JOURNAL OF BACTERIOLOGY, June 1969, p. 970-978
Vol. 98, No. 3

Characteristics of a New Sterol-nonrequiring *Mycoplasma*

JOSEPH G. TULLY AND SHMUEL RAZIN

Laboratory of Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014, and Department of Clinical Microbiology, The Hebrew University—Hadassah Medical School, Jerusalem, Israel

Received for publication 1 April 1969

Two *Mycoplasma* strains recovered from tissue culture environments were found to grow in complex media devoid of serum or serum fractions containing cholesterol and in a cholesterol-free synthetic medium. Neither strain was capable of synthesizing pigmented carotenoids, although these compounds are present in, and characteristic of, other sterol-nonrequiring mycoplasmas. Serological tests and an analysis of their cell protein patterns obtained by gel electrophoresis indicated that the isolates were similar to each other but distinct from other sterol-nonrequiring serotypes, *Mycoplasma laidlawii* and *M. granularum*, as well as from sterol-requiring species. The existence of *Mycoplasma* other than *M. laidlawii* and *M. granularum* without sterol requirements suggested the need for some taxonomic changes in this group of organisms.

Members of the *Mycoplasmatales* are usually grouped into those species with nutritional requirements for cholesterol and those capable of growing in the absence of this component. The latter group, until recently, consisted solely of those strains identified serologically as *Mycoplasma laidlawii*. All *M. laidlawii* strains, regardless of whether they have been recovered from saprophytic environments or animal hosts, have been observed to grow in a medium devoid of animal serum or serum fractions containing cholesterol (14, 23, 25). They also differ from the sterol-requiring *Mycoplasma* in their ability to synthesize pigmented carotenoids (13, 20).

The cultivation of *M. granularum* strains in a serum-free medium was recently reported (25). Although some partial serological relationships were noted between these strains and *M. laidlawii*, a distinction among sterol-nonrequiring *Mycoplasma* could be made by some serological results and by differences in the electrophoretic patterns of their cell proteins. *M. granularum* strains also produced carotenoid pigments (25).

During the study reported above (25), two mycoplasma cultures recovered from tissue cell lines were examined, and, on the basis of growth in a medium devoid of serum, were initially thought to be *M. laidlawii* strains. Their serological characteristics, however, did not conform to those of *M. laidlawii*. This report presents further serological, physiological, and gel electrophoretic comparisons of these cultures to other sterol-requiring and -nonrequiring mycoplasmas.

MATERIALS AND METHODS

Mycoplasma. The S-410 and S-743 strains were recovered from two murine leukemia tissue culture cell lines (5, 6) by Charlotte Friend. The cell cultures were grown in basal media (Eagle) with Earle's balanced salt solution, supplemented with 15% fetal calf serum. The S-410 strain was recovered from the 61st passage of one cell line, and the S-743 was recovered from the 58th passage of another line. The PG-8 and PG-9 strains of *M. laidlawii* and the BTS-39 strain of *M. granularum* were included for comparative purposes. Some experiments were conducted with a wider collection of sterol-nonrequiring strains, whose origin and history were given earlier (25), and with two sterol-requiring strains, *M. gallisepticum* A5969 and *M. mycoides* var. *capri* PG-3.

Culture medium. Growth characteristics and colonial morphology of the new strains were examined on standard *Mycoplasma* media containing seven parts of Mycoplasma Broth Base (BBL), one part fresh 25% yeast extract, two parts unheated horse serum, and bacterial and fungal inhibitors (7). A serum fraction medium was also employed in which the broth base was increased to nine parts, 1% PPLO Serum Fraction (Difco) was substituted for horse serum, and the only inhibitor consisted of 500 units of penicillin per ml. Serum-free medium was prepared by omitting the PPLO Serum Fraction in the latter preparation. This medium was employed for growth of S-410 and S-743 mycoplasma antigens utilized in both the indirect fluorescent antibody (FA) test and the preparation of specific antisera in rabbits. The

organisms were also tested for growth in the partially-defined medium of Razin and Cohen (14) modified by replacing the bovine serum albumin fraction V with albumin (bovine) fatty acid poor (Calbiochem, Los Angeles, Calif.) and by the addition of Tween 80 to a final concentration of 0.005%. All cultures were maintained aerobically. Trypticase Soy Broth (BBL) was also used to test for possible reversion of the sterol-nonrequiring growth to bacterial.

Assessment of osmotic fragility. The procedure described by Razin (10) was followed. Results were expressed as percentage lysis in 0.06 M NaCl, calculated according to the formula: [(OD in 0.25 M NaCl − OD in 0.6 M NaCl)/(OD in 0.25 M NaCl)] × 100.

Fermentation tests. Procedures for determining the ability of *Mycoplasma* strains to ferment glucose and mannose when grown in serum-free media were previously given (25).

Ultrafiltration tests. Filterability of the S-410 and S-743 strains was estimated by serial filtration through graded cellulose ester filters by the method of Morowitz et al. (9), with some modifications (24).

Carotenoid biosynthesis. Tests for the production of carotenoid pigments by the S-410 and S-743 strains were performed according to the procedure of Razin and Cleverdon (13), and as previously outlined in tests on *M. laidlawii* and *M. granularum* (25). The amount of carotenoid pigments in cells was expressed as absorbancy at 438 nm times 1,000 per mg of cell protein. Incorporation of sodium acetate-*1*-^{14}C (The Radiochemical Centre, Amersham, England) into polar and nonpolar lipids of the cells was tested according to Rottem and Razin (19).

Cholesterol incorporation. The ability of *Mycoplasma* strains to incorporate cholesterol from the growth medium was tested in a manner similar to that reported by Argaman and Razin (1). Organisms grown for 24 to 48 hr at 37 C in 250-ml volumes of a modified Edward medium (10), containing 2% PPLO Serum Fraction and 0.1 μc of cholesterol-*4*-^{14}C (The Radiochemical Centre, Amersham, England) were harvested, washed twice in 0.25 M NaCl, and total cell protein was determined according to Lowry et al. (8). The cell lipids were extracted with chloroform and methanol, their radioactivity was assayed, and the total cholesterol was determined (1). An analysis for cholesterol was also carried out on lipids of cells grown in modified Edward medium (10) without PPLO Serum Fraction, but with 0.1% of albumin (bovine) fatty acid poor. The cells used as inocula were washed twice in 0.25 M NaCl. Growth was carried out in 1-liter volumes of medium for 48 hr at 37 C. The organisms were harvested and washed and their lipids extracted. Analysis for cholesterol in the lipids was done by thin-layer chromatography using Silica Gel G (0.25-mm thick) chromatoplates. The developing solvent was benzene-diethyl ether-acetic acid-ethyl alcohol (50:40:0.2:2, v/v). Cholesterol spots were detected with the Liebermann-Burchard spray reagent (26). The minimal amount of cholesterol detected with this reagent was 1 μg included in a spot of 4-mm diameter.

Effect of cholesterol on growth. The organisms were grown at 37 C for 48 hr in 100-ml quantities of Edward medium (10) without PPLO Serum Fraction. The medium was supplemented with 0.1% albumin (bovine) fatty acid poor and with various amounts of cholesterol dissolved in Tween 80. Final concentration of Tween 80 in the medium was 0.01%. The inoculum for each bottle consisted of a 5-ml culture of cells grown in the serum-free medium. Organisms were harvested and washed twice in 0.25 M NaCl, and their protein content was determined according to Lowry et al. (8).

Inhibition of growth by digitonin. The method of Razin and Schefer (*in preparation*) was used. Growth inhibition was tested in tubes containing 3 ml of Edward medium (10) with 3% horse serum and various concentrations of digitonin. Each tube received 0.2 ml of a 1:1,000 dilution of a 24-hr culture. Results were read after 48 hr of incubation at 37 C and expressed as the minimal concentration of digitonin inhibiting visible growth.

Polyacrylamide gel electrophoresis of cell proteins. For polyacrylamide gel electrophoresis, procedure described by Razin (12) was followed. Cell proteins were solubilized in phenol-acetic acid-water (2:1:0.5, w/v/v) and run in polyacrylamide gels containing 5 M urea and 35% acetic acid.

Serological techniques. *Mycoplasma* strains and antisera were compared in an indirect FA technique similar to that previously reported (25). A direct FA test of *Mycoplasma* colonies grown on agar (3), previously utilized in a study on sterol-nonrequiring mycoplasmas (25), was also included. Antisera prepared to *Mycoplasma* serotypes and strains listed in Table 1 were tested against S-410 and S-743 strain antigens. Complement-fixation tests among sterol-nonrequiring mycoplasmas were performed with unheated antigens prepared from organisms grown in serum-free broth for 24 hr and concentrated 20- to 40-fold by centrifugation at 27,000 × *g* for 1 hr. We employed 8 units of antigen and read the tests after overnight fixation at 4 C in microcomplement-fixation plates. The PG-8 antigen was tested only at a 1:35 dilution (veronal buffer) because of strong anticomplimentary activity; 11 other *M. laidlawii* antigens were also anticomplimentary.

Growth inhibition by immune sera. Growth inhibition tests were performed with the general technique of Clyde (2) against two lots of hyperimmune antisera to *M. laidlawii* PG-9, one antiserum to *M. laidlawii* PG-8, two antisera to *M. granularum* BTS-39, and one antiserum to the S-743 strain. Fluid cultures of the above strains were passed in serum-free broth and 48-hr-old cultures diluted 1:100, 1:200, and 1:300 before plating in 0.2-ml quantities to serum-free agar plates. The plates were incubated 1 to 2 hr before the addition of antiserum-saturated discs. All plates were read after 18 to 24 hr at 37 C and again after an additional 24 hr at room temperature.

RESULTS

Cultural and biochemical characteristics. The S-410 and S-743 isolates grew abundantly on the standard *Mycoplasma* medium containing either 20% horse serum or 1% PPLO Serum Fraction

TABLE 1. *Mycoplasma employed in antisera preparation for indirect immunofluorescent serological tests*

Mycoplasma serotypes	Strain	Mycoplasma serotypes	Strain
Human		Avian	
M. hominis	PG-21[a]	*M. gallisepticum*	S 6
M. fermentans	PG-18[a]	*M. gallinarum*	PG-16
M. salivarium	PG-20[a]	*M.* sp. (C)	859
M. pneumoniae	FH[a]	*M.* sp. (D)	887
M. orale 1	CH 19299[a]	*M.* sp. (F)	1197
M. orale 2	CH 20247[a]	*M. iners* (G)	640
M. orale 3	DC 333	*M. meleagridis* (H)	886
M. lipophilum	MaBy[a]	*M.* sp. (I)	Iowa 695
M. sp.	Navel[a]	*M. anatis*	1340
Caprine and Ovine		Bovine	
M. arginini	BBL-88[a]	*M. bovigenitalium*	PG-11
M. sp.	B 3	*M. bovirhinis*	PG-43
M. sp.	Goat 189	*M.* sp.	Calf 188
M. sp.	BBL G-145[a]	*M.* sp.	Conn.
M. sp.	KS-1	Swine	
M. sp.	HRC S-058	*M. hyorhinis*	BTS-7, GDL[a]
Canine		*M. granularum*	BTS-39
M. spumans	PG-13[a]	Feline	
M. canis	PG-14[a]	*M. felis*	Cat 27
M. Maculosum	PG-15[a]	*M. gateae*	Mart
M. sp.	PG-24[a]	*M.* sp.	LL
Murine		*M.* sp.	Bennett
M. neurolyticum	Type A[a]	Miscellaneous	
M. pulmonis	PG-34[a]	*M. laidlawii*	PG-8[a]
M. arthritidis	PG-6, PG-27[a]	*M. laidlawii*	PG-9[a]

[a] Strains in which fluorescein-conjugated antisera were used in direct test on agar colonies.

at 30 or 37 C. The growth of S-410 was always heavier than that of S-743. The turbidity associated with the initial transfer of each culture to serum-free medium, as well as with subsequent passages in this environment, indicated that sterol was not a required component for growth. Broth cultures of each cloned strain at the 16th passage level were transferred to plates of the three media. Colonial variations of the S-743 strain on media containing horse serum, serum fraction, or without serum, after 5 days incubation at 37 C, are depicted in Figures 1 to 3. Colonies on horse serum exhibited the classical "fried egg" structure while those appearing on serum fraction or serum-free media usually were smaller and showed no peripheral growth around the colony center. After 7-days incubation, a few colonies on both serum fraction and serum-free media showed peripheral growth. Much the same pattern of colonial variation was noted with the S-410 strain. *M. laidlawii* strains grown repeatedly in the absence of serum usually showed typical "fried egg" colonies on all media after 3 to 4 days. Growth of S-410 and S-743 mycoplasmas on serum-free broth and agar also occurred at 30 C and at approximately the same rate as *M. laidlawii* strains. Both the S-410 and S-743 strains were able to grow in the partially-defined medium of Razin and Cohen (14), which is free of cholesterol. Their growth in this medium as judged by turbidity and by viable counts was inferior to that of the oral strain of *M. laidlawii* and about equivalent to *M. granularum* BTS-39. The maximal titer of *M. laidlawii* in this medium was about 1×10^9 colony-forming units (CFU)/ml, whereas S-410, S-743, and *M. granularum* only reached a titer of 2×10^8 CFU/ml. *M. mycoides* var. *capri* PG-3 and *M. gallisepticum* A5969 did not grow in this sterol-free medium. The two tissue culture isolates fermented glucose within 24 hr when cultivated in either serum fraction or serum-free media. Mannose was not fermented at 7 days. The two *M. laidlawii* strains and *M. granularum* strain exhibited fermentation patterns similar to the tissue culture mycoplasmas.

In an attempt to determine whether S-410 and S-743 strains were the L-phase of a bacterium, each strain was passed 15 times in a Trypticase Soy Broth medium without penicillin. Both strains grew in this medium with a very light turbidity and were successfully passed at 2- to 3-day intervals at 37 C. Gram stains did not reveal bacterial forms at any time during this series of transfers, and platings to trypticase soy agar resulted in the appearance of small, micro-

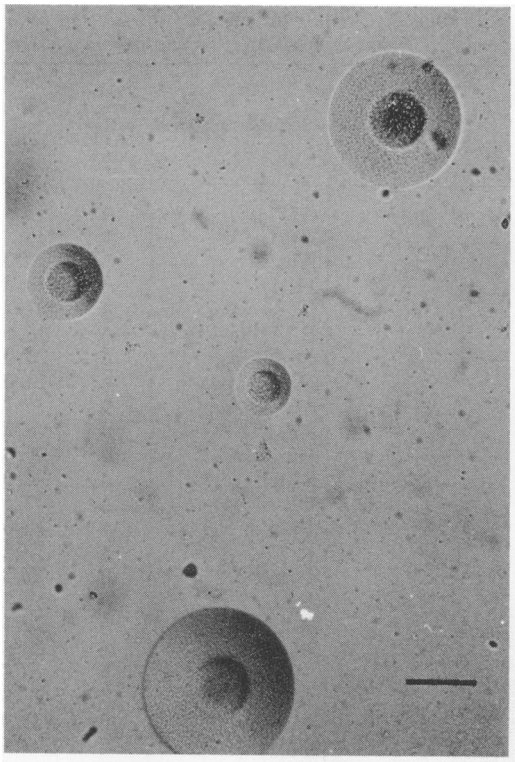

FIG. 1. *Colonial morphology of S-743 strain on horse serum medium after 5 days of incubation. × 105. All markers represent 100 μm.*

scopic colonies, similar to those observed on serum-free *Mycoplasma* media containing penicillin.

Ultrafiltration properties. The S-410 and S-743 cultures passed through 0.80-, 0.45-, and 0.30-μm membrane filters without significant decrease in titer. A 10- to 100-fold decrease occurred after passage through 0.22-μm filters and both strains were retained by 0.10-μm filters. *M. laidlawii* and *M. granularum* strains also passed through 0.22-μm filters but were completely retained by filters having an average pore diameter of 0.10 μm.

Osmotic fragility. The S-410 and S-743 strains resembled *M. laidlawii* in being very sensitive to osmotic lysis. The per cent lysis values in 0.06 M NaCl were 48 for S-410, 50 for S-743, and 42% for the oral *M. laidlawii* strain.

Carotenoid tests. Tissue culture mycoplasmas did not produce carotenoid pigments when grown in modified Edward medium or in serum-free medium supplemented with sodium acetate. Standard *M. laidlawii* strains A and B as well as a large collection of sterol-nonrequiring related strains including *M. granularum* produced carotenoid pigments (Table 2). The absence of pigmented carotenoids does not rule out the presence of nonpigmented, more saturated, carotenoids. Since acetate is the primary precursor for polyterpene biosynthesis (22), its incorporation into lipids of the S-410 and S-743 strains was investigated. Of the four strains tested (Table 3), *M. laidlawii* oral strain incorporated the largest amount of ^{14}C-acetate into its lipids, an appreciable part of the label being found in the nonpolar lipid fraction which includes the carotenoids. *M. granularum* was less active than *M. laidlawii* in acetate incorporation, whereas the two tissue culture isolates exhibited an even lower ability to incorporate acetate. The low radioactivity values found in the nonpolar lipid fraction of S-410 and S-743 indicated the absence or, at most, a weak ability for polyterpene biosynthesis in these strains.

Incorporation of cholesterol. The capacity of S-410 and S-743 strains, and other parasitic and

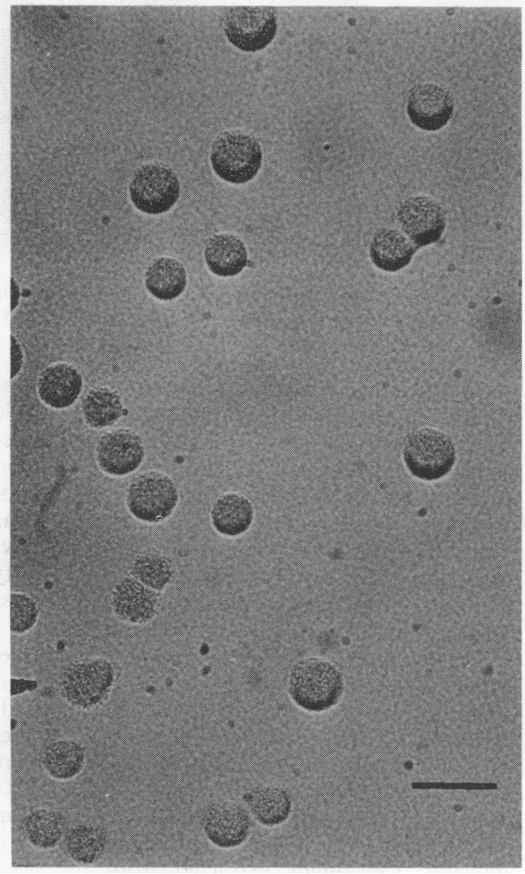

FIG. 2. *Colonial morphology of S-743 strain on PPLO serum fraction medium after 5 days of incubation. × 105.*

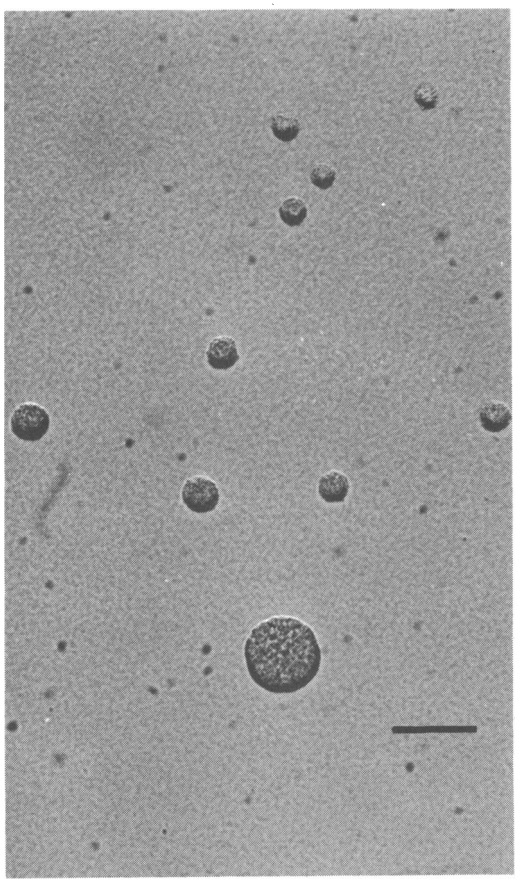

FIG. 3. *Colonial morphology of S-743 strain on serum-free medium after 5 days of incubation.* × *105.*

saprophytic mycoplasmas, to incorporate cholesterol from the growth medium was tested (Table 4). The tissue culture isolates bound more cholesterol than *M. laidlawii* but less than *M. gallisepticum* or *M. mycoides* var. *capri*. The amount of total cholesterol in *M. mycoides* var. *capri* was much higher than expected from the radioactivity values. This results from the marked ability of this strain to incorporate the unlabeled cholesterol esters from the growth medium (1).

Growth of the S-410 and S-743 in Edward medium devoid of serum or serum fraction was much poorer than that of *M. laidlawii*. Yields measured as milligrams of cell protein per liter of medium after 48-hr incubation at 37 C were 80 mg for *M. laidlawii* oral strain, 15 mg for S-410, 8 mg for S-743, and 45 mg for *M. granularum*; *M. mycoides* var. *capri* failed to grow in this medium. Cell lipids extracted from the total cell yield of the four strains were analyzed for cholesterol. Only traces of cholesterol (less than 10 μg) were found in the lipids of *M. laidlawii*.

TABLE 2. *Carotenoid pigment production by sterol-nonrequiring mycoplasmas*

Mycoplasma serotype	Strain designation	Electrophoretic pattern classification[a]	Carotenoids[b]
M. laidlawii	A(PG-8)	A	37.3
	B(PG-9)	B	35.2
	Algen	A	22.0
	Mist	A	10.5
	L	A	37.7
	B-15(PG-10)	A	15.6
	J-18S	A	26.0
	Haig 179-L	A	17.0
	643-N	B	36.1
	3M-152(PG-47)	B	8.2
	H3-10	B	26.8
	STR	A	4.0
	OR	A	42.5
	Flamm 583	A	16.0
	R-4	A	13.2
	Granoff 8	B	17.5
	TC7277	B	17.7
	R-1	A	24.4
	PG-5	A	20.8
	31B	A	26.8
	39B	A	20.8
	46B	B	25.0
	48B	B	27.5
	58B	B	29.2
	B4	B	17.5
M. granularum	BTS-39		18.5
Tissue culture isolates	S-410		0
	S-743		0

[a] Based on the resemblance of the electrophoretic pattern of cell proteins to the pattern of either strain A or B of *M. laidlawii*.

[b] Optical density at 438 nm × 1,000 per mg of cell protein.

TABLE 3. *Incorporation of ^{14}C-acetate into lipids of sterol-nonrequiring mycoplasmas*[a]

Strain	Yield of organisms[b]	Radioactivity (counts/min)	
		Polar lipid fraction	Nonpolar lipid fraction
M. laidlawii oral	10.0	21,217	1,739
M. granularum	7.3	4,558	350
S-410	9.2	3,030	152
S-743	6.5	2,340	120

[a] Organisms were grown in 100 ml of Edward medium containing 2% PPLO serum fraction and 1 μc of sodium acetate-*1*-^{14}C.

[b] Expressed as milligrams of cell protein.

TABLE 4. *Incorporation of cholesterol by sterol-requiring and -nonrequiring Mycoplasma*[a]

Mycoplasma strain	Cell protein	Radioactivity in cell lipids	Radioactivity/mg of cell protein	Total cholesterol in lipid	Cholesterol[b]
	mg	*counts/min*	*counts/min*	*μg*	
M. laidlawii (oral)	30.0	17,200	573	352	11.7
S-410	21.5	18,500	860	322	15.0
S-743	16.5	19,600	1,187	337	20.4
M. gallisepticum	20.0	42,800	2,140	600	30.0
M. mycoides var. *capri*	35.0	89,000	2,542	3,000	85.0

[a] Organisms were grown in 250 ml of Edward medium containing 2% PPLO serum fraction and 0.1 μc of cholesterol-4-^{14}C.

[b] Expressed as micrograms per milligram of cell protein.

Cholesterol could not be detected in the lipids of the other three organisms, but, as expected, much smaller quantities of lipids were available for analysis.

Effect of cholesterol on growth. Tween 80, but not cholesterol, improved growth of the tissue culture isolates in the serum-free medium, indicating the lack of an adequate supply of fatty acids in this medium (Table 5). Cholesterol and Tween 80 had no significant effect on the growth of *M. laidlawii* and *M. granularum* in the serum-free medium.

Inhibition of growth by digitonin. Razin and Schefer (*in preparation*) have shown that the sensitivity of microorganisms to growth inhibition by digitonin parallels their capacity to incorporate cholesterol into their plasma membranes. Growth of the four sterol-nonrequiring strains, *M. laidlawii*, *M. granularum*, S-410, and S-743, was inhibited by 500 μg of digitonin per ml, whereas that of the sterol-requiring *M. mycoides* var. *capri* and *M. gallisepticum* was inhibited by 30 μg of digitonin per ml medium. These results are in accordance with the higher cholesterol content of the "parasitic" mycoplasmas.

TABLE 5. *Effect of cholesterol and Tween 80 on growth of the mycoplasmas in a serum-free medium*

Cholesterol (μg/ml)	Cell protein[a]			
	M. laidlawii oral strain	*M. granularum* BTS-39	S-410	S-743
0[b]	8.8	5.4	1.7	2.2
0[c]	9.4	6.2	12.4	10.0
1.2	10.0	6.2	12.4	8.8
5.0	10.4	7.6	13.0	10.2
10.0	10.6	6.8	14.4	10.1

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

[b] Serum-free medium without cholesterol and Tween 80.

[c] Serum-free medium without cholesterol but with 0.01% Tween 80.

Electrophoretic patterns of cell proteins. Comparison of the cell protein patterns obtained by polyacrylamide gel electrophoresis is shown in Fig. 4. Although the patterns of the PG-8 and PG-9 strains of *M. laidlawii* and the *M. granularum* strain are different, there is a basic similarity in the lower parts of the gel. The S-410 and S-743 strains have patterns identical to one another but show a completely different pattern from that seen with the other sterol-nonrequiring

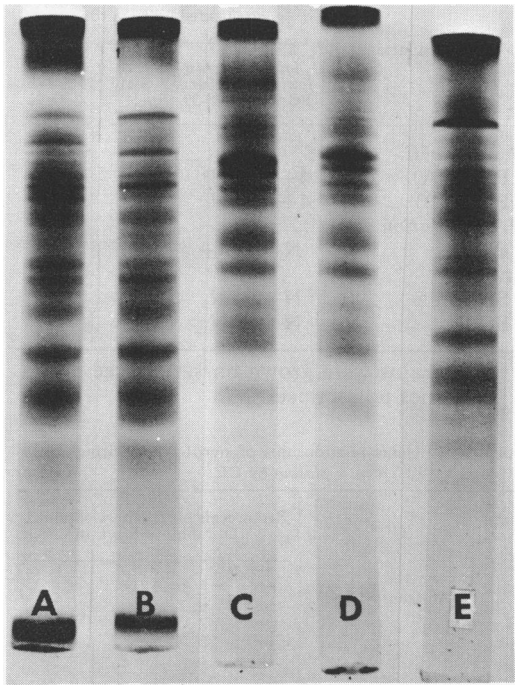

FIG. 4. *Electrophoretic patterns of Mycoplasma cell proteins: A, M. laidlawii (A), PG-8; B, M. laidlawii (B), PG-9; C, Mycoplasma sp. S-410; D, Mycoplasma sp. S-743; and E, M. granularum BTS-39 (ATCC 19168).*

mycoplasmas. With the exception of *M. granularum* strains, all the carotenoid-producing, sterol-nonrequiring strains included in our collection exhibited patterns very similar or even identical with the patterns of either strain A or B of *M. laidlawii* (Table 2).

Serological characteristics. The tissue culture *Mycoplasma* strains were compared in indirect immunofluorescence tests against antisera to other established *Mycoplasma* serotypes and numerous unclassified mycoplasmas (Table 1) without evidence of a serological relationship. In addition, the direct fluorescence test, performed on agar colonies of S-410 and S-743 with fluorescein-tagged antisera to several *Mycoplasma* serotypes (Table 1), also showed no relationship. Direct FA tests were performed on agar colonies of the sterol-nonrequiring mycoplasmas (Table 6). The results of complement-fixation (CF) tests among the sterol-nonrequiring mycoplasmas (Table 7) confirmed the relatedness of the S-410 and S-743 strains and their antigenic distinction from other sterol-nonrequiring serotypes. The slight cross-reaction observed between *M. laidlawii* and *M. granularum* in this test was also noted earlier with the indirect FA procedure (25). The persistent anticomplimentary activity of numerous *M. laidlawii* antigens necessitated dilution of the PG-8 antigen with a consequent decrease in the homologous CF titer. However, the results of reciprocal tests fail to show any serological relationship among the S-410 or S-743 strains and *M. laidlawii* or *M. granularum*.

TABLE 6. *Direct immunofluorescence tests on agar colonies of sterol-nonrequiring mycoplasmas*

Mycoplasma strains[a]	Fluorescence against fluorescein-conjugated antisera to:			
	M. laidlawii PG-9	*M. granularum* BTS-39	S-410	S-743
M. laidlawii				
PG-8 (A)	4+	N[b]	N	N
PG-9 (B)	4+	N	N	N
M. granularum				
BTS-39	N	4+	N	N
Untyped				
S-410	N	N	4+	4+
S-743	N	N	4+	4+

[a] All strains were grown on serum-free media.
[b] Colonies not stained.

TABLE 7. Antigenic relationships of sterol-nonrequiring *Mycoplasma* by CF

Antigens	Reciprocals of CF titers obtained on rabbit antisera to			
	M. laidlawii PG-8	*M. granularum* BTS-39	S-410	S-743
M. laidlawii PG-8	128	<8	<8	<8
M. granularum BTS-39	64	>512	32	32
S-410	32	32	128	256
S-743	16	16	128	>512

Growth-inhibition tests. The S-410 and S-743 strains were not inhibited by hyperimmune rabbit sera to three different lots of *M. laidlawii* or to two different lots of *M. granularum* antisera. Both PG-8 and PG-9 strains of *M. laidlawii* showed growth inhibition zones of 3 to 4 mm against either PG-8 or PG-9 antisera. Inhibition zones of 2 to 3 mm were observed in homologous *M. granularum* tests. Reciprocal growth-inhibition tests between *M. granularum* and *M. laidlawii* strains and antisera were completely negative. The slight inhibition of *M. granularum* strains by a commercial *M. laidlawii* antiserum observed earlier (25), could not be confirmed with the three *M. laidlawii* antisera employed in the present study. Antiserum to S-743, showing 3- to 4-mm inhibition zones against S-743 and S-410 strains, did not inhibit the growth of *M. laidlawii* or *M. granularum*.

DISCUSSION

The two mycoplasmas recovered from tissue cultures possess many of the cultural and biochemical properties of the other sterol-nonrequiring mycoplasmas, *M. laidlawii* and *M. granularum*. However, the absence of pigmented carotenoids in these strains appears to be a major feature in their separation and differentiation from other mycoplasmas capable of growing in the absence of sterols. The lack of any serological relationship to other established *Mycoplasma* serotypes and the differences observed between electrophoretic patterns of cell proteins of the tissue culture isolates and those of other sterol-requiring and nonrequiring mycoplasmas offer additional evidence that these strains are distinct from previously described *Mycoplasma*.

The incorporation of cholesterol into the cell membrane of the *Mycoplasma* has received a great deal of attention, not only from a physiological standpoint but from some taxonomic interest, since membranes of other bacteria do not contain this constituent. Most mycoplasmas, except *M. laidlawii*, require cholesterol for growth and incorporate cholesterol of the growth medium into their membrane lipids (1, 13, 17).

It was recently proposed that *M. granularum* should be classified as a *Mycoplasma* without cholesterol requirements (25). Although *M. laidlawii* strains do not require cholesterol for growth, they are able to incorporate it from the medium, generally in much smaller quantities than the parasitic *Mycoplasma* (11). The S-410 and S-743 mycoplasmas described here incorporate larger amounts of cholesterol from the growth medium than *M. laidlawii*, but smaller quantities than two representative parasitic *Mycoplasma* serotypes. However, the tissue culture isolates, like *M. laidlawii* and *M. granularum* strains, could grow in cholesterol-free media. Cholesterol could not be detected in cells grown under these conditions, indicating the inability of the organisms to synthesize it. The poor growth of the tissue-culture isolates in the serum-free Edward medium results apparently from an inadequate supply of essential fatty acids. The addition of Tween 80 corrected this deficiency. Addition of cholesterol, on the other hand, had no effect on growth, a confirmation of our thesis that these strains do not require cholesterol. Growth of *M. mycoides* var. *capri* in the same medium was markedly improved by increasing the cholesterol concentration (*see* Table 4 in reference 11).

Another unique aspect of the sterol-nonrequiring mycoplasmas has been their ability to synthesize carotenoid pigments in the cell membrane (13, 17, 20, 25). The exact function of carotenoids in these mycoplasmas has not been established. Some believe they may fulfill a function analogous to that of cholesterol (20, 22), although *M. laidlawii* has been grown in a medium devoid of cholesterol without evidence of carotenoid biosynthesis (15). Carotenoids also apparently neither interfere with the cholesterol uptake in *M. laidlawii* (13) nor affect the osmotic fragility of the cells (16). *M. laidlawii* carotenoids were recently shown to protect the adenosine triphosphatase activity of the cell membrane against photodynamic destruction. The pigments may thus play a role in the protection of cells against the damaging effects of solar radiation (18). The S-410 and S-743 mycoplasmas were unable to synthesize carotenoid pigments under conditions adequate for their synthesis by *M. laidlawii* and *M. granularum*. Moreover, the very low incorporation of radioactive acetate into nonpolar lipids also indicates the inability of the tissue culture isolates to synthesize nonpigmented carotenoids. Hence it appears that the S-410 and S-743 mycoplasmas are able to grow without cholesterol in the absence of carotenoid synthesis. These findings lend support to the idea (15) that carotenoids might not be essential for *Mycoplasma* growth in a sterol-free medium, contradicting the hypothesis (21) that carotenoids in sterol-nonrequiring mycoplasmas fulfill analogous functions to those of sterols in the sterol-requiring mycoplasmas.

The occurrence of other *Mycoplasma* without sterol requirements but serologically distinct from *M. laidlawii* and *M. granularum* provides additional support for the creation of a new genus in the Order *Mycoplasmatales* (4, 25). This genus could then include all sterol-nonrequiring mycoplasmas and provide for further speciation into those strains with distinct serological characteristics, those with other unique properties of lipid metabolism, such as lack of pigmented carotenoids, and those strains possessing additional nutritional and biochemical distinctions. In this regard, we feel it inappropriate to assign a species designation to the described *Mycoplasma* until such time as the sterol-nonrequiring mycoplasmas might be reclassified and further isolates and possible hosts of these strains identified. The S-743 culture was deposited in the American Type Culture Collection (ATCC 25176).

ACKNOWLEDGMENTS

We thank Charlotte Friend for the two tissue culture isolates and the numerous investigators who supplied mycoplasmas for antisera preparation. We are also grateful for the assistance of M. F. Barile and R. H. Purcell in supplying samples of *M. laidlawii* and *M. granularum* growth-inhibiting antisera, R. A. Del Giudice for furnishing conjugated antisera, and H. C. Turner and W. L. Cline for the complement-fixation tests. C. W. Renshawe, N. G. Ramsburg, and M. Wormser provided valuable technical assistance.

LITERATURE CITED

1. Argaman, M., and S. Razin. 1965. Cholesterol and cholesterol esters in *Mycoplasma*. J. Gen. Microbiol. 38:153–168.
2. Clyde, W. A., Jr. 1964. *Mycoplasma* species identification based upon growth inhibition by specific antisera. J. Immunol. 92:958–965.
3. Del Giudice, R. A., N. F. Robillard, and T. R. Carski. 1967. Immunofluorescence identification of *Mycoplasma* on agar by use of incident illumination. J. Bacteriol. 93:1205–1209.
4. Edward, D. G., E. A. Freundt, R. M. Chanock, J. Fabricant, L. Hayflick, R. M. Lemcke, S. Razin, N. L. Somerson, and R. G. Wittler. 1967. Recommendations on nomenclature of the order *Mycoplasmatales*. Science 155:1694–1696.
5. Friend, C., M. C. Patuleia, and E. de Harven. 1966. Erythrocyte maturation *in vitro* of murine (Friend) virus-induced leukemic cells. Nat. Cancer Inst. Monogr. 22:505–520.
6. Friend, C., M. C. Patuleia, and J. B. Nelson. 1966. Antibiotic effect of tylosin on a *Mycoplasma* contaminant in a tissue culture leukemic cell line. Proc. Soc. Exp. Biol. Med. 121: 1009–1010.
7. Hayflick, L. 1965. Tissue cultures and mycoplasmas. Texas Rept. Biol. Med. 23:285–303.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
9. Morowitz, H. J., M. E. Tourtellotte, and M. E. Pollack. 1963. Use of porous cellulose ester membranes in the primary iso-

lation and size determination of pleuropneumonia-like organisms. J. Bacteriol. 85:134–136.
10. Razin, S. 1963. Osmotic lysis of *Mycoplasma*. J. Gen. Microbiol. 33:471–475.
11. Razin, S. 1967. The cell membrane of *Mycoplasma*. Ann. N. Y. Acad. Sci. 143:115–129.
12. Razin, S. 1968. *Mycoplasma* taxonomy studied by electrophoresis of cell proteins. J. Bacteriol. 96:687–694.
13. Razin, S., and R. C. Cleverdon. 1965. Carotenoids and cholesterol in membranes of *Mycoplasma laidlawii*. J. Gen. Microbiol. 41:409–415.
14. Razin, S., and A. Cohen. 1963. Nutritional requirements and metabolism of *Mycoplasma laidlawii*. J. Gen. Microbiol. 30: 141–154.
15. Razin, S., and S. Rottem. 1967. Role of carotenoids and cholesterol in the growth of *Mycoplasma laidlawii*. J. Bacteriol. 93:1181–1182.
16. Razin, S., M. E. Tourtellotte, R. N. McElhaney, and J. D. Pollack. 1966. Influence of lipid components of *Mycoplasma laidlawii* membranes on osmotic fragility of cells. J. Bacteriol. 91:609–616.
17. Rothblat, G. H., and P. F. Smith. 1961. Nonsaponifiable lipids of representative pleuropneumonia-like organisms. J. Bacteriol. 82:479–491.
18. Rottem, S., L. Gottfried, and S. Razin. 1968. Carotenoids as protectors against photodynamic inactivation of the adenosine triphosphatase of *Mycoplasma laidlawii* membranes. Biochem. J. 109:707–708.
19. Rottem, S. and S. Razin. 1967. Uptake and utilization of acetate by *Mycoplasma*. J. Gen. Microbiol. 48:53–63.
20. Smith, P. F. 1963. The carotenoid pigments of *Mycoplasma*. J. Gen. Microbiol. 32:307–319.
21. Smith, P. F. 1963. The role of sterols in the growth and physiology of pleuropneumonia-like organisms. p. 518–525. *In* N. E. Gibbons (ed.), Recent progress in microbiology, vol. 8, University of Toronto Press, Toronto.
22. Smith, P. F., and C. V. Henrikson. 1966. Growth inhibition of *Mycoplasma* by inhibitors of polyterpene biosynthesis and its reversal by cholesterol. J. Bacteriol. 91:1854–1858.
23. Tourtellotte, M. E., H. J. Morowitz, and P. Kasimer. 1964. Defined medium for *Mycoplasma laidlawii*. J. Bacteriol. 88:11–15.
24. Tully, J. G. 1965. Biochemical, morphological, and serological characterization of *Mycoplasma* of murine origin. J. Infec. Dis. 115:171–185.
25. Tully, J. G., and S. Razin. 1968. Physiological and serological comparisons among strains of *Mycoplasma granularum* and *Mycoplasma laidlawii*. J. Bacteriol. 95:1504–1512.
26. Waldi, D. 1965. Spray reagents for thin-layer chromatography, p. 483–502. *In* E. Stahl (ed.), Thin-layer chromatography. Academic Press Inc., New York.