/ml). The lipid mixture was added to the growth medium as an ethanolic solution. In some experiments 25 μg of egg-PC, 1,2-dipalmitoyl PC (DPPC), 1,2-dioleoyl PC (DOPC), 1-palmitoyl-2-oleoyl PC (POPC), or 1-oleoyl-2-palmitoyl PC (OPPC) per ml was added as an ethanolic solution. All PC preparations were obtained from Sigma. Final concentrations of ethanol in the growth medium did not exceed 0.2%. To label membrane lipids, we added 0.002 μCi of [1-^{14}C]palmitate (50 to 60 Ci/mol) or 0.002 μCi of [1-^{14}C]oleate (50 to 60 Ci/mol) (both products of the Radiochemical Centre, Amersham, England) per ml of medium. The organisms were harvested after 20 to 24 h of incubation at 37°C by centrifugation at 12,000 × *g* for 15 min. The cells were washed once with 0.25 M NaCl at 4°C. For membrane isolation, cells were disrupted by either osmotic lysis or ultrasonic irradiation (25).

Measurement of endogenous phospholipase. The endogenous phospholipase activity of *M. gallisepticum* membranes

TABLE 1. Incorporation of radioactive fatty acids into PCs of various mycoplasmas[a]

Organism	PC in polar lipid (% of total)	Radioactivity (% of total radioactivity in lipids) in PC from cells grown with:	
		[^{14}C]palmitate	[^{14}C]oleate
M. gallisepticum	32.6	40.5	2.0
M. pulmonis	20.4	25.0	6.5
M. pneumoniae	11.8	13.5	2.0
M. capricolum	28.5	4.0	3.0
M. mycoides subsp. *mycoides*	27.1	2.0	3.0
M. hominis	14.5	3.0	3.5
M. arginini	22.1	2.0	1.5

[a] The organisms were grown in medium containing 4% horse serum with either [^{14}C]palmitate or [^{14}C]oleate (0.002 μCi/ml). Lipid analyses were performed as described in Materials and Methods. The data represent the mean values of three experiments with different batches of organisms.

was determined by measuring the release of radioactive fatty acids from membrane polar lipids. Intact membranes (2 mg of protein) or membranes solubilized with sodium dodecyl sulfate, octyl-β-D-glucoside (both from Sigma), or Triton X-100 were incubated with a reaction mixture containing 0.4 M sucrose, 50 mM Tris, 2.5 mM calcium chloride, and 2.5 mM magnesium chloride adjusted to pH 7.5. After incubation at 37°C for up to 4 h, free fatty acids were extracted (9) and assayed for radioactivity. Phospholipase A activity was presented as the percentage of the total radioactivity in membrane lipids.

Lipid analyses. Lipids were extracted from intact cells or isolated membranes by the method of Bligh and Dyer (2). Polar lipids were separated on silica gel HR plates developed first at room temperature with petroleum ether (bp 40 to 60°C)-acetone (3:1, vol/vol) and then at 4°C with chloroform-methanol-water (65:25:4, vol/vol/vol). Lipid spots were detected with iodine vapor, scraped from the plates, and analyzed for radioactivity and lipid phosphorus. Methyl esters of fatty acids were prepared as described before (25), and the esters were subjected to gas-liquid chromatography in a Packard model 437 chromatograph equipped with a polar column (100 by 0.2 cm, packed with 10% SP 2330 on 100/120-mesh Chromosorb W-HP from Supelco, Belfonte, Pa.). Fatty acid methyl esters were identified by their retention times relative to those obtained with standard mixtures of fatty acid methyl esters (Supelco).

Analytical methods. Protein was determined by the method of Lowry et al. (17). Total lipid phosphorus was determined as described by Ames (1) after digestion of the material with ethanolic magnesium nitrate. Radioactivity in the lipid extracts and in the spots from thin-layer plates was measured with 5 ml of scintillation liquid (40% [vol/vol] Lumax [obtained from Lumac BV, Schaesberg, The Netherlands] in toluene). Samples were counted in a Packard Tri-Carb scintillation spectrometer. Sodium dodecyl-sulfate slab gel electrophoresis was performed by the method of Laemmli and Favre (16), using 12.5% acrylamide gels.

TABLE 2. Fatty acid composition of PCs from various *Mycoplasma* species[a]

Organism	Fatty acid content (mol%)			
	16:0[b]	18:0	18:1	18:2
M. gallisepticum	48.5	45.0	3.0	3.0
M. pulmonis	25.0	66.5	2.0	2.0
M. pneumoniae	48.5	39.0	8.0	2.6
M. capricolum	20.5	38.5	20.0	22.0

[a] The organisms were grown in medium containing 4% horse serum. Lipid analyses were performed as described in Materials and Methods.

[b] The first number indicates the chain length, and the second number is the number of double bonds.

RESULTS

Incorporation and modification of exogenous PC by mycoplasmas. When grown in medium containing horse serum, many *Mycoplasma* species incorporate significant amounts of PC into their cell membranes (Table 1). Uptake of PC by the *Spiroplasma* species strains BNR1, MQ1, PBS1, B31, and BC3 was very low (data not shown), reaching levels of 2 to 7% of the total lipids. PC was, however, not detected in *Mycoplasma* or *Spiroplasma* species grown in a medium in which horse serum was replaced with delipidated bovine serum albumin, a fatty acid mixture, and cholesterol (6, 25). Table 1 also shows that when the cells were grown with radioactive fatty acid, the PC in some of the *Mycoplasma* species and in all of the *Spiroplasma* species (data not shown) remained unlabeled, suggesting that the exogenous PC was incorporated unchanged from the growth medium. In *M. gallisepticum*, *M. pulmonis*, and *M. pneumoniae*, however, the PC incorporated was labeled by the radioactive palmitate but not by the radioactive oleate. The labeling intensities (counts per minute per mole of PC P_i) in these three organisms were almost the same, and the fatty acid composition of the PC fraction was highly saturated, differing from that of the PC fraction obtained from *M. capricolum* (Table 2). The PC from *M. capricolum* had almost identical amounts of saturated and unsaturated fatty acids. A simple calculation indicates that the PC fraction from *M. gallisepticum*, *M. pulmonis*, and *M. pneumoniae* must contain at least 87, 83, and 75% disaturated species, respectively.

Endogenous phospholipase activity in *M. gallisepticum*. Heating the growth medium to 56°C for 1 h or replacing it with bovine serum albumin, fatty acids, cholesterol, and egg-PC had no effect on the formation of disaturated PC by

TABLE 3. Effect of detergents on endogenous phospholipase A activity of *M. gallisepticum* membranes[a]

Detergent (%)	Free fatty acid released (% of total) after incubation for:	
	2 h	4 h
None	6 ± 2	14 ± 4
Sodium dodecyl sulfate		
0.05	2 ± 1	12 ± 3
0.5	<1	<1
Octyl-β-D-glucoside		
0.05	5 ± 2	15 ± 4
0.5	27 ± 2	38 ± 3
Triton X-100		
0.05	26 ± 3	34 ± 6
0.5	<1	2 ± 1

[a] [^{14}C]palmitate-labeled membranes (2 mg of protein) were incubated for 2 or 4 h at 37°C with or without detergent in a reaction mixture containing 0.4 M sucrose, 25 mM $CaCl_2$, 25 mM $MgCl_2$, and 50 mM Tris buffer (pH 7.5). Phospholipase A activity was determined as described in Materials and Methods, and the results are expressed as the percentage of radioactive free fatty acid released.

TABLE 4. Effect of chloramphenicol on incorporation of radioactive fatty acid into *M. gallisepticum* lipids[a]

Prep	A_{640}	Cell yield (100 ml)		Radioactivity in polar lipid		
		Protien (mg)	Lipid P_i (nmol)	Total (cpm/μmol of P_i)	PG[b] (% of total)	PC (% of total)
Zero time cells	0.16	3.7	360	0	0	0
Treated cells	0.18	4.0	856	4,390	95.0	5.0
Untreated cells	0.26	8.2	1,072	5,710	69.5	30.5

[a] Cells were grown in a medium containing 4% horse serum to the mid-exponential phase of growth (zero time cells). [^{14}C]palmitate (0.002 μCi/ml) was then added to the culture, and the culture was divided into two parts. To one part chloramphenicol (100 μg/ml) was added; the other part served as a control. The cultures were then incubated for an additional 4 h at 37°C, and cells were harvested and analyzed as described in Materials and Methods.
[b] PG, Phosphatidylglycerol.

M. gallisepticum cells. Our attempts to demonstrate an endogenous phospholipase activity are presented in Table 3. Isolated membrane preparations showed very little activity even after prolonged incubation periods (4 h). The activity was measured with [^{14}C]palmitate-labeled membrane. As in *M. gallisepticum*, the saturated fatty acids are incorporated preferentially into position 2 (25), and the release of radioactivity into the fatty acid fraction suggests a phospholipase A_2 activity. A negligible release of radioactive fatty acids from complex lipids was also obtained with [^{14}C]oleate-labeled membrane. Table 3 also shows that treating the isolated membrane preparations with mild detergents enabled us to demonstrate reasonable levels of endogenous phospholipase A activity. Most pronounced were the activities obtained after treating the membranes with 0.5% octyl glucoside or 0.5% Triton X-100. The phospholipase activity was almost completely inhibited at higher concentrations of Triton X-100. Phospholipase activity was not detected in the soluble fraction obtained after ultrasonic treatment of *M. gallisepticum* intact cells. Furthermore, the addition of the soluble fraction (0.1 to 10 mg of protein per mg of membrane protein) to the membrane preparations did not enhance fatty acid release. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Triton X-100 (0.05%)-treated and untreated membranes revealed that the profile of the treated membrane preparation differed only slightly from the profile of the untreated membranes, primarily with respect to a decrease in the staining intensities of two polypeptide bands having apparent molecular weights of 77,000 and 72,000.

Effect of chloramphenicol on membrane lipid composition. Treating *M. gallisepticum* with chloramphenicol resulted in almost immediate arrest of cell growth (Table 4). Protein synthesis in the treated cells was almost completely inhibited. Yet, the treated cells continued to incorporate radioactive palmitate into complex lipid fractions for at least four additional hours, resulting in an increase in the lipid content of the cell membrane fraction. Lipid analysis of the chloramphenicol-treated cells revealed that the incorporation of [^{14}C]palmitate into phosphatidylglycerol (the only de novo-synthesized phospholipid) was unaffected but that the incorporation of radioactive palmitate into PC was markedly inhibited (Table 4).

Table 5 shows that the amount of exogenous PC incorporated into *M. gallisepticum* cells and the extent of modification of the incorporated PC was affected by the fatty acid composition of the exogenous PC molecules added to the growth medium. After 4 h of incubation at 37°C, substantial amounts of all of the PC species analyzed were incorporated. The incorporation of egg-PC, DOPC, and POPC was highest, whereas OPPC and DPPC were incorporated to a lesser extent. The PCs from cells grown with POPC, OPPC, DOPC, or egg-PC were modified by the organism as indicated by the considerable amount of radioactivity incorporated into the PCs and by the fatty acid profile of the PC fraction (Table 5). As was shown before (25), the cells modify the exogenous PC by binding a saturated fatty acid to position 2 of the glycerol backbone of lyso-PC molecules derived from the PC of the growth medium. Thus, modification of PC molecules having an unsaturated residue at position 2 resulted in an increase in the saturated fatty acid composition (Table 5).

When the various PC species were added to chloramphenicol-treated cells, the incorporation of [^{14}C]palmitate into the PC fractions was inhibited by 70 to 80%, but the cells

TABLE 5. Incorporation of various PC species into *M. gallisepticum* cells in the presence and absence of chloramphenicol[a]

PC in medium (25 μg/ml)	Characteristics of the PC fraction incorporated into:											
	Untreated control cells						Chloramphenicol-treated cells					
	Lipid P_i (% of total phospholipids)	Radioactivity (% of total phospholipids)	Fatty acids (mol%)				Lipid P_i (% of total phospholipids)	Radioactivity (% of total phospholipids)	Fatty acids (mol%)			
			16:0[b]	18:0	18:1	18:2			16:0	18:0	18:1	18:2
Egg-PC	52.0	20.5	66.0	15.0	6.0	3.5	35.5	6.0	35.0	17.5	23.0	14.5
DOPC	52.0	28.0	37.0	2.0	60.5	<0.5	36.0	6.5	11.0	<0.5	87.5	<0.5
DPPC	34.0	4.0	98.0	<0.5	<0.5	<0.5	30.5	3.0	99.0	<0.5	<0.5	<0.5
POPC	48.5	28.5	83.5	<0.5	14.5	<0.5	26.5	3.5	56.5	<0.5	42.0	<0.5
OPPC	40.5	16.0	51.0	<0.5	47.5	<0.5	31.0	5.0	50.0	<0.5	48.5	<0.5

[a] Cells were grown in a medium containing 0.5% delipidated bovine serum albumin, palmitic and oleic acids (10 μg of each per ml), and cholesterol (20 μg/ml) to the mid-exponential phase of growth (A_{640} = 0.15 to 0.20). The cultures were then divided into two parts. To one part chloramphenicol (100 μg/ml) was added; the other part served as a control. [^{14}C]palmitate (0.002 μCi/ml) and the various PC species (25 μg/ml) were then added to the cultures, and the cultures were incubated for an additional 4 h at 37°C. Cells were harvested, and their lipids were analyzed as described in Materials and Methods.
[b] The first number indicates the chain length, and the second number is the number of double bonds.

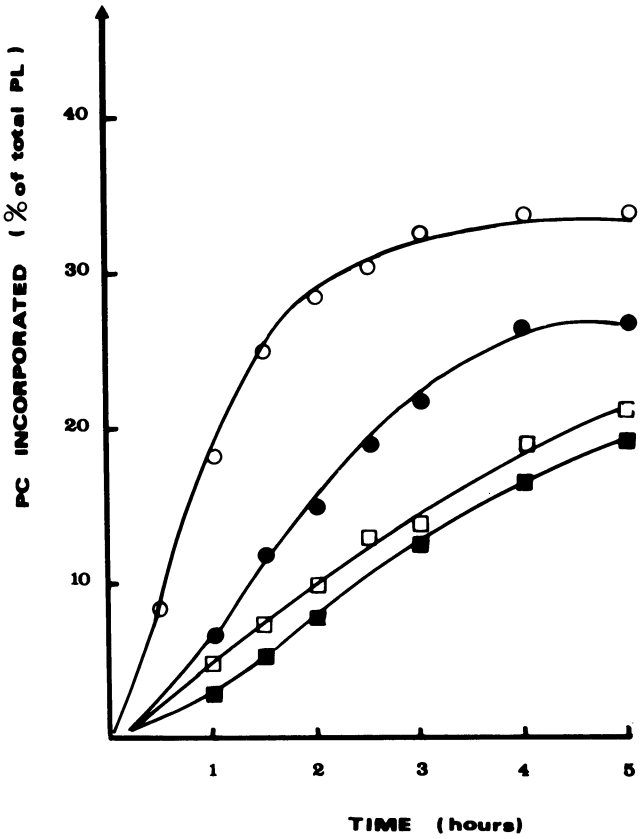

FIG. 1. Effect of chloramphenicol on the rate and extent of incorporation of PC. The experiment was performed as described in Table 5. Symbols: open symbols, untreated cells; closed symbols, chloramphenicol (100 μg/ml)-treated cells; ○, ●, egg-pC; □, ■, DPPC. PL, Phospholipid.

continued to incorporate exogenous PC, although to a lesser extent. The fatty acid profile of the PC from chloramphenicol-treated cells was more unsaturated than the profile of fatty acids from control untreated cells and resembled, to a large extent, the fatty acid composition of the PC molecules added to the growth medium (Table 5). The activity of the endogenous phospholipase in Triton X-100-treated membrane preparations from cells treated with chloramphenicol was the same as that from control cells grown without chloramphenicol (data not shown). Figure 1 shows the rate and extent of incorporation of egg-PC and DPPC into *M. gallisepticum* cells. Egg-PC was incorporated at a higher rate than DPPC. Similarly, the rate and extent of egg-PC incorporation was markedly affected by chloramphenicol, whereas the incorporation of DPPC was not. DOPC was incorporated to the same extent and at the same rate as egg-PC (data not shown).

DISCUSSION

The modification of exogenous PC by *Mycoplasma* and *Spiroplasma* species was followed by measuring the preferential incorporation of radioactive palmitate into the membrane PC fraction and by fatty acid analysis. Our previous study with *M. gallisepticum* (25) showed that the exogenous PC incorporated by this organism is modified by the insertion of a saturated fatty acid at position 2 of the *sn*-glycerol-3-phosphate. As a result, radioactive saturated (but not unsaturated) fatty acids are incorporated into the membrane PC, whereas in the de novo-synthesized phospholipid both saturated and unsaturated fatty acids are incorporated (25). The preferential incorporation of palmitate was found in three of seven *Mycoplasma* species but in none of the *Spiroplasma* strains that were included in our study. Despite the limited number of species, preliminary conclusions can be reached with some caution. In most *Mycoplasma* and *Spiroplasma* species PC is incorporated unchanged from the growth medium. In *M. gallisepticum*, *M. pneumoniae*, and *M. pulmonis* the PC incorporated is modified to a disaturated PC. These three organisms have several common features including peculiar structural features (14), the ability to adhere to inert surfaces as well as to erythrocytes and tissue culture cells (14, 19), and a gliding motility (3, 19). It seems that these organisms, regarded as the flask-shaped mycoplasmas (14), also share a unique biochemical property, the modification of incorporated PC to a disaturated species.

A deacylation-reacylation enzymatic sequence will require the hydrolysis of the ester bond at position 2 of the PC of the growth medium by an enzyme present in either *M. gallisepticum* cells or the growth medium. Since heating the medium to 56°C or replacing it with bovine serum albumin and lipid precursors had no effect on the disaturated PC formed, we attempted to demonstrate an endogenous phospholipase A activity that will hydrolyze the ester bond at position 2 of the exogenous PC. Phospholipase activity seems to be uncommon in mycoplasmas (19, 23). However, lysophospholipase activity was described in *Acholeplasma laidlawii* (31) and *M. gallisepticum* (9), and a phospholipase A was postulated in *M. hominis* (26) and in the *Spiroplasma* species (6). Our result showing very low endogenous phospholipase A activity in native *M. gallisepticum* membranes and a three- to fourfold increase after treating the membranes with mild detergent suggests that a conformational change is required to activate the enzyme. Such a change may be induced in the isolated membranes by the detergents and in the intact cells by a soluble control protein (13). We cannot, however, exclude entirely the possibility that the endogenous phospholipase activity detected after detergent treatment was due to an effect of the detergents on the endogenous substrate(s).

Brief chloramphenicol treatments under conditions that completely arrested protein synthesis have no effect on the de novo-synthesized polar lipids of *A. laidlawii* (12). As a result, the lipid-to-protein ratio in the treated organisms was dramatically increased. Our results show that in *M. gallisepticum* as well, the de novo synthesis of phosphatidylglycerol was not affected by chloramphenicol, but the modification of PC was almost completely inhibited. As the phospholipase A activity in chloramphenicol-treated and untreated cells was identical, it seems that the effect of chloramphenicol is due to either the inhibition of the synthesis of a regulating protein or an effect on the translocation of PC. PC is first incorporated into the outer leaflet of the bilayer but has to be translocated to the inner leaflet where biosynthesis takes place. The effect of chloramphenicol on the translocation of cholesterol in *M. capricolum* cells was described before (4).

The incorporation of exogenous PC by *M. gallisepticum* seems to be uncoupled from the modification of the PC molecules. This conclusion is based on our findings that (i) DPPC was incorporated by the cell although this molecule is not modified and (ii) egg-PC, DOPC, POPC, and OPPC were incorporated by chloramphenicol-treated *M. gallisepticum* cells in which modification was inhibited to a large extent. In

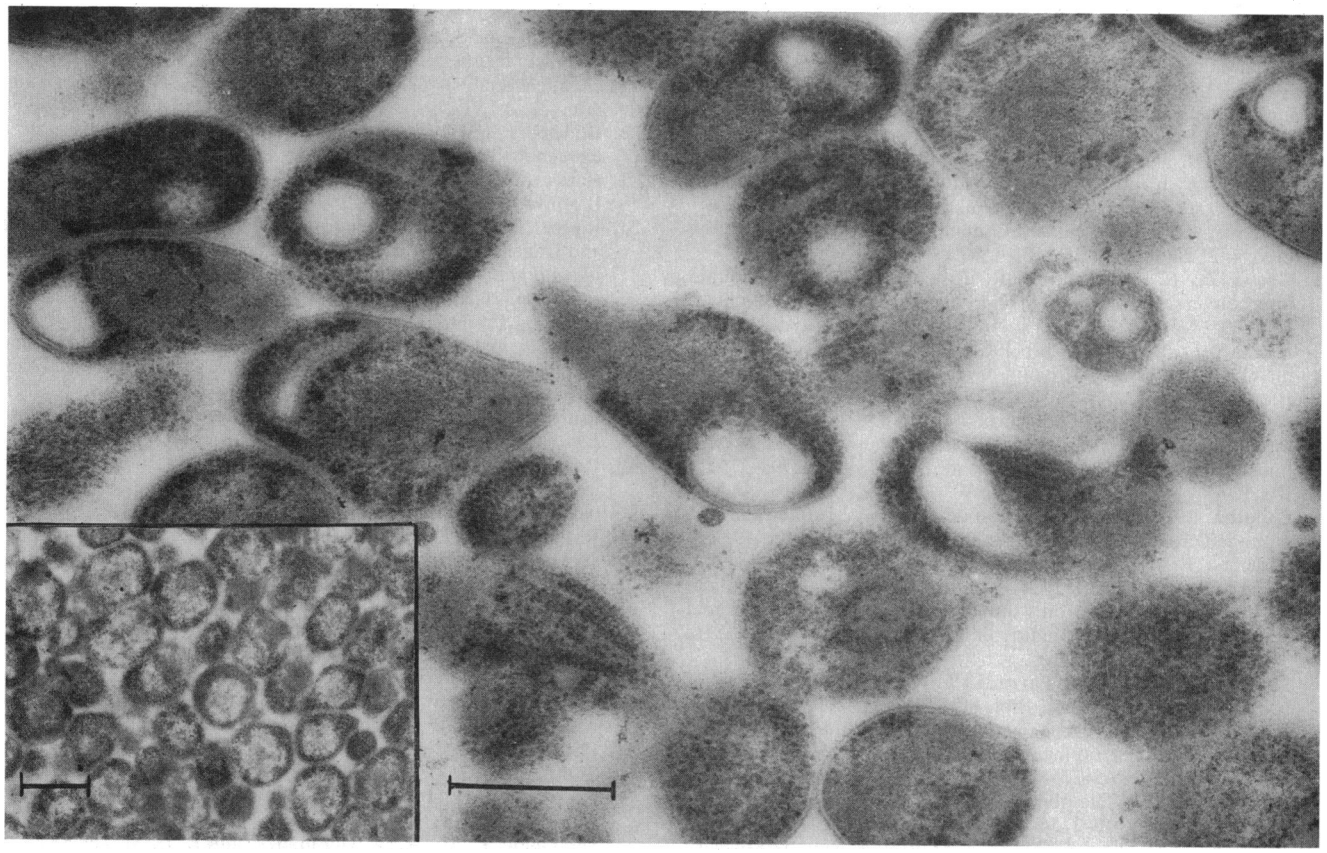

FIG. 2. Thin sections of *M. gallisepticum* cells grown with DPPC. The cells were grown with 20 μg of DPPC per ml to the mid-exponential phase, and transmission electron microscopy was performed as described before (24). Inset, Control cells grown without exogenous PC. Bar, 0.5 μm.

the absence of modification, however, the incorporation of PC was at a lower rate and to a lesser extent. The inability of cells to modify DPPC is not due to the saturated fatty acid that occupies position 2 in the DPPC molecule, since OPPC also containing a palmitic acid residue at position 2 was modified. The finding that DPPC is preferentially accumulated in the outer half of the bilayer (24) strongly suggests that DPPC remains unchanged owing to the inability of the cells to translocate it from the outer to the inner leaflet of the bilayer where phospholipid biosynthesis presumably occurs.

The accumulation of DPPC in the outer half of the bilayer has a pronounced effect on cell morphology, mainly, the appearance of intracytoplasmic membrane structures (Fig. 2) that seem to be a result of an invagination of the cell membrane (24). The association of membrane PC with intracytoplasmic membranes was noted in many bacterial systems (10) and was attributed to the accumulation of nonbilayer lipids (5) (mainly unsaturated phosphatidylethanolamine) in the outer half of the bilayer. In our study, both the incorporated PC and the de novo-synthesized phosphatidylglycerol adopted a lamellar structure (7). Furthermore, intracytoplasmic membranes were detected only with exogenous DPPC, which was shown to be incorporated assymetrically into the outer half of the bilayer (24). It seems that a preferential localization of a disaturated phospholipid in the outer leaflet of the bilayer disturbs the structural and functional equilibrium between the two leaflets leading to membrane invaginations (15). In egg-PC-grown cells, the PC is apparently translocated to the inner leaflet of the bilayer and modified to a disaturated PC that is accumulated in the inner leaflet (24, 25). Such accumulation does not promote the formation of intracytoplasmic membranes (24) but leads to an increase in membrane permeability (28).

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