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Cholesterol Requirement of Mycoplasmas

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Cholesterol requirement for growth of mycoplasmas was tested in a serum-free medium supplemented with albumin, L-arginine, palmitic acid, and various concentrations of cholesterol dissolved in Tween 80. In cases in which Tween 80 was shown to inhibit growth, the test medium was supplemented with cholesterol dissolved in ethanol. Of the 31 species examined, all but *Mycoplasma laidlawii*, *M. granularum*, and *Mycoplasma* species strain S-743 exhibited a growth response to cholesterol. No requirement for cholesterol could be shown with the stable L-phase variants of *Streptobacillus moniliformis* and *Proteus* species. The results provide experimental support for the view that the large majority of the established *Mycoplasma* species require cholesterol for growth.

Requirement of cholesterol is regarded as one of the most important criteria distinguishing the *Mycoplasma* species from the *Schizomycetes* (2, 4). Yet, this important characteristic has directly been tested and confirmed for only a few of the over 30 *Mycoplasma* species established so far (2, 5, 7, 8, 12, 13, 16, 20). The necessity for testing the requirement for cholesterol in all the established *Mycoplasma* species and in proposed new species became apparent with the recent discovery of mycoplasmas which do not require cholesterol for growth and which differ from the long-known sterol-nonrequiring *M. laidlawii* strains (21, 22). Edward and Freundt (3) have proposed the division of the *Mycoplasmatales* into two families: one for those dependent on sterol for growth, and the other for those not dependent on sterol. As the serum component of mycoplasma media is essentially the only source for cholesterol, growth of mycoplasmas in a serum-free medium may well be taken to indicate their independence on cholesterol for growth. However, since the serum also supplies the long-chain fatty acids required by many mycoplasmas (9), the failure of a strain to grow in the serum-free medium does not always mean that it depends on cholesterol. Thus the new sterol-nonrequiring strains (S-410 and S-743) isolated from tissue cultures grew very poorly in a serum-free medium, but this poor growth was shown to be the result of an inadequate supply of fatty acids rather than of cholesterol (22). It seemed therefore of importance to devise a simple method to test for the cholesterol requirement of mycoplasmas and to apply it to a wide and representative series of species to establish their classification within the two new families. The method selected was based on that described by Razin (8) in which the growth response to cholesterol was determined in a serum-free medium supplemented with albumin and various concentrations of cholesterol dissolved in Tween 80. This method, or some modification of it, has now been shown suitable for the determination of cholesterol requirement in a wide variety of *Mycoplasma* species, and can thus be proposed as a standard technique for establishing this property in new isolates before taxonomic designations are offered.

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MATERIALS AND METHODS

Organisms and culture medium. The organisms tested and their strain designation appear in Tables 1 and 2. The inoculum used for testing the growth response to cholesterol was grown in a medium composed of 2.1% Mycoplasma Broth Base (BBL), 10% Fresh Yeast Extract (Microbiological Associates, Bethesda, Md.), 0.5% glucose, 0.42% L-arginine-HCl, 500 units of penicillin G per ml, and 1% Serum Fraction (Difco, code 0441). For growing *M. pneumoniae*, the Serum Fraction was replaced by 20% horse serum (T. C. Select, BBL).

Medium for testing cholesterol requirement. The medium described above without Serum Fraction or horse serum served as the basal serum-free medium. This basal medium was supplemented with 0.5 or 1.0% bovine serum albumin fraction V (Armour Pharmaceutical Co., Chicago, Ill.), 10 μg of palmitic acid per ml, 0.01% Tween 80, and various concentrations of cholesterol. The basal medium containing bovine serum albumin (fraction V), palmitic acid, and Tween 80 has been shown to be free of detectable amounts of cholesterol (12). The albumin supplement was pre-

pared as a 10% stock solution in distilled water, adjusted to *p*H 7.5 with 1 N NaOH, and sterilized by filtration through a 0.45-μm membrane filter. Palmitic acid was added to the basal medium as an ethanolic solution (10 mg/ml), and cholesterol was added dissolved in Tween 80. A stock solution of cholesterol (20 mg/ml) was prepared in ethanol. Since cholesterol crystallized from this solution in the cold, it had to be warmed before use. A stock solution of 10% Tween 80 (Practical Grade, J. T. Baker Chemical Co., Phillipsburg, N.J.) was prepared in distilled water and sterilized by steam at 121 C for 15 min. Different volumes up to 1 ml of the cholesterol solution were added to 1 ml volumes of the Tween 80 solution. Ethanol was used to bring the final volume of all mixtures to 2 ml. The turbid mixtures were then carefully warmed until they cleared. Portions (0.2 ml) of these solutions were immediately transferred to flasks containing 100 ml of basal medium supplemented with albumin and palmitic acid. A maximal level of 20 μg of cholesterol per ml of medium could be obtained by this method. However, very frequently part of the cholesterol at this level crystallized immediately upon its introduction to the growth medium. Preincubation of the medium at 37 C and heating of the pipette used to transfer the concentrated cholesterol solution helped to decrease or even to prevent crystallization. Growth of each organism was tested in eight flasks containing 100 ml of the basal medium with various supplements as described in Table 1. The volume of the inoculum usually amounted to 1 or 2% of the total volume of the growth medium. However, with some very slow-growing mycoplasmas an increase in the size of inoculum was found necessary. The organisms were harvested when growth in the flasks containing the higher cholesterol concentrations showed fairly good turbidity. With some strains, a 3-day incubation period at 37 C was sufficient, whereas for others incubation periods up to 10 days were required. As growth of several of the mycoplasmas was found to be inhibited by 0.01% Tween 80, this detergent had to be omitted in some tests. For these strains, cholesterol was dissolved in ethanol, and 0.4-ml quantities were added to 100 ml of the serum-free medium. Part of the cholesterol crystallized and formed an opalescent turbidity at concentrations higher than 5 μg/ml. The organisms were collected by centrifugation at 34,000 × *g* for 15 min and washed twice with 0.25 M NaCl containing 0.01 M $MgCl_2$. The amount of cell protein in the washed organisms was determined according to the method of Lowry et al. (6). When the amount of cell protein in any of the flasks with added cholesterol did not reach an arbitrary level of 1 mg, the experiment was repeated by using a larger inoculum or a higher albumin concentration, or both. If growth did not improve, Tween 80 was omitted from the medium and cholesterol was added dissolved in ethanol alone.

RESULTS

Tables 1 and 2 show that all the *Mycoplasma* species included in our study, apart from *M. laidlawii*, *M. granularum*, and *Mycoplasma* species strain S-743, showed a growth response to increasing concentrations of cholesterol in the serum-free medium. Growth of these strains in the basal medium without cholesterol was either very poor or totally absent. *M. mycoides* var. *capri* and *M. gallisepticum* exhibited some limited growth in the absence of added cholesterol, provided albumin and palmitic acid were incorporated in the basal serum-free medium. With some slow-growing mycoplasmas, the size of inoculum had to be increased above the usual level of 1 or 2%. This increase supplemented the basal medium with small quantities of the Serum Fraction, enabling some limited growth with no added cholesterol. The case of *M. pneumoniae* may serve as an extreme example to illustrate this point. The strain available to us required horse serum and did not grow with Serum Fraction. Furthermore, a 10% inoculum was found to give best results in the cholesterol requirement test. The large inoculum introduced about 2% horse serum into the medium, enabling very limited growth in the unsupplemented basal medium. Nevertheless, this limited growth did not mask the growth response of this *Mycoplasma* to cholesterol (Table 1). An inoculum of 10% was also essential for testing the growth response of *M. lipophilum* MaBy to cholesterol, as this strain grew very poorly in the serum-free medium even when supplemented with Serum Fraction (Tables 1 and 2).

M. laidlawii and *M. granularum* grew very well in the unsupplemented serum-free medium, whereas strain S-743 grew rather poorly in this medium. The addition of palmitic acid, Tween 80, and albumin to the basal medium brought the growth of strain S-743 up to the level obtained with Serum Fraction, again indicating its requirement for fatty acids (22). Of the two stable L-phase variants included in the present study, only the *Proteus* L-phase grew well in the unsupplemented basal medium. The addition of cholesterol did not improve growth but rather inhibited it at the higher concentrations. The L-phase of *Streptobacillus moniliformis* did not grow in the unsupplemented basal medium. Bovine serum albumin and palmitic acid supported growth equivalent to that obtained with 1% Serum Fraction. However, Tween 80 caused a partial growth inhibition which was not relieved by the addition of cholesterol (Table 1).

Though with many mycoplasmas the yield of cell protein obtained at optimal cholesterol concentrations reached, or even surpassed, the yield obtained in the Serum Fraction medium, growth was usually faster in the latter. The prolonged lag in growth observed in the cholesterol series could be traced in many cases to the inhibitory effect of Tween 80. Some mycoplasmas, such as

TABLE 1. *Effect of cholesterol on growth of mycoplasmas and bacterial L-phase organisms in serum-free medium with Tween 80*

Organism and strain designation	Inoculum (% of growth medium)	Incubation period (days)	Cell protein[a]							
			Cholesterol (μg/ml) added to serum-free medium[b]					Other additives to basal serum-free medium		
			0	1	5	10	20	No additives	0.5% Albumin and 10 μg of palmitic acid per ml	1% Serum fraction
Mycoplasma salivarium PG-20	1	5[c]	0.10	0.50	1.50	0.93	0.92	0.10	0.10	3.30
M. fermentans PG-18	2	7	0.01	0.15	3.10	4.33	4.53	0.01	0.01	2.87
M. pneumoniae FH	10	9	0.82	1.01	2.90	2.25	2.60	0.25	0.77	2.25
M. orale 1 CH 19299	2	5[c]	0.08	0.44	0.67	1.05	1.25	0.02	0.07	1.80
M. orale 2 CH 20247	2	3[c]	0.25	1.48	3.23	4.89	4.35	0.02	0.07	3.53
M. orale 3 DC 333	2	4[c]	0.41	1.85	3.25	4.33	4.95	0.62	0.10[d]	2.63
M. lipophilum MaBy	10	7[c]	0.16	0.70	1.09	0.48	0.65	0.16	0.12[d]	0.80
Mycoplasma sp. Navel	2	6	0.01	0.25	1.37	1.04	3.33	0.01	0.09	1.73
M. neurolyticum type A	1	7	0.03	0.06	4.58	12.40	3.05	0.03	0.03	5.15
M. pulmonis PG-34	5	2	0.28	0.85	3.78	4.25	4.25	0.23	0.69[d]	7.95
M. arthritidis PG-6	1	7	0.02	0.22	1.60	1.60	1.60	0.05	0.05	2.18
M. bovigenitalium PG-11	1	7	0.01	0.04	1.00	0.99	0.97	0.01	0.04	3.18
M. bovirhinis PG-43	2	3	0.03	2.15	4.58	5.07	6.38	0.03	0.57	7.13
M. hyorhinis BTS-7	4	6	0.01	0.66	2.40	2.28	5.25	0.01	0.23[d]	5.88
M. mycoides var. *capri* PG-3	1	3	1.19	2.45	2.60	4.35	7.15	0.02	1.10	7.73
M. canis PG-14	1	7	0.02	0.05	1.03	5.15	2.83	0.02	0.02	7.30
M. maculosum PG-15	2	7	0.03	0.13	4.73	2.70	3.43	0.03	0.03	3.38
M. edwardii PG-24	1	4	0.01	0.10	1.10	1.65	2.15	0.01	0.01	7.60
M. felis Cat 27	1	7	0.06	0.52	0.99	1.01	1.18	0.01	0.07	4.67
M. gallisepticum S6	4	3	1.92	4.48	10.00	9.18	10.15	0.09	1.17[d]	10.18
M. gallinarum PG-16	1	5	0.50	1.05	5.35	5.75	5.10	0.10	0.20	2.55
M. iners 640	2	5	0.08	0.52	2.90	3.20	3.50	0.04	0.08	4.40
M. meleagridis 886	2	5	0.04	0.31	1.13	1.20	0.98	0.04	0.04	1.34
M. anatis 1340	2	5	0.13	2.13	3.86	4.08	4.40	0.05	0.13	5.70
M. laidlawii PG-8	1	3	14.60	20.50	15.20	16.20	20.50	10.10	13.40	17.50
M. granularum BTS-39	1	3	7.50	7.68	6.45	6.95	6.75	7.35	8.05	13.20
Mycoplasma sp. S-743	1	3	10.43	10.45	10.45	10.45	11.43	2.51	4.95	9.38
Streptobacillus moniliformis L_1 Rat 30 (ATCC 14075)	2	5	3.85	4.68	2.95	3.26	4.65	0.02	6.65[d]	5.85
Proteus sp. An (ATCC 14220)	2	8	14.75	15.60	18.90	12.30	2.18	14.20	15.60[d]	11.28

[a] Expressed in milligrams per 100 ml of medium.

[b] Medium supplemented with 0.5% albumin and 10 μg of palmitic acid per ml. Cholesterol was dissolved in Tween 80. Final concentration of Tween 80 was 0.01%.

[c] Grown in a 95% nitrogen + 5% carbon dioxide atmosphere.

[d] Albumin concentration in this series was raised to 1%.

M. hominis PG-21, *M. arginini* BBL-88, *M. gateae* Mart, and *M. spumans* PG-13 proved to be particularly sensitive to growth inhibition by Tween 80, as demonstrated by their failure to grow in the Serum Fraction medium when 0.01% Tween 80 was present. The growth response to cholesterol of these strains, and several strains that did grow in the presence of Tween 80, was therefore tested in the absence of the detergent (Table 2). Growth was usually faster and heavier in the series containing cholesterol dissolved in ethanol alone than in the series where it was dissolved in Tween 80.

DISCUSSION

Perhaps the most distinctive of the nutritional requirements of *Mycoplasma* are for lipids and lipid precursors needed for membrane synthesis. Here the major difficulty is to provide the lipid materials in an aqueous medium and in an assimilable, nontoxic, form resembling that found in serum. Thus cholesterol has been supplied in

TABLE 2. *Effect of cholesterol on growth of mycoplasmas in serum-free medium without Tween 80*

Mycoplasma species and strain designation	Inoculum (% of growth medium)	Incubation period (days)	Cell protein[a]							
			Cholesterol (μg/ml) added to serum-free medium[b]						Other additives to basal serum-free medium	
			0	1.0	2.5	5.0	10.0	20.0	0.4% Ethanol	1% Serum fraction
M. hominis PG-21	1	1	0.02	0.90	1.78	1.78	2.15	2.85	0.01	2.68
M. arginini BBL-88	1	1	0.11	1.04	2.00	2.55	2.85	3.00	0.02	3.15
M. gateae Mart	1	1	0.02	1.18	3.02	3.33	3.78	4.00	0.01	2.73
M. spumans PG-13	1	1	0.02	1.27	3.65	3.25	3.28	3.33	0.01	2.65
M. lipophilum MaBy	10	6[c]	0.05	0.09	0.02	0.41	0.41	0.70	0.05[d]	0.84
M. orale 3-DC 333	2	4[c]	0.09	1.70	1.95	2.40	2.13	3.48	0.09	3.05
M. fermentans PG-18	2	2	0.02	1.60	3.60	4.93	6.03	5.45	0.01	2.20
M. bovigenitalium PG-11	2	2	0.14	2.43	2.43	2.30	2.43	2.73	0.01	2.73
M. maculosum PG-15	2	3	0.01	1.20	3.28	6.08	3.70	3.93	0.01	3.10

[a] Expressed in milligrams per 100 ml of medium.

[b] Medium supplemented with 0.5% albumin and 10 μg of palmitic acid per ml. Cholesterol was dissolved in ethanol. Final concentration of ethanol in medium was 0.4%.

[c] Grown in a 95% nitrogen + 5% carbon dioxide atmosphere.

[d] Ethanol concentration in this series was reduced to 0.2%.

mycoplasma media together with a carrier—a thermostable defatted serum fraction C (14) or a similar serum lipoprotein (19). It is still more difficult to furnish an adequate supply of the long-chain fatty acids required, as small quantities of unesterified fatty acids cause rapid lysis of mycoplasmas. We overcame this problem by solubilizing the hydrophobic cholesterol molecules in Tween 80. The mixed micelles of Tween 80 and cholesterol remain in fine dispersion in the aqueous growth medium, providing an easily accessible supply of cholesterol. Moreover, Tween 80 may also serve as a water-soluble, less-toxic supply of oleic acid, as it can be slowly hydrolyzed by the mycoplasma lipase (11, 18). Palmitic acid has also been added to the growth medium since some mycoplasmas require a supply of both saturated and unsaturated fatty acids (17). Bovine serum albumin has been added to the medium to serve as a buffer, binding the free fatty acids and liberating minute nontoxic quantities of these substances (10, 15). The addition of L-arginine to the growth medium was found to be of advantage for the growth of the mycoplasmas possessing the arginine dihydrolase pathway (1).

Our results indicate that the L-phase of *S. moniliformis*, and some of the mycoplasmas, were inhibited by Tween 80 at the concentration employed (0.01%). Unfortunately this concentration of Tween 80 was found essential for keeping the high cholesterol concentration in solution. The toxicity of Tween 80 can apparently be traced to its detergent properties, since the wall-less mycoplasmas and L-phase variants are very sensitive to lysis by detergents. However, Tween 80 is a relatively weak detergent, and, at the concentration used in the test, it affected only a few of the organisms. The addition to the medium of cholesterol dissolved in ethanol in place of Tween 80 enabled the demonstration of the requirement for cholesterol in those mycoplasmas sensitive to Tween 80.

The results of the present study which encompass almost all the known *Mycoplasma* species provide experimental support for the view that the large majority of mycoplasmas require cholesterol for growth. The L-phase variants of *S. moniliformis* and *Proteus* species, which like mycoplasmas were found to incorporate substantial amounts of exogenous cholesterol into their cell membrane (12), do not depend on cholesterol for growth.

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