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Interaction of Albumin and Phospholipid:Cholesterol Liposomes in Growth of *Mycoplasma* spp.

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***Mycoplasma* spp., sterol and fatty acid auxotrophs, are conventionally grown in complex media containing high concentrations of serum. Serum supplies the required lipids, but its presence complicates studies on the metabolism and antigenicity of mycoplasmas as well as the membrane dynamics of these organisms. In the present work, fetal bovine serum was replaced with dilipidated albumin and liposomes containing high concentrations of cholesterol. The liposomes were produced from phosphatidylcholine which contained other lipid species, including phosphatidylethanolamine, phosphatidylglycerol, and cholesterol. Other liposomes containing cholesterol and one phospholipid yielded significantly less growth of *Mycoplasma gallisepticum*, indicating that several phospholipids are required to achieve growth levels comparable to those obtained with complex medium. The sources and concentrations of cholesterol, albumin, phosphatidylcholine, and other phospholipids and the interactions among them were important affectors of mycoplasmal growth. Optimal lipid and albumin conditions established for *M. gallisepticum* were then used to propagate five diverse *Mycoplasma* spp. to growth levels which equalled or surpassed those obtained with medium containing 17% fetal bovine serum.**

Mycoplasma spp. require cholesterol and fatty acids for growth, because they lack the de novo pathways for their synthesis (9). In media for the cultivation of mycoplasmas, cholesterol is commonly supplied in serum or a serum fraction, which also contains ample quantities of phospholipids and albumin. However, the use of serum (or egg yolk [28]) places additional ill-defined components into already complex formulations, and, in fact, there are indications that a high serum level, 20% for example, may inhibit growth (32). Studies of pathogenesis may also be complicated by serum (39), since the source of the serum influences the extent of cytopathogenicity accompanying *Mycoplasma pneumoniae* infection of human lung fibroblasts.

There is evidence that culture medium components, particularly proteins from sera, are tightly adsorbed to mycoplasmas, and repeated washings do not remove them (4, 34). Therefore, cross-reactions among mycoplasmas or between these microbes and the tissues which they infect may result from contaminations of mycoplasma antigens with medium components (3, 34, 35, 41).

Replacement of serum with defatted albumin, sterols, phospholipids, and fatty acids (5, 23) has greatly simplified the composition of the growth medium. These substitutions are particularly helpful for investigations of mycoplasma lipid metabolism (7) and membrane chemistry (12, 18), cases in which a defined lipid composition is essential.

In our studies with serum-free medium, the SP4 basal medium was used, since it is superior to other formulations for primary isolation of mycoplasmas (33, 37) and has been used almost exclusively for the recovery of spiroplasmas from plants and insects (36). The mycoplasmas tested grew to high concentrations in SP4 medium containing liposomes and albumin and achieved titers equal to those in SP4 medium with serum. Six species were propagated in this medium, indicating that it may be useful as a general serum-free formulation for the mycoplasma groups. A phospholipid-defined medium containing liposomes and defatted albumin also supported high growth levels of *Mycoplasma gallisepticum*. The liposome bilayer components and the fatty acid content of albumin were important effectors of mycoplasma growth.

MATERIALS AND METHODS

Organisms. *Mycoplasma gallisepticum* S6, *M. fermentans* PG18, *M. arginini* G230, *M. pulmonis* N-3, *M. hyorhinis* GDL, and *M. arthritidis* PG27 were obtained from our stock culture collection. Each organism was serially transferred at least 10 times in medium containing liposomes and albumin before determinations of growth in serum-free medium. Cultures were maintained by subculturing glass-adherent (5) or broth-grown organisms.

Medium formulations. Three variations of the SP4 formulation (38) were used to grow mycoplasmas. SP4-FBS contained 17% (vol/vol) fetal bovine serum (FBS), SP4-LIP contained albumin and crude (impure) phospholipid-cholesterol liposomes, and SP4-LD was prepared by depleting SP4 medium of endogenous lipids and then adding phospholipid-cholesterol liposomes and albumin.

All SP4 basal media contained 3.5 g of mycoplasma broth base (BBL Microbiology Systems, Cockeysville, Md.), 10.0 g of tryptone, and 5.3 g of peptone (both from Difco Laboratories, Detroit, Mich.) in 621 ml of water. They were modified by inclusion of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Calbiochem-Behring Corp., La Jolla, Calif.) buffer at a final concentration of 0.05 M. Each base was adjusted to pH 7.5, autoclaved, cooled, and then supplemented with 0.002% (wt/vol) phenol red; 0.5% (wt/vol) glucose; 5% (vol/vol) 10× CMRL 1066 with

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glutamine (GIBCO Laboratories, Grand Island, N.Y.); 2% (wt/vol) yeastolate (final concentration, 10% [vol/vol]; Difco) and 25% yeast extract solution (final concentration, 0.9% [vol/vol]) as prepared by the method of Johnson and Somerson (15); and either 17% (vol/vol) FBS (Microbiological Associates, Bethesda, Md.) or liposomes and albumin.

SSR_2-liposome (SSR_2-LIP [5]) and Hayflick media (11) were used in some comparisons with SP4 formulations. The Hayflick broth contained 10% (vol/vol) horse serum (KC Biologicals, Lenexa, Kans.). Agar media for all formulations contained 10 g of Noble agar (Difco) per liter in basal medium. After addition of supplements, 8 ml of agar medium was poured into plastic petri dishes (15 by 60 mm). The agar plates with Hayflick medium contained 20% (vol/vol) heat-inactivated horse serum.

Lipid depletion of SP4-LD. Lipids were extracted from the yeast products and from the powdered basal medium components routinely present in SP4 medium. For the aqueous extractions, 2% (wt/vol) yeastolate (Difco) and 25% (wt/vol) yeast extract were combined at the proportions present in SP4 medium, and their lipids were extracted three times by the procedure of Bligh and Dyer (2). Before the second extraction, the pH of the sample was lowered to 5.0 to remove the remaining lipid salts, and after extraction, the pH was readjusted to 7.0. The remaining methanol was removed by bubbling with nitrogen gas for 16 h at 56°C. The volume of the sample was adjusted to its original level with the addition of sterile distilled water. The delipidized yeast extract-yeastolate was sterilized by passage through a membrane filter (pore size, 0.20 μm).

For lipid extraction of basal medium components, mycoplasma broth base, tryptone, and peptone powders were combined and pulverized with a mortar and pestle. This mixture was then extracted three times with chloroform-methanol (2:1) as described by Razin and Rottem (24). The delipidized base was lyophilized to remove all residual chloroform and methanol.

Preparation of liposomes. Liposomes were prepared by sonication as described previously (5). Lipids were solubilized in 2 ml of chloroform, the solution was placed at 40°C, and contents were evaporated to dryness under nitrogen. Five milliliters of sterile distilled water was added, and the vial was capped and placed in an ultrasonic bath. After sonication, 5 ml of sterile 0.4 M sucrose was added, and the liposome suspension was gently vortexed. The suspensions were either used immediately or stored in the dark at 4°C for up to 4 weeks. Liposomes were negatively stained with ammonium molybdate and sized and classified with a Hitachi HU-12 electron microscope. Examination of crude phosphatidylcholine (PC)-cholesterol liposomes by electron microscopy revealed multilamellar vesicles which ranged in size from 0.1 to 1.0 μm.

In making liposomes, lipids were weighed into screw-cap vials (diameter, 21 mm; capacity, 4 drams [ca. 15.5 g]) Defined liposomes of chromatographically pure phospholipids and cholesterol were produced with 25 mg of PC (type VE), 15 mg of dipalmitoyl phosphatidylethanolamine (PE), 10 mg of phosphatidylglycerol, 10 mg of diphosphatidylglycerol, 5 mg of sphingomyelin (SPH), and 30 mg of cholesterol in a final volume of 10 ml. Liposomes of less precise content were composed of 30 mg of cholesterol and 66 mg of crude PC and, unless otherwise noted, were made in all experiments with phospholipid and cholesterol at 1:1 molar ratios.

The liposome preparations did not require filtration before addition to media to ensure sterility. While inclusion of albumin in liposomes increases their stability (14), growth yields in such preparations in SP4-LIP did not increase; therefore, albumin was added separately as an aqueous solution.

Serum substitutes. Cholesterol, albumin, and phospholipids were used as substitutes for serum. Crude PC, vegetable lecithin obtained from Mann Research Laboratories, New York, N.Y., contained a variety of lipid impurities. Thin-layer chromatographic analysis of this crude PC incorporated into PC-cholesterol liposomes revealed six major lipid components. PC, PE, phosphatidylglycerol, and cholesterol were identified; the first two were the predominant components. Authentic diphosphatidylglycerol and SPH comigrated in the vicinity of the remaining two phospholipids. There were several compounds with R_f values less than 0.10 which did not stain as phospholipids. All other lipids were acquired from Sigma Chemical Co., St. Louis, Mo. Cohn fraction V and fatty acid-free fraction V bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) were sterilized by passage through a filter (pore size, 0.20 μm) and added to media as either 10 or 20% (wt/vol) aqueous solutions. Unless indicated serum-free medium contained 150 μg of cholesterol per ml, 330 μg of phospholipid per ml, and 1.2% fatty acid-free fraction V albumin.

Quantitation of mycoplasma growth. To quantitate mycoplasmas attached to glass, total glass-adherent mycoplasma (GAM) protein was determined with the Coomassie brilliant blue G-250 microassay (Bio-Rad Laboratories, Richmond, Calif.) as previously described (5). For determinations of growth in broth cultures, organisms were quantitated as CFU per milliliter. Broth medium containing organisms was serially diluted and plated onto agar medium of identical composition. All platings were performed in triplicate. Colonies were stained with the lipid-protein stain (6) and then were counted with the aid of a bacterial colony counter.

Attempts at isolation from clinical specimens. Clinical specimens positive for *M. pneumoniae* were kindly provided by J. Tully, National Institutes of Health, Cancer Research Facility, Frederick, Md. Diphastic (agar-broth) media were prepared as previously described (37); either SP4-FBS or SP4-LIP was used as the broth phase. Each of nine samples was inoculated into both diphastic media, and the vials were incubated at 37°C. When a decline or increase in pH was observed, 0.1 ml of broth was immediately plated onto agar medium of the same formulation as the broth. If no pH change occurred, the broth was inoculated onto agar medium every 2 to 3 weeks.

Thin-layer chromatography. Lipids were analyzed by thin-layer chromatography. Samples and standards were solubilized in chloroform, spotted on silicic acid hard-layer (HL) plates (Analtech, Newark, Del.), and developed in chloroform-methanol-acetone-acetic acid-water (120:20:40:20:10). Lipids were visualized by using I_2 vapors and identified by comigration with authentic lipids and by specific staining. To aid in identifications, we used the Dittmer and Lester molybdate reagent for phospholipids, ninhydrin stain for PE, sulfuric acid-acetic acid reagent for cholesterol and cholesterol esters, napthol stain for glycolipids, Dragendorff reagent for PC, and the indicator bromocresol green for fatty acids, all as described by Kates (17).

RESULTS

Growth of *M. gallisepticum* in SP4-LIP broth and agar media. Since *M. gallisepticum* had been grown serum-free in an SSR_2 medium formulation containing liposomes (5), we determined whether the SP4 formulation with liposomes and

albumin would yield the same results. Organisms repeatedly subcultured in SSR_2-LIP served as inoculum. We compared the growth of *M. gallisepticum* in broth cultures of SP4-LIP, SP4-FBS, and Hayflick medium with that obtained in SSR2-LIP.

The amounts of growth in the two broth media containing liposomes were comparable (Table 1). SP4-FBS and Hayflick medium yielded higher titers than those observed in SP4-LIP and SSR2-LIP.

Agar formulations which contained liposomes instead of serum supported the growth of *M. gallisepticum*. Regardless of which medium was used, the colonies displayed the classical fried egg morphology associated with mycoplasmas. Colonies appearing on liposome media were also equal in size to those on SP4-FBS agar. Apparently, the immobilization of liposomes did not affect either property of the organisms. Due to the translucence of agar with liposomes, stained colonies were more easily observed than were unstained colonies.

Ratios of lipids in liposomes. Decreasing the quantity of crude PC and cholesterol before sonication while maintaining a 1:1 molar ratio did not affect the growth of *M. gallisepticum*, provided the final concentration of lipid in the medium was equal to control levels of 150 μg of cholesterol per ml and 330 μg of phospholipid per ml lipid. However, more growth resulted when PC-cholesterol (1:1) vesicles were included in medium than with vesicles at 2:1 or 1:2 ratios (unpublished data).

Growth curves of *M. gallisepticum* grown in SP4 media. We compared the growth kinetics of *M. gallisepticum* in medium containing serum with that obtained with medium containing liposomes (Fig. 1). A large inoculum was used, and there was no lag in growth with SP4-FBS. At each sampling, higher titers were obtained with SP4-FBS until the onset of the decline phase, which preceded that observed in SP4-LIP. Organisms grown in SP4-LIP did not reach the same growth level as that observed in SP4-FBS until approximately 15 h later. While the overall growth responses with the two media were similar, the maximum titer obtained with SP4-FBS was higher than that obtained with SP4-LIP; however, this difference was not statistically significant. The generation times were 3.6 h for SP4-FBS and 4.3 h for SP4-LIP.

Effect of cholesterol and albumin levels. In previous work, significantly higher growth yields of mycoplasmas were obtained when the cholesterol concentration was increased from 100 to 150 μg/ml (5). By using the SP4 formulation, the concentrations of cholesterol and albumin were altered to determine the effect on growth of mycoplasmas attached to glass.

With cholesterol at 50 μg/ml, increase of the albumin levels from 0.2 through 0.8% dramatically improved GAM protein levels (Fig. 2A). In another experiment (Fig. 2B), increasing the cholesterol to 100 μg/ml with the albumin at 0.8 or 1.2% gave no increase in GAM protein. However, the formulation with 150 μg of cholesterol per ml and 1.2% (wt/vol) albumin produced the highest yield, with a growth level approaching that obtained in serum-containing medium (Fig. 2B). In other experiments, further increases of albumin and cholesterol concentrations above these levels did not significantly increase growth yields.

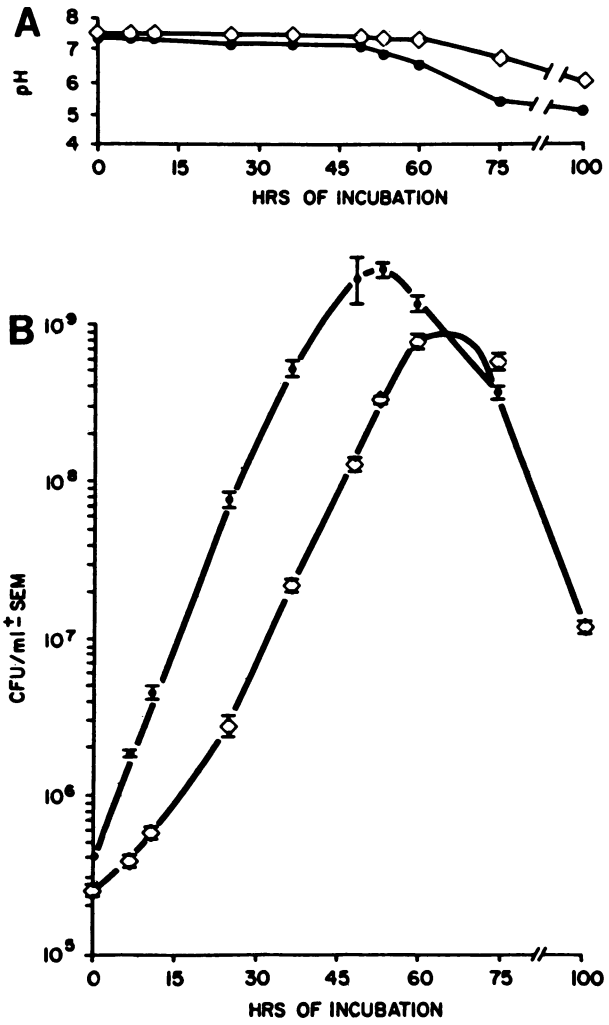

FIG. 1. pH (A) growth (B) curves of *M. gallisepticum* in SP4-LIP (◇) and SP4-FBS (●). A 50-ml portion of broth medium (at 37°C) was inoculated with 1.1×10^7 CFU of *M. gallisepticum* that had been transferred more than 20 times in SP4-LIP. In the serum-free conditions, the PC/cholesterol ratio in liposomes was 1:1, and the cholesterol concentration was 150 μg/ml. Each point represents the mean plus or minus the standard error of the mean (horizontal bars.). At each sampling, 0.7 ml of broth was removed to monitor pH.

TABLE 1. Initial growth of *M. gallisepticum* in SP4-LIP medium[a]

Medium	Growth (10^9 CFU/ml)	pH
SSR_2-LIP	1.1 ± 0.06	6.67
SP4-LIP	0.9 ± 0.09	7.02
SP4-FBS	3.5 ± 0.10	6.48
Hayflick	2.7 ± 0.15	7.12

[a] Each formulation was tested in duplicate in two separate experiments. Each bottle containing 25 ml of prewarmed medium was inoculated with 0.2 ml from a 5.0-ml SSR_2-LIP broth culture and incubated statically at 37°C for 48 h. CFU determinations (mean plus or minus the standard error of the mean) were calculated from triplicate platings of each culture.

Importance of albumin impurity. In the previous experiments, fatty acid-free fraction V albumin served as the serum protein component in liposome medium. Since it was reported (8) that there is less attachment of *M. pneumoniae* to glass in the presence of essentially fatty acid-free albumin than when normal Cohn fraction V albumin is used, we compared the growth yields when these albumins were incorporated into liposome medium. Significantly higher growth yields of GAMs resulted when Cohn fraction V

TABLE 2. Effect of albumin purity on *M. gallisepticium* growth in SP4-LIP

Medium, albumin[a]	Growth (μg of GAM protein)[b]	No. of replicates
SP4-FBS	260 ± 10	9
SP4-LIP		
Fatty acid-free	223 ± 19	15
Normal	343 ± 19	14

[a] SP4-LIP contained either normal or fatty acid-free fraction V albumin.
[b] Each value is a mean plus or minus the standard error.
[c] Determinations were obtained from five experiments. One-way analysis with Scheffé post hoc multiple comparisons (29) revealed that results obtained with fraction V albumin differed significantly from those obtained with fatty acid-free fraction V albumin ($P < 0.10$) and with FBS ($P < 0.05$).

TABLE 3. Growth of several mycoplasmas in SP4 media[a]

Mycoplasma sp.	Growth (CFU/ml of broth medium) in:	
	SP4-FBS	SP4-LIP
M. hyorhinis	1.25×10^9	1.25×10^9
M. pulmonis	7.70×10^6	7.60×10^8
M. fermentans	1.35×10^9	1.10×10^9
M. gallisepticum	1.60×10^9	1.15×10^9

[a] Media were evaluated as described in footnote *a* of Table 1. Each medium was inoculated with 0.2 to 1.0 ml of a 5-ml SP4-LIP culture. Fraction V albumin was used in SP4-LIP. Incubation times ranged from 40 to 64 h. Standard errors determined from replicate colony counts ranged from 0.01 to 0.3; only the amount of growth of *M. pulmonis* was significantly different ($P < 0.05$) between the two media.

albumin was used (Table 2). In fact, GAM protein levels achieved with media containing fraction V albumin significantly exceeded those obtained with 17% FBS.

Additional *Mycoplasma* sp. grown in SP4-LIP. After the optimal cholesterol and albumin conditions were established for *M. gallisepticum*, we determined whether SP4-LIP could serve as a serum-free medium for diverse mycoplasma species. *Mycoplasma* species representing the three recognized metabolic subgroups (*M. hyorhinis*, *M. gallisepticum*, and *M. pulmonis* from the glucose-fermenting group; *M. arginini* and *M. arthritidis* from the arginine-hydrolyzing group; and *M. fermentans*, which both ferments glucose and hydrolyzes arginine) were serially transferred in SP4-LIP. All six species were grown in broth and on agar media of SP4-LIP formulations. These organisms were subcultured through at least 10 passages. No attempt was made to cultivate spiroplasmas or ureaplasmas in liposome-containing medium.

By using broth cultures, growth of the fermenting mycoplasmas in medium with FBS was compared with that in medium containing liposomes. *M. hyorhinis* and *M. fermentans* grew to equal levels in both media, while *M. gallisepticum* reached slightly higher titers in medium containing FBS (Table 3). In contrast to the other species, *M. pulmonis* reached a titer two logs higher in liposome medium compared with SP4-FBS.

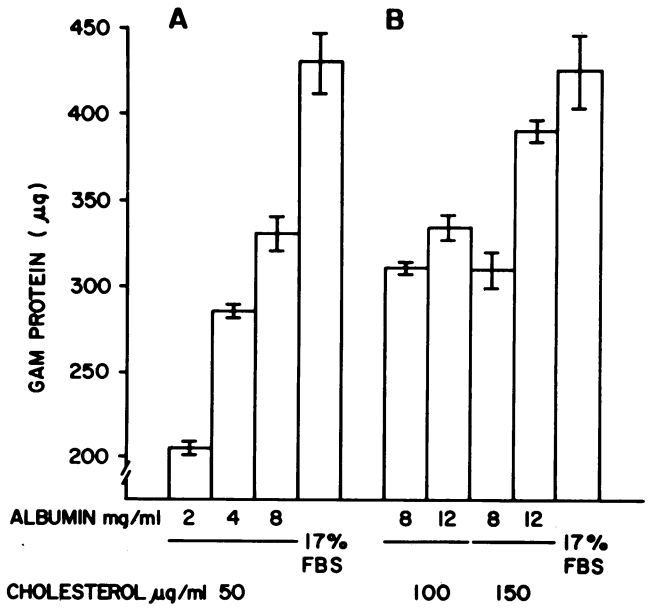

FIG. 2. Effect of cholesterol and albumin concentration on *M. gallisepticum* growth. Each value is the mean result of triplicate cultures.

Primary isolation of mycoplasmas with SP4. SP4-LIP was also evaluated for usefulness for the primary isolation of *M. pneumoniae*. Nine clinical specimens were inoculated into SP4-LIP and SP4-FBS. With SP4-FBS, mycoplasmas were isolated from some of the specimens previously determined as positive for *M. pneumoniae*. No isolates were obtained with SP4-LIP, suggesting that it may be unsuitable for primary isolation.

Growth promotion of defined liposomes in SP4-LIP. To determine if one of the predominant lipids could replace crude PC, liposomes of pure SPH, pure PC, and PE, all with the cholesterol/phospholipid ratio at 1:1, were added individually to SP4 media. These media were then compared for growth promotion with medium containing crude PC-cholesterol. Organisms were cultivated attached to glass, as well as in broth medium. In both conditions, growth levels were higher in the medium with liposomes containing crude PC-cholesterol (Table 4). Additional experiments with pure PC-cholesterol liposomes (i.e., soybean and egg PC) gave results for glass-attached cultures similar to those shown for SPH-cholesterol and PE-cholesterol.

Growth of *M. gallisepticum* in SP4-LD. We combined the lipid-defined and lipid-free SP4 medium components with a solvent-extracted basal medium and yeast products to formulate a lipid-depleted medium. The addition of fatty acid-free albumin and liposomes of defined phospholipid content

TABLE 4. Comparison of the effects of crude PC-cholesterol liposomes and pure phospholipid-cholesterol liposomes on the growth of *M. gallisepticum*

Phospholipid in phospholipid-cholesterol liposomes	Growth of organisms[a]	
	Attached to glass (μg of GAM protein)	In broth culture (CFU/ml)
Soybean PC	NT[b]	3.7×10^7
Egg PC	NT	2.3×10^8
Crude PC	294 ± 2	1.1×10^9
PE	147 ± 2	NT
SPH	101 ± 21	NT

[a] Growth was assayed as described in Materials and Methods. Fatty acid-free albumin was present at 1.2% (vol/vol). Values for GAM proteins are expressed as means plus or minus the standard error of the mean. For broth-grown organisms, standard errors, determined as CFU, ranged from 0.05 to 0.2.
[b] NT, Not tested.

TABLE 5. Growth of *M. gallisepticum* in SP4-LD

Formulation	GAM protein (μg)[a]
SP4-LIP (crude PC-cholesterol liposomes)	239 ± 16
SP4-LD (crude PC-cholesterol liposomes)	164 ± 9
SP4-LD[b]	251 ± 14
SP4-LD plus oleate (10 μg/ml)[c]	174 ± 11
SP4-LD (phospholipid liposomes, no cholesterol)	56 ± 12
SP4-LD (no albumin)	63 ± 10

[a] Results are combined values from triplicate cultures of two separate experiments plus or minus the standard error of the mean.

[b] SP4-LD contained PC, PE, phosphatidylglycerol, diphosphatidylglycerol, SPH, cholesterol liposomes, and 1.2% defatted bovine serum albumin unless indicated otherwise.

[c] Oleate was included in the liposome suspension.

yielded a medium, SP4-LD, of known phospholipid composition. The thin-layer chromatographic patterns of lipids extracted from SP4-LD containing liposomes and albumin were identical, within detectable limits, to those obtained from solubilized liposomes alone (data not shown), confirming that SP4-LD was essentially lipid defined.

M. gallisepticum was transferred four times in SP4-LD and used as inoculum. SP4-LD supported levels of growth which equalled and were essentially no different from ($P > 0.5$) those obtained in SP4-LIP (Table 5). The addition of 10 μg of oleate per ml to SP4-LD decreased growth yields. Also, delipidized SP4 medium containing crude PC-cholesterol liposomes yielded growth levels lower than those obtained wih SP4-LD, which had not been extracted with solvents ($P < 0.0005$). As expected, cholesterol and albumin were both required to obtain growth in SP4-LD.

DISCUSSION

Johnson and Somerson (15) could not grow *M. gallisepticum* or *M. fermentans* with albumin and an ethanolic cholesterol suspension in the place of serum in the culture medium. With the SP4 culture formulation containing liposomes and albumin instead of serum, these mycoplasmas and others were cultivated at levels which equalled or surpassed those obtained in medium containing serum. In media with cholesterol-containing liposomes and albumin, organisms could be grown (i) in broth, (ii) attached to glass surfaces, and (iii) on agar medium. Since *M. gallisepticum* is a phospholipid auxotroph (27), the evidence suggests that the mixture of phospholipids in our PC-cholesterol liposomes may be critical in supporting the growth of these organisms in SP4-LIP.

In our experiments, liposomes were practical carriers of cholesterol, fatty acids, and phospholipids. While the concentration of lipids in the liposome suspension was not critical, a high level of cholesterol in culture medium and a 1:1 molar ratio of PC to cholesterol were important in obtaining optimal growth levels. Kahane et al. (16) used pure PC-cholesterol liposomes and 0.5% albumin to grow *M. hominis*. However, they found that growth, as measured by optical density, was partially inhibited when liposomes were added to give cholesterol concentrations of 30 μg/ml or higher. In our work, the optimal concentration of cholesterol was 150 μg/ml, with albumin at 1.2% (wt/vol) (Fig. 2). Possibly, under cultural conditions favoring large amounts of growth, some of the albumin enhances the availability of cholesterol. In turn, the increased cholesterol presented to the mycoplasmas might be used to neutralize and perhaps scavenge peroxide or other toxic products of oxidative metabolism.

Possibly, albumin served several roles under our culture conditions, and both the final concentration and the presence or absence of associated fatty acids affected mycoplasmal growth attached to a glass surface. Fraction V albumin dramatically increased the growth yields over those obtained with fatty acid-free V albumin. One explanation is that lipid-poor albumin complexes with free fatty acids present in the medium, effectively depleting the medium of available fatty acids (25). A second explanation, suggested by Feldner et al. (8), is that fatty acid-free albumin may bind directly to the mycoplasma membrane, making attachment to glass difficult. Nevertheless, in the present work, high growth yields were obtained with delipidated albumin in SP4-LD, a medium essentially devoid of free fatty acids.

Recent evidence (30) for an albumin-cholesterol interaction in cultured human fibroblasts has been indicated by an enhanced uptake, in the presence of serum albumin, of [^{3}H]cholesterol from PC vesicles. This reinforces the suggestion of Rodwell (26), which is indirectly supported here, that albumin may complex with cholesterol, thus enhancing its uptake by mycoplasma cells. Also, in work on *M. gallisepticum* (19), it has been suggested that the ability of albumin to sequester free fatty acids and lysophospholipid impurities may aid in the formation of segregated membrane domains protective against phospholipid hydrolysis and help to maintain cellular integrity.

Thin-layer chromatography of the crude PC included in SP4-LIP revealed various lipid species. When these phospholipids were tested for growth-promoting activity, liposomes of PC-cholesterol, PE-cholesterol, or SPH-cholesterol did not support growth to levels obtained with crude PC-cholesterol vesicles. This difference may be explained in several ways. The affinity of cholesterol for phospholipids varies according to the specific polar headgroup (40) and the overall melting temperature of the phospholipid. This could effect cholesterol exchange between the vesicles and the mycoplasma membrane. Also, Razin (22) has demonstrated that cholesterol uptake from PC-cholesterol vesicles by mycoplasmas is dependent on the membrane fluidity of the organisms. Therefore, transfer of phospholipid and cholesterol is dependent on the compositions of both the liposome bilayer and the mycoplasma membrane. Due to heterogeneity in phospholipid class and fatty acid content, crude PC-cholesterol liposomes may exchange lipid components with the mycoplasmas more readily than do pure phospholipid-cholesterol liposomes.

In the experiments with SP4-LD, the lipid content of the defined liposomes was based on the content of crude PC. Five phospholipids were included in these phospholipid-cholesterol liposomes, but other combinations or subsets of this group may support equivalent or superior growth levels.

Unextracted SP4 medium with crude PC-cholesterol liposomes and delipidated medium with defined phospholipid-cholesterol liposomes promoted equivalent growth levels, suggesting that any minor growth factor which is removed through extraction by lipid solvents is unimportant in the SP4 basal component or yeast products. Certainly, some undetected free fatty acids are present in SP4-LD. Since mycoplasmas are fatty acid auxotrophs which lack phospholipase activity (31), free fatty acids must be exogenously incorporated to serve as substrates for phospholipid synthesis. However, Rottem and coworkers, under stringent assay conditions, have recently detected phospholipase A_2 activity in *M. gallisepticum* (personal communication). Our work

supports the contention that *M. gallisepticum* either incorporates phospholipids into their membranes in toto to satisfy their fatty acid requirement or does indeed have phospholipase activity.

The SP4 medium as described by Tully et al. (38) is considered a superior formulation for the growth of mycoplasmas and spiroplasmas. In the present work, the high growth-promoting activity of SP4-LD for *M. gallisepticum* suggests it may be useful for future studies of mycoplasmal nutrition, lipid metabolism, membrane dynamics, and enzyme localization and purification.

At least two components of our liposome delivery system, i.e., cholesterol (13) and sucrose (1), are known mediators of cell fusion. Nicolau and Rottem (20) encapsulated the *Escherichia coli* (pBR322)-encoded β-lactamase gene in liposomes and demonstrated enzyme activity in transfected *Mycoplasma capricolum* cells. They concluded that β-lactamase activity is transferred after fusion of the liposomes and the mycoplasma membranes. Fusion of *Acholeplasma laidlawii* and PC vesicles has also been reported (10). These findings suggest the possibility that liposome cholesterol and phospholipids are incorporated by mycoplasma membranes by similar fusion events. In SP4-LIP, liposomal cholesterol, fatty acids, and sucrose must come into intimate contact with the mycoplasma membrane, which would favor exchange of lipids by membrane fusion rather than by exchange by such proposed mechanisms (21) as endocytosis, adsorption to the cell surface, or simple exchange of membrane lipids between the two outer leaflets.

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