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Possible Association of Segregated Lipid Domains of *Mycoplasma gallisepticum* Membranes With Cell Resistance to Osmotic Lysis

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Freeze-fracturing of cholesterol-rich *Mycoplasma gallisepticum* membranes from cells grown in a medium containing horse serum revealed particle-free patches. The patches appeared in cells quenched from either 4 or 37°C. Particle-free patches also occurred in membranes of cells grown in a serum-free medium supplemented with egg-phosphatidylcholine but not in membranes of cells grown with dioleoylphosphatidylcholine. The appearance of particle-free patches was attributed to the presence of disaturated phosphatidylcholine (PC) molecules in *M. gallisepticum* membranes, which were synthesized by the insertion of a saturated fatty acid at position 2 of lysophosphatidylcholine derived from exogenous PC present in the growth medium. Consequences of the synthesis of the disaturated PC also included a decrease in osmotic fragility and the ability of the cells to be permeated by K^+. Electron paramagnetic resonance and fluorescence polarization measurements revealed that the fluidity of the lipid domain in the protein-rich *M. gallisepticum* membranes was almost identical to that of an aqueous dispersion of *M. gallisepticum* membrane lipids. Furthermore, the electron paramagnetic resonance spectra of the membranes were single-component spectra showing no indication of immobilized regions. The possibility that the osmotic resistance of *M. gallisepticum* cells is associated with the particle-free patches rather than with a restricted membrane fluidity caused by membrane proteins is discussed.

Lacking a cell wall and bound by a single membrane, most mycoplasmas behave as ideal osmometers and are sensitive to osmotic shock (5, 12, 15). Yet a few species, such as *Mycoplasma gallisepticum* and *M. pneumoniae*, were found to be much more resistant to osmotic lysis than were other mycoplasmas (16, 17). Therefore, for isolation of the cell membrane of *M. gallisepticum*, ultrasonic treatment or mechanical presses rather than osmotic lysis must be employed (17). Attempts to correlate the resistance of *M. gallisepticum* cells with the unusually high protein content of its cell membrane have previously been made (25). In other systems, membrane proteins were found to restrict the freedom of motion of the hydrocarbon chains of membrane phospholipids (8). It was therefore suggested that in *M. gallisepticum* a restricted fluidity of the membrane lipids interferes with the swelling and subsequent lysis of the cells (16).

A study of the physical and ultrastructural features of *M. gallisepticum* membranes seems, therefore, to be an essential step in elucidating the basis of the peculiar osmotic resistance of this organism. This report shows that *M. gallisepticum* membrane proteins had little effect on the physical state of the membrane lipids but that membrane lipids existed at physiological temperature as segregated lipid domains, as visualized by freeze-fracturing. Owing to this phase segregation, a rapid release of internal solutes occurred, reducing the internal osmotic pressure so as to cushion the osmotic shock.

MATERIALS AND METHODS

Growth of organisms and isolation of membranes. *M. gallisepticum* (strain A5969) and *M. capricolum* (California kid) were grown in an Edward medium (18) supplemented with 4% horse serum. In some experiments, the horse serum was replaced by 0.5% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.), cholesterol (10 μg/ml), oleic and palmitic acid (15 μg of each per ml), and 50 μg of either egg phosphatidylcholine (egg-PC) or dioleoylphosphatidylcholine (DOPC), both products of Sigma. All of the lipids were added as an ethanolic solution. The final concentration of the added ethanol did not exceed 0.1%.

To label membrane lipids, we added 0.002 μCi of [1-^{14}C]palmitate (59 Ci/mol; New England Nuclear Corp., Boston) per ml of medium. The cultures were incubated at 37°C for 14 to 22 h, and growth was observed by measuring the absorbance of the culture at 640 nm. Most experiments were performed with cultures at the mid-exponential phase of growth (absorbance at 640 nm, 0.2). The cells were harvested by centrifugation at 12,000 × *g* for 20 min, washed once, and suspended in a 0.25 M NaCl solution. Membranes were prepared by ultrasonic treatment of the washed cell suspensions (23).

Osmotic fragility measurements. The swelling of *M. gallisepticum* and *M. capricolum* cells was determined spectrophotometrically. Washed cell suspensions were diluted in 0 to 0.25 M NaCl solutions to an absorbance at 500 nm of 0.5 to 0.6. The cell suspensions were then incubated at 37°C for 15 min, and cell swelling was observed by measuring the absorbance at 500 nm of the cell suspensions. Results were also expressed as percent lysis in 0.025 M NaCl solution.

Potassium content. Cell suspensions (containing 1 to 5 mg of cell protein) were pipetted onto 0.5 ml of silicone oil (Contour Chemical Co., Woburn, Mass.) in 1.5-ml plastic Microfuge tubes and centrifuged at 12,800 × *g* for 2 min. Under these conditions, the cells passed through the silicone oil, forming a pellet at the bottom of each tube. The pellets were extracted by boiling with 1 ml of 0.1 M HCl for 10 min. The cell residue was removed by centrifugation, and potassium content in the supernatant fluid was determined by flame photometry.

Lipid analysis. Lipids were extracted from intact cells or isolated membranes by the method of Bligh and Dyer (2). The dried lipids were weighed and redissolved in chloroform. The total membrane lipids were chromatographed on Silica gel HR (E. Merck AG, Darmstadt, Germany) plates. The plates were first developed at room temperature with petroleum ether-acetone (3:1, vol/vol) and then at 4°C with chloroform-methanol-water (65:25:4, vol/vol/vol). Lipid spots were detected by iodine vapor and identified as described previously (23).

For determination of radioactivity in the lipid spots, the spots were scraped off the plates into scintillation vials containing 10 ml of toluene scintillation liquor, and radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Rockville, Md.). Fatty acid methyl esters were prepared by heating the lipid samples in 14% boron trifluoride in methanol (Sigma) at 72°C for 15 min. The resulting methyl esters were extracted with *n*-hexane and analyzed by gas-liquid chromatography on a Perkin-Elmer instrument (model 900) equipped with a 1.8-m column of 10% sp 2330 (Supelco, Belfonte, Penn.).

Analytical methods. Protein was determined by the method of Lowry et al. (10). Total phosphorous in the lipid fraction was determined by the method of Ames (1) after digestion of the sample with an ethanolic solution of $Mg(NO_3)_2$.

Fluorescence polarization measurements. Membrane microviscosity was determined by the fluorescence polarization technique; 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe. Membranes (1 mg of protein per ml) in a 0.25 M NaCl solution were mixed with an equal volume of 2 μM DPH and incubated at 37°C for 30 min. Polarization values were measured at various temperatures in an Elscint microviscosimeter model MV-1A, and microviscosity (η) was calculated by the method of Shinitzky and Inbar (28).

Electron paramagnetic resonance (EPR) spectrometry. One-milliliter membrane suspensions (1 mg of protein per ml) were spin labeled as previously described (21) with 2 μl of a 2.5 mM solution of *N*-oxyl-$4^{1'}$-$4^{1'}$ dimethyloxazolidine derivatives of 5-ketostearic acid (5-doxylstearate) or 12-ketostearic acid (12-doxylstearate), both products of Syva (Palo Alto, Calif.). The labeled membranes were sedimented by centrifugation at 34,000 × *g* for 30 min, washed once with 2 ml of a cold 0.25 M NaCl solution, and resuspended in 50 μl of a 0.25 M NaCl solution. The membranes were analyzed in a Varian E-9 spectrometer equipped with a temperature control accessory. The order parameters were calculated by the method of Gaffney (7). The values of the order parameters are related to the mean angular deviations of the labeled fatty acid chain from its average orientation in the membrane. Low values of the order parameters are associated with higher freedom of motion of membrane lipids.

Freeze-fracturing electron microscopy. Freeze-fracturing was performed as previously described (30). The cells, suspended in a 0.25 M NaCl solution containing 30% glycerol, were incubated at 4 or 37°C for 1 h and then treated with 2% glutaraldehyde for 1 h. Finally, they were frozen in liquid Freon. The samples were fractured at −110°C, replicated with platinum carbon, and examined with an electron microscope.

RESULTS

Osmotic fragility of *M. gallisepticum* cells. Figure 1 shows that *M. gallisepticum* cells suspended in hypotonic NaCl solutions and incubated at 37°C showed only a slight decrease in absorbance at 500 nm. A marked decrease in absorbance at 500 nm, apparently due to cell swelling, was observed for *M. capricolum* cells under the same conditions (Fig. 1). The tendency of *M. capricolum* to swell was greatest for early-exponential-phase cells and slightest for stationary-phase cells.

Composition of *M. gallisepticum* membranes. Membranes obtained by ultrasonic treatment contained only 180 μg of lipids per mg (dry weight) of membrane, with a protein/lipid ratio of about 4.0. Analysis of *M. capricolum* membranes revealed a much higher lipid content (300 μg of lipids per mg [dry weight] of membrane) and a protein/lipid ratio of about 1.5. Lipid contents similar to that of *M. capricolum* are found in membrane preparations of a variety of mycoplasmas (20, 29). *M. gallisepticum* membrane lipids had a high cholesterol/phospholipid molar ratio (0.90 ± 0.4 mol of cholesterol per mol of phospholipids) and a simple phospholipid composition. The major phospholipids found in cells grown in a horse serum-containing medium were phosphatidylglycerol (PG) de novo synthesized by the cells (~40% of total), sphingomye-

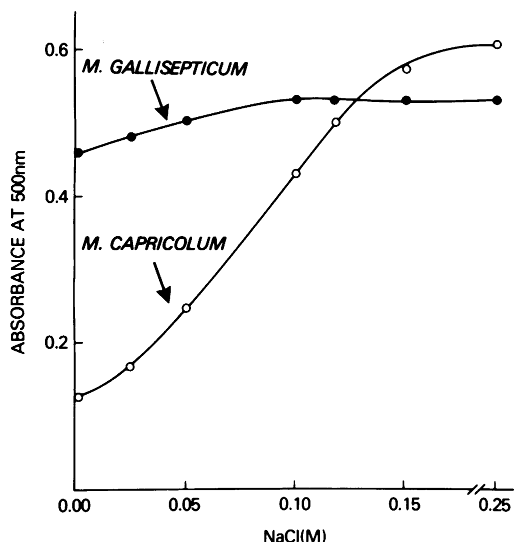

FIG. 1. Swelling of *M. gallisepticum* and *M. capricolum* cells. Washed mid-exponential-stage cells were suspended in various NaCl solutions and incubated at 37°C for 15 min. Cell swelling was measured spectrophotometrically.

lin incorporated unchanged from the growth medium (~15% of total), and a disaturated phosphatidylcholine (PC) (~25% of total). The disaturated PC differs from the position-1-saturated, position-2-unsaturated PC found in the growth medium. Disaturated PC is synthesized by the insertion of a saturated fatty acid at position 2 of a lysophosphatidylcholine (lyso-PC) derived from exogenous PC present in the growth medium by a deacylation-reacylation enzymatic sequence (23).

Physical state of *M. gallisepticum* membranes. The microviscosity of the hydrophobic core of *M. gallisepticum* membranes and the order parameters (S) of spin-labeled fatty acids incorporated into the membranes are presented in Fig. 2 in Arrhenius plots of η and S versus $^{0}k^{-1}$. The plots revealed a clear temperature dependence with activation energies of 4.6 kcal (1.925×10^4 J)/mol for the microviscosity and 1.1 kcal (4.60×10^3 J)/mol and 2.4 kcal (1.1×10^4 J)/mol for the order parameters of 5-doxylstearate and 12-doxylstearate, respectively. At each temperature tested, the microviscosity of the native membranes and the order parameter of 5-doxylstearate incorporated in the native membranes were about the same as those of a lipid dispersion of total membrane lipids. The order parameter of 12-doxylstearate in the membranes was, however, lower than that in the lipid dispersion. The EPR spectra of both 5-doxylstearate- and 12-doxylstearate-labeled *M. gallisepticum* membranes at each temperature were homogeneous single-component spectra showing no immobilized regions. This suggests that in *M. gallisepticum* membranes, the lipids form a continuous bilayer with relatively little lipid-protein interactions.

Freeze-fracture electron microscopy. Close observation of freeze-fractured replicas of *M. gallisepticum* revealed several unique features of the cell membranes. As in other cytoplasmic membranes, the inner fracture face was found to be studded with intramembranous particles ~5 to 10 nm in diameter (Fig. 3 and 4A and B). When cells were grown in horse serum-containing medium, the inner fracture face displayed particle-free patches (Fig. 3). Patching occurred with membranes quenched from 4 as well as 37°C.

Controlled alterations in content and composition of *M. gallisepticum* membrane PC. The PC content and composition of *M. gallisepticum* cells can be changed by varying the serum content of the growth medium or by replacing the serum with chemically defined PC molecules (23). Replacing the horse serum component of the growth medium with bovine serum albumin, cholesterol, and free fatty acids had only a slight effect on cell growth, but the PC content of the

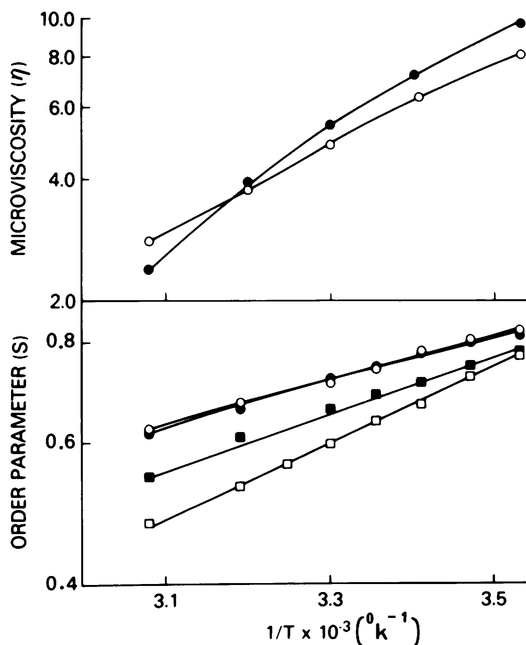

FIG. 2. Arrhenius plots of η values (in poise) calculated from fluorescence polarization measurements of DPH incorporated into *M. gallisepticum* membranes and measurements of S values calculated from EPR spectrometry data for *M. gallisepticum* membranes (open symbols) and aqueous dispersions of *M. gallisepticum* lipids (closed symbols). Membranes and dispersions were spin labeled with 5-doxylstearate (●, ○) or 12-doxylstearate (■, □).

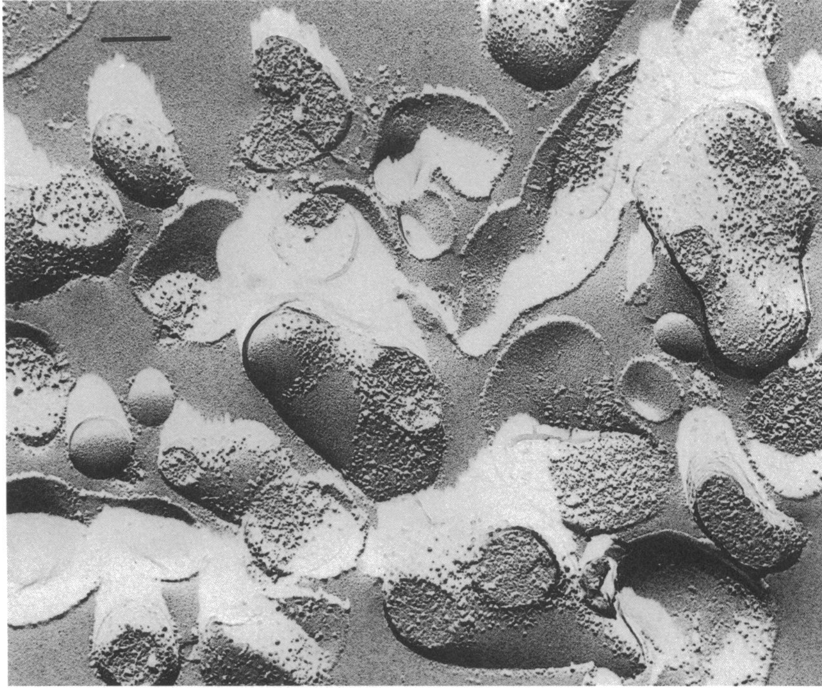

FIG. 3. Replica of freeze-fractured *M. gallisepticum* cells grown in Edward medium (18) containing 4% horse serum. The cells were quenched from 37°C. Bar represents 0.2 μm.

cell membrane was markedly reduced from 25 to 35% to 0 to 10%. Cells grown in a serum-free medium incorporated egg-PC or DOPC added to the growth medium (Table 1). Both PC molecules were incorporated to the same extent (~28% of total phospholipids; result based on phosphorous determination). Both molecules were modified by the organism, as indicated by the considerable amount of radioactivity that was found in the PC fraction (Table 1). As was shown previously (23), *M. gallisepticum* cells modify the exogenous PC by binding a saturated fatty acid to position 2 of the glycerol backbone of lyso-PC. Since the PC molecules added to the growth medium markedly differed in their fatty acid residues acylated to position 1, a major difference in the fatty acid composition of the membrane PC was expected between cells grown with egg-PC and cells grown with DOPC. The fatty acid profile of PC from these membranes is presented in Table 2. When cells were grown with egg-PC, in which position 1 is occupied primarily by a saturated fatty acyl chain, the incorporation of palmitate into position 2 resulted in a disaturated PC. When cells were grown with DOPC, which has an oleic acid residue at position 1, the incorporation of palmitate into position 2 resulted in a position-1-unsaturated, position-2-saturated PC.

Effect of alterations in PC on ultrastructure and osmotic properties. Particle-free patches abundant in membranes of *M. gallisepticum* cells grown with horse serum (Fig. 3) were also found in membranes of cells grown in a serum-free medium supplemented with egg-PC (50 μg/ml) (Fig. 4A). Such patches were, however, not found in membranes of cells grown in a serum-free medium supplemented with 50 μg of DOPC per ml (Fig. 4B) or in membranes of cells grown with no added phospholipids (data not shown).

Cells grown in either horse serum or a serum-free medium supplemented with egg-PC were partially resistant to lysis in a hypotonic NaCl solution (0.025 M). Cells grown in a serum-free medium supplemented with DOPC were found to be much more fragile. The higher fragility of DOPC-grown cells was observed throughout a temperature range of 15 to 37°C (data not shown). Small changes in K^+ content were found among the various cell preparations suspended in an isoosmotic NaCl solution (0.25 M). However, when cell preparations were suspended in a hypotonic NaCl solution (0.025 M), marked differences in intracellular K^+ were observed. In cells grown with DOPC, the K^+ concentration was 2 to 3 times higher than that of cells grown with serum or egg-PC.

DISCUSSION

The effect of membrane proteins on the freedom of motion of the hydrocarbon chains of

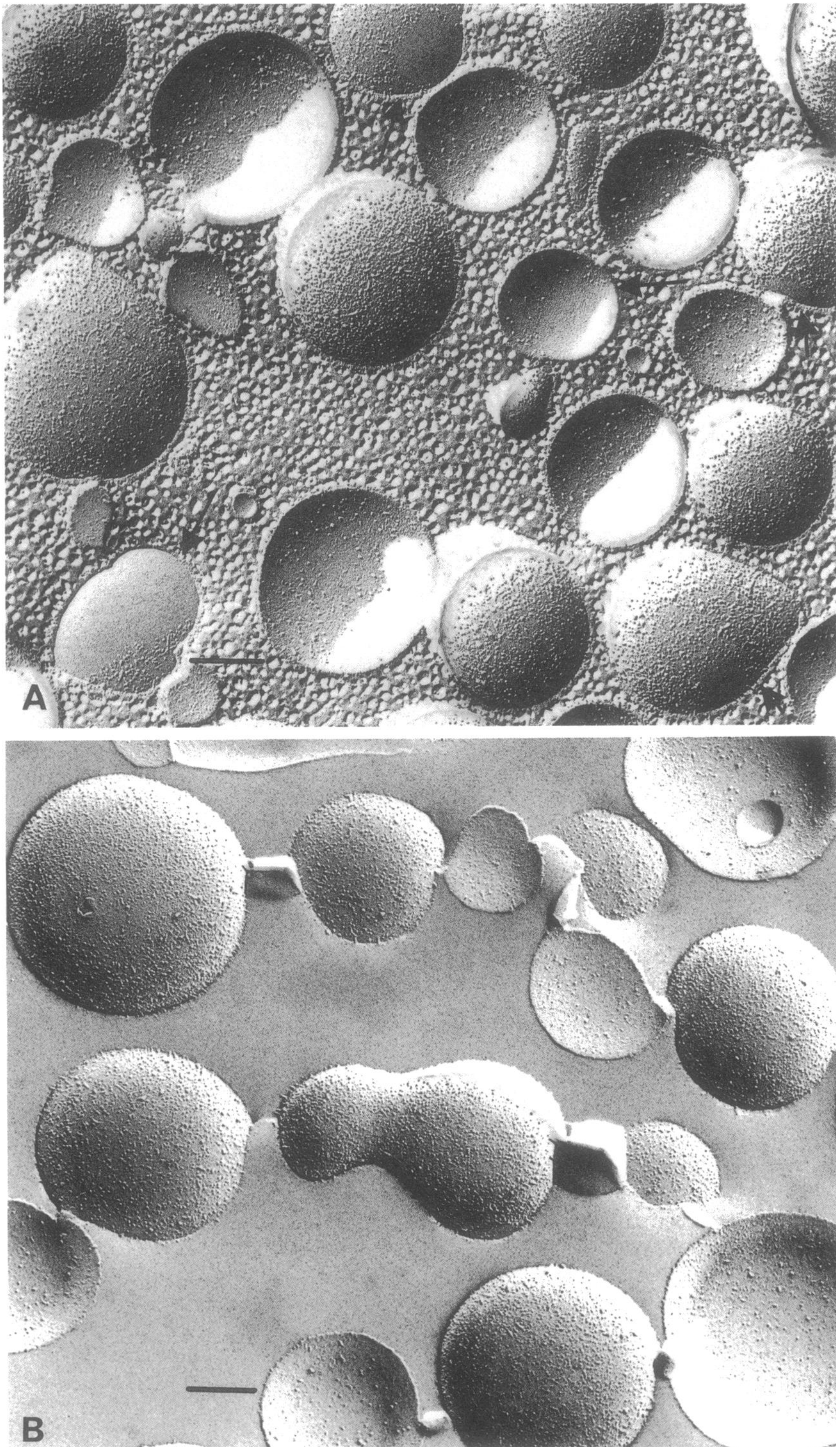

FIG. 4. Replica of freeze-fractured *M. gallisepticum* cells grown in a serum-free medium containing egg-PC (A) or DOPC (B). The cells were quenched from 37°C. Arrows indicate particle-free patches. Bar represents 0.2 μm.

TABLE 1. Incorporation of [^{14}C]palmitate into phospholipids of *M. gallisepticum* cells[a]

Membrane phospholipid	Radioactivity in phospholipids of cells grown with a 50-μg/ml concn of:					
	Egg-PC			DOPC		
	dpm	Lipid phosphorus (dpm/nmol)	% Total	dpm	Lipid phosphorus (dpm/nmol)	% Total
Sphingomyelin	350	120	<0.1	480	110	0.2
PC	35,240	2,600	27.9	28,450	2,200	25.9
PG	83,180	6,800	65.2	68,300	6,800	62.3

[a] Cells were grown to an absorbance at 640 nm of 0.22 in an Edward medium containing 0.5% bovine serum albumin, cholesterol, oleic and palmitic acids (15 μg of each per ml), 0.01 μCi of [^{14}C]palmitate per ml, and 50 μg of either egg-PC or DOPC per ml. Lipids were separated and analyzed as described in the text.

membrane phospholipids was first noted in mycoplasmas (21, 24) and later was found in a variety of membrane systems (8). This effect was attributed primarily to proteins that penetrate deeply into the lipid bilayer and interact with membrane lipids. The lipid-protein interactions can also be demonstrated by EPR spectra of spin-labeled fatty acids incorporated into the membranes. These spectra may be two-component spectra, one component from the label, exhibiting motion characteristics of a lipid bilayer, and one component from the label immobilized by its binding to a lipid-protein interface (8, 32). Although the protein content of *M. gallisepticum* membranes is unusually high, we found that the spectra of spin-labeled fatty acids incorporated into these membranes were single-component spectra. Furthermore, both EPR and fluorescence polarization measurements revealed very similar freedoms of motion in the lipid domain of *M. gallisepticum* membranes and in protein-free lipid dispersions made from these membranes (Fig. 2). These findings suggest that in *M. gallisepticum* membranes, proteins do not restrict the fluidity of membrane lipids. Most membrane lipids seem to be organized in a bilayer-like structure, with the hydrocarbon chains mainly associated with one another rather than with proteins.

Our freeze-fracturing studies showed that particle-free patches appeared on the fracture faces of *M. gallisepticum* membranes from cells that contain a high cholesterol/phospholipid molar ratio (0.9 mol of cholesterol per mol of lipid phosphorus). Temperature-induced patching of intramembrane particles and the formation of particle-free patches have been reported for the membranes of many procaryotes and have been attributed to the order-disorder transition (9, 27, 31). However, particle-free patches are not found in cholesterol-rich membranes (6, 26). This is consistent with the role of cholesterol as a bilayer fluidizer eliminating the order-disorder phase transition (4, 19). Thus, membrane proteins of cholesterol-rich *M. mycoides* subsp. *capri* cells remain randomly dispersed even at 4°C, whereas in a strain adapted to grow on low cholesterol levels, extensive patching takes place (26). The particle-free patches which appeared in the cholesterol-rich *M. gallisepticum* membranes indicate the existence of segregated lipid domains in the membrane of this organism, a cholesterol-poor phospholipid domain capable of undergoing order-disorder transition, and a

TABLE 2. Fatty acid composition of the PC preparations from *M. gallisepticum* cells grown with egg-PC or DOPC[a]

Fatty acid[b]	Fatty acid content (mol%) of PC from cells grown with:	
	Egg-PC	DOPC
14:0	1.0	<0.5
16:0	82.0	47.0
18:0	16.0	1.5
18:1	<0.5	50.8
18:2	<0.5	<0.5

[a] Cells were grown in Edward medium (18) containing 0.5% bovine serum albumin, cholesterol, oleic and palmitic acids (15 μg of each per ml), and 50 μg of either egg-PC or DOPC per ml. The lipid analyses were performed as described in the text.

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

TABLE 3. Osmotic fragility and K^+ content of *M. gallisepticum* cells grown with various preparations[a]

Cells grown with:	% Lysis in 0.025 M NaCl	K^+ concn (mM) in cells incubated in:	
		0.25 M NaCl	0.025 M NaCl
Horse serum	18	172	28
Egg-PC	23	148	41
DOPC	44	156	94

[a] The cells were grown in Edward medium (18) containing 4% horse serum or in media in which horse serum was replaced with 0.5% bovine serum albumin cholesterol, oleic and palmitic acids (15 μg of each per ml), and 50 μg of either egg-PC or DOPC per ml. Percent lysis and K^+ content were determined as described in the text.

cholesterol-rich lipid domain which remained in a fluid state and contained the intramembranous particles.

Particle-free patches ordinarily imply lateral-phase separation and free diffusion of the particles presumably made of proteins. The particles were excluded from the more-solid portions of the membrane, which consisted of lipids enriched in saturated fatty acids. The patching in *M. gallisepticum* membranes was associated with the synthesis of a disaturated PC. The disaturated PC may have formed 20 to 35% of the total lipids of the membrane. The disaturated PC was synthesized by the insertion of a saturated fatty acid at position 2 of lyso-PC, which was derived from exogenous PC of the growth medium by what appears to be a deacylation-reacylation enzymatic sequence. Disaturated PC molecules will therefore be synthesized by *M. gallisepticum* cells grown with PC having a saturated fatty acid at position 1 (such as egg-PC or the PC of the horse serum of the growth medium) but not by cells grown with dioleoylphosphatidylcholine, in which a position-1-unsaturated, position-2-saturated PC molecule is synthesized. The existence of both fluid and solid domains provides the sites for increased permeation of ions and small molecules (3, 11, 14). This increased permeation is due to the formation of disordered boundaries at the interface between the fluid and solid regions within the bilayer (13). Consistent with this hypothesis are our findings correlating the existence of segregated domains in *M. gallisepticum* membranes and the decreased K^+ content and osmotic fragility of the cells. Likewise, it was recently shown (22) that *M. gallisepticum* cells, grown in a serum-containing medium, swell after prolonged incubation periods in an isoosmotic solution of NaCl, KCl, choline chloride, or tetramethylammonium chloride, apparently because the cells can be permeated by these solutes. The leakage of small molecules from *M. gallisepticum* cells eventually reduces the internal osmotic pressure so as to minimize osmotic swelling and subsequent cell lysis.

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