A Test for Measuring Growth Responses of Mollicutes to Serum and Polyoxyethylene Sorbitan

DAVID L. ROSE,^{1†} JOSEPH G. TULLY,^{1*} JOSEPH M. BOVÉ,² AND ROBERT F. WHITCOMB³

Mycoplasma Section, Frederick Cancer Research and Development Center, National Institute of Allergy and Infectious Diseases, Frederick, Maryland 21702¹; Laboratoire de Biologie Cellulaire et Moléculaire, Institut National de la Recherche Agronomique, and University of Bordeaux II, 33883 Villenave d'Ornon Cedex, France²; and Insect Biocontrol Laboratory, U.S. Department of Agriculture, Beltsville, Maryland 20705³

A test is described that is useful for characterizing mollicutes in terms of the ability to maintain growth in medium containing 15 to 20% fetal bovine serum or in serum-free media with or without 0.04% Tween 80 (polyoxyethylene sorbitan). Representative *Acholeplasma* species maintained growth in serum-free medium, and about half of the strains tested grew well in Tween 80-supplemented medium. Representative *Mycoplasma* and *Entomoplasma* species did not maintain growth in either serum-free medium alone or when Tween 80 was added. *Spiroplasma* species and group representatives generally failed to sustain growth in serum-free medium with or without Tween 80, but at least four of the spiroplasmas tested maintained growth in serum-free medium. The representative *Mesoplasma* species grew in serum-free media only when Tween 80 was added, as did *Mycoplasma lactucae*. Although the test has obvious determinative uses for members of the class *Mollicutes*, it does not supplant the conventional methodology for assaying the cholesterol requirements of these organisms.

Tests for determination of sterol or cholesterol requirement for growth have been important procedures in taxonomic assignments of newly isolated mollicutes (6). The most widely employed procedure measures total organism protein in cell pellets from strains grown in 100-ml volumes of serum-free broth media containing additions of various quantities of cholesterol (13, 19). This basic test was later modified to include growth on solid medium containing similar additives (4). Both procedures have certain drawbacks. In the broth method, a large amount of medium is required and cultivated organisms must be harvested by laborious centrifugation procedures. In the agar method, potential carryover of serum or cholesterol in the inoculum frequently presents a problem in interpretation of growth.

Recent studies on mollicutes from insect and plant hosts have revealed a large cluster of mollicutes with unusual nutritional requirements (7, 25, 27). Although these organisms do not grow on conventional serum-free broth medium, as has been demonstrated for classic *acholeplasmas* (13, 17, 21), the plant-insect organisms do grow in serum-free broth medium containing small concentrations of Tween 80 (polyoxyethylene sorbitan) (20). These mollicutes grow equally well in serum-free base medium supplemented with 0.04% Tween 80 and in serum-containing medium (1, 7, 24). In earlier studies, three strains in this group were assigned to the genus *Acholeplasma*, as *Acholeplasma florum* (7), *A. entomophilum* (24), or *A. seiffertii* (1). Difficulties with this placement emerged when a recent phylogenetic analysis (28) suggested that these insect-plant organisms are evolutionarily distant from *acholeplasmas*. More recent studies have shown also that *A. florum* has at least some of the components of a phosphotransferase sugar transport system and utilizes UGA as a tryptophan codon (8). These characteristics align it, and presumably other organisms isolated from insects or plants, more closely with the spiroplasmas than with the *acholeplasma* lineage (28). A recently proposed

revised classification of the class *Mollicutes* (22) provides a taxonomic scheme that is in relatively close accord with current phylogenetic data. In this proposal, the three sterol-nonrequiring insect and plant strains previously described as *Acholeplasma* species would be transferred to a new genus, *Mesoplasma*, in recognition of their ability to grow in Tween 80-supplemented medium. Nonhelical, sterol-requiring insect and/or plant mollicutes that are unable to grow in this medium would be removed from the genus *Mycoplasma* (30) and transferred to a new genus, *Entomoplasma*.

We report here a test procedure that permits more convenient assessment of the serum requirements of mollicutes, including methodology to identify mollicutes that exhibit growth promotion with Tween 80 supplements. The procedure involves the ability to carry the test organism through a series of 23 serial 10-fold dilutions in three media: serum-free broth alone, serum-free broth with 0.04% Tween 80, and a control broth containing 15 to 20% fetal bovine serum. The responses of a representative collection of various species and unclassified strains within the class *Mollicutes* are recorded here.

MATERIALS AND METHODS

Mollicutes. Fifty-seven mollicutes representing species, putative species, or strains within the established genera (*Acholeplasma*, *Mycoplasma*, *Entomoplasma*, *Mesoplasma*, and *Spiroplasma*) of the class *Mollicutes* were examined (see Tables 1 to 4). All cultures were from the collection in the Mycoplasma Section of the National Institute of Allergy and Infectious Diseases.

Culture media and growth conditions. Formulations and preparation of the three test media utilized in the study were as follows (all in a final volume of 400 ml).

(i) **Serum-free broth.** The basal medium contained 8 g of mycoplasma broth base (BBL Microbiology Systems, Cockeysville, Md.), 8.0 ml of a 0.1% aqueous solution of phenol red, 2.0 ml of 1 N sodium hydroxide, and 338 ml of deionized water. The base broth was sterilized at 121°C for 20 min and cooled, and the following filter-sterilized ingredients were added aseptically: 40 ml of fresh 15% aqueous yeast extract

* Corresponding author.

† Present address: 8602 Cinnamon Creek, San Antonio, TX 78240.

(GIBCO, Grand Island, N.Y.), 10 ml of 10% bovine albumin (essentially fatty acid free; catalog no. A-6003; Sigma Chemical Co., St. Louis, Mo.) (sterilized through a 220-nm-pore-size filter), 4 ml of a 50% glucose solution, 4 ml of a 50% fructose solution, 2 ml of a 42% arginine solution, and 2.0 ml of a 100,000-U/ml potassium penicillin solution. The final pH was 7.4 to 7.5.

(ii) **Serum-free broth with 0.04% Tween 80.** The base medium and final formulation were similar to those of serum-free broth, except that the final medium was supplemented with 1.6 ml of a sterile (450-nm membrane-filtered) 10% aqueous solution of Tween 80 (catalog no. P-1754; Sigma Chemical Co.).

(iii) **Serum-containing broth.** The base medium and final formulation were also similar to those of serum-free broth, except that the deionized water content in the base medium was reduced to 278 ml and 60 to 80 ml of sterile fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah) was added. The final control medium contained 15 to 20% serum.

An additional serum-containing control medium capable of supporting good growth of the test organism may also be required. In the tests described here, the SP-4 broth formulation (29) was used to verify growth of various mollicutes in serum-free broth alone or Tween 80-containing formulations.

Mollicutes derived from insect or plant sources, including *Spiroplasma*, *Entomoplasma*, and *Mesoplasma* species and a few *Acholeplasma* species, were cultivated at 30 to 32°C. Animal-derived *Mycoplasma* and *Acholeplasma* species were grown at 37°C. *Mycoplasma mobile* 163K was grown at room temperature (25 to 27°C).

Tween 80 preparations. In addition to Sigma Tween 80, three other commercial preparations of Tween 80 were examined: Tween 80 Practical (catalog no. X257-07; Baker Chemical Co., Phillipsburg, N.J.), Tween 80 (catalog no. 27,436-4; Aldrich Chemical Co., Milwaukee, Wis.), and Surfact-Amps 80 (a specially purified 10% solution; catalog no. 28328; Pierce Chemical Co., Rockford, Ill.).

Test procedure. (i) **Inoculum.** It is desirable to grow test inocula in a medium formulation containing the smallest quantity of serum that will support a minimum growth yield of about 10^7 CFU/ml (or an equivalent number of color changing units per milliliter). This approach minimizes carryover of serum in the inoculum that might have some slight influence on the inability of sterol-requiring mollicutes to grow in serum-free media. Most strains in this study grew adequately in media containing 10% fetal bovine serum, and all were purified by conventional triple-cloning techniques (18).

(ii) **Test dilutions.** Broth media were distributed in 1.8-ml volumes in sterile 1 dram (1 fluidram = 3.697 ml) screw-cap glass vials. A series of seven vials of each of the three broth media was used for initial screening of each test organism. The first vial in each series received a 0.2-ml inoculum, and serial 10-fold dilutions were made from that vial for each organism in each broth medium, continuing through a 10^{-7} dilution. Vials in each medium series were incubated at 30, 32, or 37°C, depending upon the optimum growth temperature for the organism. Each medium series should have an uninoculated vial of medium for color and turbidity control.

At 2- to 3-day intervals, the vials were observed for changes in the pH indicator (acidic or alkaline shifts) and for increases in turbidity by comparison with uninoculated control medium. The phenol red indicator in the uninoculated broth should exhibit slight orangeness (pH 7.5). Acid produced from growth of fermenting mollicutes results in a

decline in the medium pH (yellowness), while ammonia produced from arginine-hydrolyzing mollicutes increases the pH (magentaness). Most fast-growing organisms capable of sustained growth induce obvious changes in the color and turbidity of the medium in the sixth or seventh vial in the initial dilution series after an incubation period of 3 to 7 days. When medium pH changes occur rapidly toward the end of the primary dilution series, further serial dilution of the culture in the last vial in the series should be made immediately. Prolonged incubation of some mollicutes in an acidic or alkaline environment can be detrimental to viability. However, considerable time should also be allowed for more slowly growing organisms or for organisms being cultivated in media devoid of serum or containing Tween 80 supplements.

At a time judged to be optimum for growth of the organism under test, usually after incubation for 5 to 7 days, a broth sample (0.2 ml) from the vial containing the highest dilution of the organism showing color or turbidity changes was serially diluted (10-fold) through seven additional vials of the test medium. This series extended the consecutive dilution of the original test inoculum from about 10^{-7} to 10^{-14} . Again, broth dilutions of the organism in the test media were incubated for 7 days and vials were read for pH and turbidity changes. Passage levels were selected on the basis of turbidity or pH change for further serial 10-fold dilution, with serial dilutions continued through a terminal dilution of 10^{-23} .

(iii) **Confirmation of growth.** The growth characteristics of a few mollicutes may present a problem in selection of the most appropriate serial dilutions for consecutive passage in the test procedure. Such difficulties may be encountered with species that have a limited ability to catabolize glucose or arginine or those that grow poorly in media with reduced serum. When changes in turbidity or pH are difficult to ascertain, it is often desirable to perform a second titration in a medium more supportive of growth (e.g., SP-4). For example, after the first 7-day incubation period, a strong color change and/or turbidity may be observed in the first two vials of a dilution series, but only a slight color change may be observed in the third or fourth vial. A broth sample (0.2 ml) from the fourth vial is then serially diluted (10-fold) through seven vials of SP-4 medium. These vials are incubated at the appropriate temperature and examined in 3 to 7 days. If growth has occurred in the broth dilution selected from the primary serial dilution series, the SP-4 titration series should show a significant increase in numbers of organisms over the expected number that would have been transferred by dilution of the initial inoculum. Specifically, if the sample selected from the initial 10^4 dilution, with a starting inoculum of 10^7 organisms, shows 10^3 or fewer organisms in the secondary SP-4 broth titration, these numbers would be expected to reflect only survival of the initial inoculum in the test medium and not newly sustained growth. With this information on growth and approximate numbers of organisms, it is possible to return to the titration in the test medium and select the proper dilution of the organism for continued passage in that particular medium.

Comparative studies with the broth-protein analysis method. Growth responses of two currently unclassified strains, representing putative *Acholeplasma* (0502) or *Mesoplasma* (PUPA-2) species, were compared by using the method described above and a standard sterol test procedure described earlier (13, 19). In the latter technique, organisms grown in various control media (including serum-free broth with addition of 0.04% Tween 80) and in media containing

TABLE 1. Growth characteristics of *Acholeplasma* species

Strain (host)	No. of 10-fold serial dilutions maintaining growth in:		
	Serum-free medium	Serum-free medium with 0.04% Tween 80	Serum
<i>A. laidlawii</i> PG9	23	23	23
<i>A. granularum</i> BTS39 ^T	23	6	23
<i>A. morum</i> 72-043 ^T	23	5	23
<i>A. oculi</i> 19L ^T	23	5	23
<i>A. axanthum</i> S743 ^T (animal)	23	23	23
<i>A. axanthum</i> H86N (animal)	23	23	23
<i>A. axanthum</i> W102 (plant)	23	23	23
<i>A. axanthum</i> W114 (plant)	23	23	23
<i>A. multilocale</i> PN525 ^T	23	4	23
<i>A. cavigenitalium</i> GP3 ^T	23	3	23
<i>Acholeplasma</i> sp. strain J233	23	23	23
<i>Acholeplasma</i> sp. strain 0502	23	23	23

selected quantities of cholesterol were sedimented by centrifugation and cell pellets were assayed for protein content.

RESULTS AND DISCUSSION

Acholeplasmas. Table 1 shows the responses of classic *Acholeplasma* species in the three test media. All of the acholeplasmas examined, including those of plant origin (*A. axanthum* W102 and W114 and unclassified strains 0502 and J233), grew well through 23 serial dilutions in serum-free broth. The plant-derived strains of *A. axanthum*, as well as the animal-derived *A. axanthum* and *A. laidlawii* strains examined, were notable for growth in serum-free media containing 0.04% Tween 80, as had been previously recorded (3, 23). Other acholeplasmas were apparently sensitive to Tween 80 at this concentration, since these strains failed to grow beyond three to six passages in media containing 0.04% Tween 80. As expected, acholeplasmas exhibited sustained growth in base medium containing 15% fetal bovine serum.

Mycoplasmas and entomoplasmas. Serum-free broth or Tween 80 supplements did not support the growth of *Mycoplasma* or *Entomoplasma* species (Table 2). The latter newly established genus contains nonhelical, sterol-requiring mollicutes from insect and plant habitats (22). The fact that several caprine or bovine *Mycoplasma* species (e.g., *M. mycoides*, *M. capricolum*, and *M. putrefaciens*) did not show sustained growth in the serum-free media provides an important control, since these organisms frequently grow well in media containing minimal amounts of animal serum (13). Other mycoplasmas of animal origin also failed to grow in serum-free medium containing 0.04% Tween 80. However, one plant-derived mollicute, previously described as *M. lactucae* (16), grew to the test endpoint in this medium (Table 2). Tests carried out at the time of the taxonomic description of this organism utilized media that contained 0.01%, rather than 0.04%, Tween 80. The failure of the lower concentration of Tween 80 to support growth was interpreted at that time as indicative of a sterol requirement. However, the results reported herein suggest that this organism should be classified in the genus *Mesoplasma*.

Spiroplasmas. Most *Spiroplasma* species (Table 3) survived only a limited number of passages in serum-free medium, with or without supplements of Tween 80. However, four strains of *Spiroplasma floricola* could be passaged

TABLE 2. Growth characteristics of sterol-requiring *Mycoplasma* and *Entomoplasma* species

Strain ^a	No. of 10-fold serial dilutions maintaining growth in:		
	Serum-free medium	Serum-free medium with 0.04% Tween 80	Serum
<i>M. mycoides</i> subsp. <i>mycoides</i> PG1 ^T (SC)	1	0	23
<i>M. mycoides</i> subsp. <i>mycoides</i> B3 (LC)	1	1	23
<i>M. mycoides</i> subsp. UM30847 (LC)	3	1	23
<i>M. mycoides</i> subsp. <i>capri</i> PG3 ^T	3	1	23
<i>M. capricolum</i> California kid ^T	3	1	23
<i>M. putrefaciens</i> KS-1 ^T	2	1	23
<i>M. arginini</i> G230 ^T	1	0	23
<i>M. fermentans</i> PG18 ^T	1	0	23
<i>M. hominis</i> PG21 ^T	1	0	23
<i>M. pirum</i> 70-159 ^T	1	1	23
<i>M. lactucae</i> 831-C4 ^T	2	23	23
<i>E. ellychniae</i> ELCN-1 ^T	1	1	23
<i>E. melaleuca</i> M1 ^T	2	1	23
<i>E. somniliux</i> PYAN-1 ^T	2	1	23
<i>M. lucivorax</i> PIPN-2 ^T	2	1	23
<i>M. luminosum</i> PIMN-1 ^T	2	1	23
<i>M. mobile</i> 163K ^T	2	1	23

^a SC, small colony form; LC, large colony form.

through 23 serial 10-fold dilutions in both the serum-free formulation and serum-free medium containing 0.04% Tween 80. Type strains of *S. apis*, *S. chinense*, and the spiroplasma group XII representative (strain DU-1) also demonstrated apparent lack of a sterol requirement. However, unlike *S. floricola*, these three spiroplasmas showed limited growth in Tween 80-containing media (Table 3), suggesting that Tween 80, at the level tested, had an inhibitory effect. Some spiroplasmas grown in the serum-free

TABLE 3. Growth characteristics of sterol-requiring *Spiroplasma* species

Strain (group)	No. of 10-fold serial dilutions maintaining growth in:		
	Serum-free medium	Serum-free medium with 0.04% Tween 80	Serum
<i>S. citri</i> Maroc ^T (I-1)	2	1	23
<i>S. melliferum</i> BC-3 ^T (I-2)	5	2	23
<i>S. floricola</i> 23-6 ^T (III)	23	23	23
<i>S. floricola</i> BNR1 (III)	23	23	23
<i>S. floricola</i> OBMG (III)	23	23	23
<i>S. floricola</i> SLH9 (III)	23	23	23
<i>S. apis</i> B31 ^T (IV)	23	5	23
<i>Spiroplasma</i> sp. strain Y32 (VI)	2	2	23
<i>Spiroplasma</i> sp. strain MQ-4 (VII)	2	2	23
<i>Spiroplasma</i> sp. strain EA-1 (VIII)	6	2	23
<i>S. culicicola</i> AES-1 ^T (X)	7	3	23
<i>Spiroplasma</i> sp. strain DU-1 (XII)	23	9	23
<i>S. sabaudiense</i> AR1343 ^T (XIII)	3	1	23
<i>S. taiwanense</i> CT-1 ^T (XXII)	2	0	23
<i>Spiroplasma</i> sp. strain TG-1 (XXIII)	2	0	23
<i>S. chinense</i> CCH ^T (XXIV)	23	3	23
<i>Spiroplasma</i> sp. strain CUAS-1 (ungrouped)	2	7	23

TABLE 4. Growth responses of *Mesoplasma* species and some unclassified insect and plant mollicutes

Strain	No. of 10-fold serial dilutions maintaining growth in:		
	Serum-free medium	Serum-free medium with 0.04% Tween 80	Serum
<i>M. florum</i> L1 ^T	7	23	23
<i>M. entomophilum</i> TAC ^T	8	23	23
<i>M. seiffertii</i> F7 ^T	8	23	23
Unclassified strains			
YJS	12	23	23
GRUA-1	8	23	23
PUPA-2	9	23	23
ELCA-2	4	23	23
CHPA-2	6	23	23
BARC 779	9	23	23
BARC 857	4	23	23
PS-1	7	23	23

formulation lost their helicity, as recorded by dark-field microscopic examination.

S. floricola (2) and *S. chinense* (5) were initially described as sterol-requiring organisms. However, procedures used to measure the cholesterol responses of both organisms involved techniques modified substantially from earlier recommendations (13). In the case of *S. floricola*, it is unlikely that the 72-h incubation period provided sufficient time for the organism to adapt to the described serum-free formulation. Likewise, the sterol need of *S. chinense* was assessed by enumerating numbers of helical forms occurring over a 4-day period in serum-free medium or in broth containing cholesterol or serum. The loss of spiroplasma helicity we observed in strains grown in serum-free broth strongly suggests that a morphological assay is inadequate for measurement of spiroplasma growth in medium devoid of serum.

Mesoplasmas. As expected, the three insect- and/or plant-derived organisms now classified as *Mesoplasma* species showed sustained growth in media containing 0.04% Tween 80 (Table 4). Limited survival of the organisms occurred in serum-free medium; cessation of growth was usually observed after seven to eight passages. Results obtained in this study with each of the three established *Mesoplasma* species were similar to those of earlier studies in which growth promotion with 0.04% Tween 80 was demonstrated with the broth-protein analysis method (1, 7, 25). Likewise, eight unclassified strains of insect and/or plant origin, each presumably representing putative *Mesoplasma* species, exhibited enhanced growth in response to 0.04% Tween 80 (Table 4).

Other aspects of the test system. A comparison of the earlier described broth-protein analysis method with the dilution technique can be made by using data presented in Table 5. In the broth-protein cholesterol test, an unclassified *Acholeplasma* species (0502) grew in serum-free medium alone or when a Tween 80 supplement and other additives were supplied. These results relate well to the sustained growth of 0502 in each of the three test media in the broth dilution series tests (Table 1). When the PUPA-2 strain, an unclassified and presumed mesoplasma, was examined by the protein analysis method, significant amounts of growth were recorded only when the strain was grown in serum-free medium containing either 0.04% Tween 80 or cholesterol

TABLE 5. Growth responses of unclassified *Mesoplasma* sp. strain PUPA-2 and unclassified *Acholeplasma* sp. strain 0502 to cholesterol or Tween 80 in broth-protein analysis cholesterol test^a

Supplement added to serum-free base medium	Cholesterol concn (µg/ml)	Amt of protein (mg/100 ml)	
		PUPA-2	0502
1% Bovine serum fraction	0 (control)	7.10	5.57
None	0	0.23	1.28
0.5% Albumin-10 µg of palmitic acid/ml	0	0.23	4.60
Albumin-palmitic acid-0.04% Tween 80	0	3.85	6.13
Albumin-palmitic acid-cholesterol	1	4.50	5.97
Albumin-palmitic acid-cholesterol	5	5.00	6.38
Albumin-palmitic acid-cholesterol	10	5.00	5.97
Albumin-palmitic acid-cholesterol	20	5.15	6.38

^a Strains were incubated at either 30°C (PUPA-2) or 37°C (0502).

(Table 5). Again, these findings agree well with the data from the serial dilution tests (Table 4), which show the organism capable of sustained growth in media supplemented with 0.04% Tween 80.

Results of limited studies in which the abilities of selected commercial Tween 80 preparations to support sustained growth of *Mesoplasma* species were compared are shown in Table 6. In general, the three preparations tested showed results identical to those obtained with Sigma polyoxyethylene sorbitan, except that the more highly purified Pierce product did not sustain growth of the ELCA-2 strain to the expected endpoint.

Addition of bovine serum albumin, at a final concentration of 0.25%, seems critical for reduction of the toxicity or inhibitory qualities of Tween 80. In Tween 80-containing serum-free broth without albumin, most mesoplasmas exhibited little fermentative activity or produced limited turbidity. These effects were dramatically reversed by addition of 0.25% bovine serum albumin. In contrast, all sterol-requiring mollicutes failed to show sustained growth in serum-free medium containing 0.25% bovine serum albumin, whether or not a 0.04% Tween 80 supplement was present.

Some mollicutes (e.g., *M. genitalium*) with fastidious growth requirements may not grow, or grow only slowly, in the serum-containing control medium. The 163K strain of *M. mobile* required more than 4 weeks to grow through the 23-dilution series in broth containing 15 or 20% fetal bovine serum. The time period for serial passage should therefore

TABLE 6. Growth responses of *Mesoplasma* species and some unclassified insect and plant mollicutes to various commercial Tween 80 preparations

Strain	No. of 10-fold serial dilutions maintaining growth in:				
	Serum-free medium without Tween 80	Serum-free medium with 0.04% Tween 80 from:			
		Sigma	Aldrich	Baker	Pierce
<i>M. florum</i> L1	2	23	23	23	23
Unclassified strains					
YJS	2	23	23	23	23
GRUA-1	2	23	23	23	23
ELCA-2	2	23	23	23	15

be selected on the basis of growth responses in serum-containing medium. Assessment of the cholesterol or serum requirements of such mollicutes is therefore difficult to establish by either the standard broth-protein analysis method or the serial dilution technique described here. In these instances, a modification in the medium formulation to show the response to graded amounts of serum has been used to show a requirement for sterol indirectly (26).

Relationship to sterol requirements of mollicutes. The significance of the test reported here in terms of the sterol requirement of mollicutes is not clear. Methods utilized previously (13) share with the present study the difficulty of ascertaining the absolute sterol contents of the reagents used in medium preparation. Because *M. mycoides* subsp. *capri*, *M. capricolum*, and closely related strains are able to grow in media with minute supplements of sterols, trace contamination of reagents could produce erroneous results. There are, in fact, some reports in the literature that Tween 80 may have a low level of sterol contamination (11). However, chemical analyses performed on some earlier serum-free formulations utilized in this study indicated that complete broth preparations containing 0.04% Sigma Tween 80 and 0.5% bovine albumin contained less than 0.1 ppm of total sterols (12). What is the mechanism of the Tween 80-induced growth enhancement of mesoplasmas? It seems unlikely that it involves a fatty acid component. Most commercial Tween 80 preparations contain about 70% oleic acid, with smaller amounts of linoleic and palmitic acids. We have been unable, in several experiments, to demonstrate enhancement of mesoplasma growth with various quantities of these specific fatty acids. Furthermore, we found that serum-free medium formulations containing 0.06 to 0.08% Tween 80 (quantities only slightly in excess of the active concentration) were inhibitory to mesoplasmas and other mollicutes. This inhibition may involve the detergent qualities of the product. It is possible that the effective levels of Tween 80 have an influence on mollicute membrane structure and function, leading to increased membrane transport of essential nutrients.

Certain findings of this study, particularly growth of some *Spiroplasma* species in serum-free broth, have complicating effects on mollicute taxonomy. For many years, the genome size of mollicutes and the growth requirement for sterol were presumed to represent profound and stable characteristics which could be trusted for classification of organisms at higher taxal levels. However, genome size has subsequently been found to be variable within higher taxa (9, 10, 14) and genome size modifications have apparently occurred several times in mollicute evolution. It appears that serum or sterol requirements, already abandoned as a taxonomic marker for members of the families *Anaeroplasmataceae* (15) and *Entomoplasmataceae* (22), must also be considered to be variable within the family *Spiroplasmataceae*. Whether careful analysis of minimal media will reveal that some entomoplasmas, anaeroplasmas, and spiroplasmas can indeed grow in the absolute absence of sterols is not clear. What is clear is that no existing test can be used to classify mollicutes at the family level on the basis of sterol requirement. However, the test described here appears to provide adequate identification and separation, at the genus level (*Acholeplasma*, *Mesoplasma*, and *Entomoplasma*), of numerous important nonhelical mollicutes recently isolated from insect and plant hosts.

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