Determination of Sterol Requirement for Mycoplasmatales

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SUMMARY

A method is described for determining the sterol requirement of a mycoplasma to permit its classification either among the sterol-requiring Mycoplasmataceae or the sterol-independent Acholeplasmataceae. A filtered washed suspension is inoculated on media with and without various concentrations of cholesterol and the development of growth noted by microscopical examination.

The sterol requirement was determined for three mycoplasmas whose growth on serum-free media for several subcultures had been claimed. *Mycoplasma anatis* and Bovine Group 7 were found to be sterol-dependent, but Bovine Group 6 grew without sterol, although under certain circumstances promotion of growth by cholesterol was noted through counteraction of an inhibitory effect.

INTRODUCTION

A requirement by mycoplasmas for sterol, in the form of cholesterol or certain other sterols, was first demonstrated by Edward & Fitzgerald (1951) who were investigating the constituent of serum needed as an enrichment of media for the growth of most strains. When this finding was confirmed (Smith, Lecce & Lynn, 1954; Smith & Lynn, 1958; Rodwell, 1963) it came to be assumed that all serum-requiring mycoplasmas had a requirement for sterol, although it is only recently that it has been determined individually for a large number of species (Razin & Tully, 1970) including the T-mycoplasmas (Rottem, Pfendt & Hayflick, 1971). As bacteria even in their L-phase do not require sterol, a requirement for sterol has been used in attempts to define the Mycoplasmatales (Edward & Freundt, 1969) and is one reason for removing this order from the Schizomycetes and establishing for it a new class (Minutes, 1967) named Mollicutes (Edward and Freundt, 1967).

Although the majority of mycoplasmas require media enriched with serum, it has long been recognized that some, the so-called 'saprophytic' strains, such as the sewage isolates of Laidlaw & Elford (1936), can grow without serum, and thus do not require sterol. Recently, in view of the taxonomic importance of sterol requirement, it has been proposed that the Mycoplasmatales be divided into two families: Mycoplasmataceae (genus *Mycoplasma*) for sterol-requiring species, and Acholeplasmataceae (genus *Acholeplasma*) for species not requiring sterol (Edward & Freundt, 1970). A method is therefore needed for determining the requirement for sterol in the investigation of any newly discovered species to permit its correct classification. It is not sufficient to rely on whether or not the organism can grow on a serum-free medium. Carry-over from one subculture to another of sufficient nutriment may permit a sterol-requiring strain to grow, feebly at least, for several subcultures on serum-free media. On the other hand, a strain not requiring sterol may fail to grow on a medium devoid of serum, because the serum was providing some other essential nutrient, e.g. fatty acids (Tully & Razin, 1969). Razin & Tully (1970) demonstrated a

requirement for sterol by 28 species of *Mycoplasma*; three species of *Acholeplasma* did not have this requirement. They grew the organisms in a fluid medium with or without various concentrations of cholesterol and measured growth chemically by estimations of protein in centrifuged deposits of the cultures. An alternative method will be described, using observation of surface growth on solid media instead of chemical estimations of fluid media.

METHODS

With one exception (PG25 strain of *Mycoplasma hominis*) the strains used in the investigation were the type strains of each species. They were obtained from the author's collection or the National Collection of Type Cultures; the strain of *M. anatis* and an additional strain of this species were kindly provided by Dr D. H. Roberts, Central Veterinary Laboratory, Weybridge, Surrey. Each strain was grown in 20 ml. of fluid growth medium (Hayflick, 1965). After 2 days incubation (4 days for *M. pneumoniae*) the culture was centrifuged for 45 min. at 3640 g in the angle head. The deposited organisms were washed twice in Bacto PPLO Broth containing 1% (w/v) Bovine Albumin Fraction V (Armour Pharmaceutical Co., Eastbourne, Sussex) and finally suspended in about 2 ml. of the same medium. The suspension was filtered through a 450 nm. Millipore filter membrane and the filtrate stored at -70° . Since in one attempt strain A82/69 of *M. anatis* failed to pass a 450 nm. filter, an 800 nm. membrane was used for this strain. Suspensions of the organisms did not lose viability and preliminary titration enabled suitable dilutions of the various strains to be chosen for the experiments.

The base for all the experimental media consisted either of Bacto PPLO Agar (Difco) or an experimental dehydrated Mycoplasma Agar Base supplied by Oxoid, to which was added 200 u. benzyl penicillin/ml., 1.25 μ g. thallium acetate/ml. and 10% (v/v) of a 25% (w/v) extract of baker's yeast (Hayflick, 1965). The method for preparing a suspension of cholesterol has been described previously (Edward & Fitzgerald, 1951); excellent stable suspensions were regularly obtained. A 10% (w/v) stock solution of Bovine Albumin Fraction V in water and a solution of palmitic acid in ethanol (10 mg./ml.) were prepared as described by Razin & Tully (1970). Plates were poured from mixtures of basal medium with 0.5% Bovine Albumin, 10 μ g. palmitic acid/ml. and various concentrations of cholesterol, namely 100, 20, 5 and 0.2 μ g./ml. Other plates were made from the same medium without cholesterol, the same medium with the addition of 1% (v/v) Bacto PPLO Serum Fraction (Difco) instead of cholesterol, the basal medium without any addition and the basal medium enriched with 20% horse serum. The total volume of additions to the basal medium were in every case made up with the broth corresponding to the source of the agar, either Difco or Oxoid.

Sets of plates were inoculated with 0.025 ml. drops of suitable dilutions of suspensions of the organisms. It was usual with any strain to test dilutions which gave both profuse and sparse growth on the plate containing serum. Plates were incubated aerobically or in an atmosphere of 10% carbon dioxide in nitrogen, according to the requirements of the strain, and observations made at intervals of two to three days until growth was maximal.

RESULTS AND DISCUSSION

Initial experiments demonstrated the necessity for the filtration step in the preparation of a suspension of the organisms. Suspensions of sterol-requiring strains that had not been filtered occasionally produced a few colonies on media without serum, serum fraction or

cholesterol. Cultures of suspensions prior to filtration showed a marked irregularity in size of colonies with some very large colonies, probably arising from clumps of organisms; presumably these clumps had enough nutrient adsorbed to them to permit a limited amount of growth on a deficient medium.

Three species of *Acholeplasma* (*Acholeplasma laidlawii*, *A. granularum* and *A. axanthum*), shown by Razin & Tully (1970) not to require cholesterol, grew well on a basal medium prepared from Oxoid Agar Base (Table 1). Growth of *A. laidlawii* and *A. granularum* was approximately equal on all the media tested, except that the colonies were slightly larger on the medium containing serum. *Acholeplasma axanthum* grew best on the basal medium alone and growth was poorer when palmitic acid and Bovine Albumin were added with or without Serum Fraction or cholesterol.

Nine species of *Mycoplasma*, whose requirement for sterol had been demonstrated by Razin & Tully (1970), together with the type species, *Mycoplasma mycoides* var. *mycoides*, were investigated. With the exception to be noted, none of the strains grew on the basal medium alone or with the addition of Bovine Albumin and palmitic acid (Table 1). Growth occurred on media containing cholesterol, usually only with the higher concentrations, there being no or diminished growth at lower concentrations. Judged by the size and number of colonies and by their time of appearance, growth on media containing cholesterol was approximately equal to that on media containing serum fraction, but inferior to that on media enriched with serum. Slight growth occurred on the basal medium alone with the lowest dilution of *M. mycoides* var. *capri*, which gave a very luxuriant growth on the serum control plate; presumably enough sterol was provided by the inoculated organisms to permit a limited amount of growth of this particularly vigorously growing organism. *Mycoplasma pneumoniae* grew only with the intermediate concentrations of cholesterol, 20 µg./ml. and 5 µg./ml., there being no growth with 100 µg./ml. or the lower concentrations. Of the strains tested the PG 18 strain of *M. fermentans* proved the most difficult to grow on media enriched with cholesterol. The result recorded to Table 1 represented the most successful experiment and suggested that the concentration of cholesterol was critical, since growth occurred only with 5 µg. cholesterol/ml. Subsequent experiments tended to confirm that too high concentrations were inhibitory, although only scanty growth was obtained.

The technique was next applied to investigate three other species whose sterol requirement had not previously been studied and which had been credited with growing in the absence of serum. The avian *Mycoplasma anatis* was reported capable of growth for several subcultures in serum-free media (Barber, 1969) as were the bovine strains N29 (PG 50) and 'Squire' (PG 49) (Barber, 1969; Al-Aubaidi, 1969). Leach also obtained slight growth of the last strain on media devoid of serum (Cottew & Leach, 1949).

Both the type strain and another strain of *Mycoplasma anatis* showed responses to the incorporation of cholesterol in the media typical of a mycoplasma (Table 1). There was no growth on the basal medium alone, but an undiluted suspension of the type strain gave slight limited growth on the medium containing Bovine Albumin and palmitic acid. This did not occur with a more dilute suspension giving a less profuse growth, but there was slight growth with only 0.2 µg. cholesterol/ml., suggesting that this strain required rather less sterol than did the others tested. The growth response to cholesterol of strain PG 50 belonging to Bovine Group 7 (Leach, 1967) was also characteristic of a mycoplasma. These findings confirm the unreliability of using growth on serum-free media, even over several subcultures, for determining sterol dependence for purposes of classification.

PG 49 ('Squire'), representative of Bovine Group 6 (Leach, 1967), grew well on the basal medium. Growth was poorer when Bovine Albumin and palmitic acid were added to the

Table 1. Growth of members of the *Mycoplasma*tales on experimental media prepared from Oxoid agar base with and without addition of cholesterol

Strain	Species	Dilution	Basal medium +20 % horse serum	Basal medium +0.5 % Bovine Albumin +10 µg./ml. palmitic acid										Basal medium alone
				+1 % PPLO serum fraction	+100 µg. cholesterol/ml.	+20 µg. cholesterol/ml.	+5 µg. cholesterol/ml.	+1 µg. cholesterol/ml.	+0.2 µg. cholesterol/ml.	No cholesterol				
PG 1	<i>Mycoplasma mycoides</i> var. <i>mycoides</i>	1/1 10 ⁻²	++	++	++	++	++	++	—	—	—	—	—	
PG 3	<i>M. mycoides</i> var. <i>capri</i>	10 ⁻³ 10 ⁻⁵	++	++	++	++	++	++	+	+	±	—	Tr	
PG 11	<i>M. bovis genitalium</i>	10 ⁻² 10 ⁻⁴	++	++	++	++	++	++	—	—	—	—	—	
PG 25 (H 23)	<i>M. hominis</i>	10 ⁻³ 10 ⁻⁴	++	++	++	++	++	++	—	—	—	—	—	
PG 34	<i>M. pulmonis</i>	10 ⁻¹	+	+	+	+	+	+	—	—	—	—	—	
PG 20	<i>M. salivarium</i>	1/1 10 ⁻¹	++	++	++	++	++	++	—	—	—	—	—	
PG 6	<i>M. arthritis</i>	10 ⁻³ 10 ⁻⁵	++	++	++	++	++	++	++	+	+	Tr	—	
PG 31	<i>M. gallisepticum</i>	10 ⁻¹	++	++	++	++	++	++	±	—	—	—	—	
PG 38 (FH)	<i>M. pneumoniae</i>	1/1	++	++	—	—	++	++	—	—	—	—	—	
PG 18	<i>M. fermentans</i>	10 ⁻¹	++	++	++	++	++	++	++	++	++	++	++	
PG 8	<i>Acholeplasma laidlawii</i>	10 ⁻³ 10 ⁻⁵	++	++	++	++	++	++	++	++	++	++	++	
NCTC 10128	<i>A. granularum</i>	10 ⁻⁵	++	++	++	++	++	++	++	++	++	++	++	
NCTC 10138	<i>A. axanthum</i>	10 ⁻⁵	++	++	++	++	++	++	++	++	++	++	++	
I340	<i>M. anatis</i>	1/1 10 ⁻³	++	++	++	++	++	++	++	++	++	Tr	—	
A 82/69	<i>M. anatis</i>	10 ⁻² 10 ⁻⁴	++	++	++	++	++	++	Tr	—	—	—	—	
PG 50 (N 29)	Bovine Group 7	10 ⁻³ 10 ⁻⁵	++	++	++	++	++	++	Tr	—	—	—	—	
PG 49 ('Squire')	Bovine Group 6	1/1 10 ⁻¹	++	++	±	±	++	++	++	+	+	+	++	

+++ = Profuse growth (> 600 colonies); ++ = moderate growth (150 to 600 colonies); + = scanty growth (30 to 150 colonies); ± = few (< 30) colonies; Tr = small number of tiny colonies.
* Number of colonies.

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basal medium, but the further addition of 5 to 100 μg . cholesterol/ml. gave growth equal to that on the basal medium alone. Cholesterol thus had a stimulating effect on growth, but it appeared to act by counteracting inhibition by the mixture of Bovine Albumin and palmitic acid. Inhibition was shown to be due to the Bovine Albumin. Addition of Bovine Albumin alone to the basal medium decreased growth, whereas the addition of palmitic acid alone did not affect growth. On a medium containing palmitic acid and twice the usual amount of Bovine Albumin (1 %) growth was very scanty. A concentration of 5 μg . cholesterol/ml. was more effective than 100 μg ./ml. in counteracting the inhibition.

Since strain 'Squire', representative of a distinct although as yet unnamed species (Cottew & Leach, 1969), is capable of growth on sterol-free medium, it must be classified as an *Acholeplasma*.

Initially, Difco PPLO Agar was used for preparing the basal medium. Growth was found to be poor or absent on the plates enriched with PPLO Serum Fraction, even when there was growth on the plates containing cholesterol. Such poor results with the Serum Fraction were unexpected and led to comparisons being made with Oxoid Agar Base. Considerably better growth, although with the exception of *Mycoplasma hominis* not as good as on horse serum media, was obtained by incorporating Serum Fraction in Oxoid Agar Base. With the Difco base there was also a failure of *Acholeplasma laidlawii* to grow on the basal medium alone, although there was growth on medium to which palmitic acid and Bovine Albumin had been added. The important addition proved to be Bovine Albumin, for there was normal growth when this alone was added to the Difco Base. It would thus appear that the batch of Difco PPLO Agar used contained a toxic material, possibly fatty acid, neutralized by albumin or other protein.

Tween 80 was used to solubilize the cholesterol by Razin & Tully (1970) working with fluid media, but was inhibitory for some strains. With solid media the inhibitory effect appeared to be greater; all the sterol-requiring strains, except *Mycoplasma hominis* and *M. mycoides* var. *capri* failed to grow on basal medium containing Bovine Albumin, palmitic acid and Serum Fraction to which 0.01 % (v/v) Tween 80 had been added. Growth of strains of *Acholeplasma* were unaffected, but Tween 80 did not have the same effect of enhancing the growth of the now laboratory-adapted strain of *Acholeplasma axanthum* as was noted by Tully & Razin (1969) using early subcultures.

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