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STANDARDIZED BACTERIOLOGIC TECHNIQUES FOR THE
CHARACTERIZATION OF MYCOPLASMA SPECIES^{1,2}

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ABSTRACT. Bacteriological methods applicable to the characterization and differentiation of Mycoplasma species were investigated. After appropriate modification and standardization, these methods were tested on 53 Mycoplasma strains comprising more than 22 species. The tests found most suitable for purposes of characterization and differentiation were: breakdown of glucose by oxidation or fermentation, hydrolysis of arginine or urea, reduction of tetrazolium, tellurite, and methylene blue, phosphatase activity, production of film and spots, hydrolysis of gelatin, digestion of casein and coagulated serum, sensitivity to optochin, and hemolysis of sheep erythrocytes.

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For taxonomic purposes a precise description of each Mycoplasma species in terms of its biochemical and physiological activities is highly desirable. In the 1950's Edward and Freundt developed the basis for most of the biochemical

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procedures currently in use (7, 8, 10, 12). Although numerous references to the reactions of various species are found in the literature, no attempt has been made to characterize under standardized conditions both the old and the new species and strains now available.

The objectives of this project were to standardize and modify, if necessary, existing bacteriologic procedures (3, 5, 7, 12, 13) and to develop new tests to extend the biochemical basis for Mycoplasma species differentiation. All of these tests were then applied to 53 strains comprising more than 22 species to determine the efficacy of the tests to characterize and differentiate mycoplasmas.

MATERIALS AND METHODS

Organisms

The organisms used, their strain designations, sources, and environmental preferences appear in Tables 1, 2, 3, 4.

Before testing for biochemical activities, all cultures were transferred daily in a standard growth broth medium until they were well adapted and produced approximately 10^8 organisms per ml during 24 hr of incubation (except for M. pneumoniae, which required 72 hr to reach this count).

Test-control organisms

The test methods were checked for optimal performance on later reruns by inclusion of control organisms known to yield consistently positive or negative reactions. The mycoplasma strains used as positive or negative test-control organisms are noted at the end of each of the test methods.

Culture media

The standard growth media to which all mycoplasmas were adapted were Heart Infusion Broth (HIB) (Difco) and Heart Infusion Agar (HIA) (Difco) enriched with horse serum and Oxoid yeast extract (Consolidated Laboratories, Inc., Chicago Heights, Ill.). The media were prepared by dissolving 25 g of dehydrated broth or 40 g of dehydrated agar in 1000 ml deionized distilled water, adjusting the pH to 7.6 with 5N NaOH, and dispensing into screw-capped bottles before autoclaving at 121°C for 15 min. The broth was enriched with 20% (v/v) sterile heated (56°C for 30 min) horse serum and 5% (v/v) of a 10+ (w/v) yeast extract stock solution prior to dispensing aseptically in volumes of 5 ml into screw-capped tubes. The yeast extract solution was prepared by making a 10% (w/v) aqueous solution from the paste,

adjusting the pH to 7.0, and sterilizing by filtration through a 0.01- μ m pore-size Seitz filter pad (Republic Seitz Filter Corp., Milldale, Conn.). Plates were prepared by adding the serum and yeast supplements in the above concentrations immediately prior to pouring the HIA into 60 x 15 mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.).

Conditions of incubation

Broth cultures of aerobic strains were incubated in air, plate cultures in candle jars, both at 37°C. Tubes and plates of anaerobic cultures were incubated at 37°C in an atmosphere of 95% H₂ + 5% CO₂.

Test media and methods

The test media employed were modifications of the standard growth media with the exception of the broth and agar media used for determination of glucose breakdown, urea and arginine hydrolysis, and oxidation of gluconate; in these the serum content was reduced to 10% (v/v). Unless specifically stated otherwise, stock reagents and substrates used in test media were prepared in deionized distilled water and sterilized by filtration through 0.01- μ m Seitz filter pads. Two sets of controls were incubated with each battery of tests as follows: (1) media controls: uninoculated media containing the appropriate test substrates, and (2) substrate controls for tests for breakdown of glucose, arginine, urea, tetrazolium, tellurite, methylene blue, and gluconate: inoculated media containing water in place of the test substrate.

Breakdown of glucose, arginine, or urea. Glucose, arginine, and urea media were prepared by adding 10 ml of horse serum, 5 ml of yeast extract stock solution, 10 ml of 10% (w/v) test substrate, and 1 ml of 0.5% (w/v) phenol red to 74 ml of HIB. Each medium was adjusted using 5N HCl or 5N NaOH to the following final pH values: glucose medium: 7.6, arginine medium: 7.0, urea medium: 7.0. Substrate controls were also prepared at pH values of 7.6 and 7.0. Each broth medium was then Seitz-filtered and dispensed aseptically in 5-ml amounts into screw-capped tubes. The test and appropriate substrate control tubes were inoculated with 1 ml of a 24-hr broth culture grown in 10% (v/v) horse serum broth. Cultures preferring anaerobic conditions were overlaid with 1.5 ml of sterile vaseline and paraffin mixture. This was prepared by mixing equal parts vaseline and paraffin (melting range: 53.5 to 56.5°C) followed by

Table 1. Differential physiologic reactions displayed by glucose positive, arginine negative mycoplasmas.

Mycoplasma species	ATCC No.	Strain	Glucose breakdown	O-F test	Arginine hydrolysis	Tetrasolium reduction	Tellurite reduction	Methylene blue reduction ^a	Phosphatase	Film and spots	Gelatin hydrolysis	Casein digestion	Serum digestion	Sensitivity to optochin	Hemolysis ^b (sheep cells)	Preferred atmosphere
ae/an ae/an ae/an																
bovirhinis	19884	PG43 (54331)	+	F	-	+/+	+/+	+/+	-	-	-	+	+	-	β r	Ae
canis	19525	PGL4 (C55)	+	F	-	-/+	+/+	-/-	-	-	-	-	-	-	β r	Ae
felis	23391	CO	+	F	-	-/+	+/+	-/+	+	+	-	-	-	-	α' r	Ae
gallisepticum	19610	PG31 (X95)	+	F	-	+/+	+/+	-/+	-	-	-	-	-	-	β	Ae
	15302	S6	+	F	-	+/+	+c/+c	-/+	-	-	-	-	-	-	β	Ae
granularum	19168	BTS39	+	F	-	$\pm$ /+	+/+	-/+	-	-	-	-	-	-	α' r	Ae
histotropicum	23115	Sabin, type C	+	F	-	-/+	+/c	$\pm$ /+	-	-	-	-	-	-	α' r	Ae
hyorhinis	17981	BTS7	+	O	-	+/+	+/+	+/+	+	-	-	-	-	+	α	Ae
	23234	PG42 (BTS7)	+	O	-	+/+	+/+	+/+	+	-	-	-	-	-	α	Ae
	23839	GDL	+	O	-	+/+	+/+	$\pm$ /+	+	-	-	-	ND	+	α	Ae

Key: +, positive reaction; $\pm$, weak positive reaction; -, negative reaction; O, oxidative reaction; F, fermentative reaction; α , alpha hemolysis; α' , alpha prime hemolysis; β , beta hemolysis; r, green ring at outer zone of hemolysis; ND, not determined; ae, aerobic; an, anaerobic.

a. Reading at 24 hr.

b. Reading at 4 days.

c. Growth inhibited by test substrate.

Table 1. (Continued)

Mycoplasma species	ATCC No.	Strain	Glucose breakdown	O-F test	Arginine hydrolysis	Tetrasodium reduction	Tellurite reduction	Methylene blue reduction ^a	Phosphatase	Film and spots	Gelatin hydrolysis	Casein digestion	Serum digestion	Sensitivity to optochin	Hemolysis ^b (sheep cells)	Preferred atmosphere
ae/an ae/an ae/an																
hyorhinae	25021	PG29 (Leach)	+	O	-	+/+	+/+	+/+	+	-	-	-	ND	-	α	Ae
	25026	PG29 (Edward)	+	O	-	+/+	+/+	+/+	+	-	-	-	ND	-	α	Ae
	25077	PG29 (Freundt)	+	O	-	+/+	+/+	+/+	+	-	-	-	ND	+	α	Ae
laidlawii	23206	PG8 (Sewage A)	+	F	-	+/+	+/+	-/+	+	-	-	-	-	-	β r	Ae
	14089	Laidlaw A	+	F	-	+/+	+/+	-/+	+	-	-	-	-	-	β r	Ae
	14192	Laidlaw B	+	F	-	+/+	+/+	-/+	-	-	-	-	-	-	β r	Ae
	23217	PG9 (Sewage B)	+	F	-	+/+	+/+	-/+	-	-	-	-	-	-	β r	Ae
neurolyticum	19988	Sabin, type A	+	F	-	-/+	+/+	-/+	-	-	-	-	-	-	α	Ae
	15049	KSA	+	F	-	-/+	+/+	-/+	-	-	-	-	-	-	α	Ae
pneumoniae	15531	FH	+	O	-	+/+	+c/+c	-/+	-	-	-	-	-	+	β	Ae
	15377	Bru	+	O	-	+/+	+c/+c	-/+	-	-	-	-	-	+	β	Ae
	15492	Mac	+	O	-	+/+	+c/+c	-/+	-	-	-	-	-	+	β	Ae
pulmonis	19612	PG34 (Ash)	+	F	-	-/+	+/+	-/+	-	+	-	-	-	-	α r	Ae
	14267	Kon	+	F	-	-/+	c/c	-/+	-	+	-	-	-	-	α	Ae
	23554	Negroni	+	O	-	+/+	+c/c	-/+	-	-	-	-	-	-	α r	Ae
sp. (goat)	15718	C30KS-1	+	F	-	+/+	+/+	+/+	-	-	-	-	-	-	α	Ae
sp. (dog)	23462	PG24 (C21)	+	F	-	-/+	+/+	-/+	-	-	-	-	-	-	β	Ae

Table 2. Differential physiologic reactions displayed by glucose positive, arginine positive mycoplasmas.

Mycoplasma species	ATCC No.	Strain	Glucose breakdown	O-F test	Arginine hydrolysis	Tetrazolium reduction	Tellurite reduction	Methylene blue reduction ^a	Phosphatase	Film and spots	Gelatin hydrolysis	Casein digestion	Serum digestion	Sensitivity to optochin	Hemolysis ^b (sheep cells)	Preferred atmosphere
ae/an ae/an ae/an																
fermentans	19989	PG18 (G)	+	F	+	-/+	- ^c / ⁺ c	-/+	+	+	-	-	-	-	-	An
	15474	GII	+	F	+	-/+	- ^c / ⁺ c	-/+	+	+	-	-	-	-	-	An
sp. (sheep)	23243	67-166	+	O	+	+/+	-/-	-/-	-	-	-	-	-	-	-	Ae

Key: +, positive reaction; $\pm$, weak positive reaction; -, negative reaction; O, oxidative reaction; F, fermentative reaction; ae, aerobic; an, anaerobic.

a. Reading at 24 hr.

b. Reading at 4 days.

c. Growth inhibited by test substrate.

sterilization in a hot air oven at 170°C for 2 to 3 hr. Tests were read at first daily, then at frequent intervals for 2 weeks. A drop of 0.5 pH unit or more in the glucose tube compared with the appropriate substrate control tube constituted a positive reaction; a rise of 0.5 pH unit or more in the arginine or urea tubes compared with the appropriate substrate control tubes constituted a positive test. The pH values were read by comparison with a set of standards ranging from pH 5.6 to 8.4. The standards were made by adding phenol red to serum-yeast-broth aliquants, adjusting the series of aliquants at 0.2 pH unit-increments, sterilizing them by filtration through 0.45- μ m pore-size Millipore membranes, (Millipore Filter Corp., Bedford, Mass.), and dispensing them in 2-ml volumes into sterile 12 x 75 mm glass tubes that were then sealed in a flame.

Test-control organisms:

Glucose breakdown:

Positive: M. bovirhinis ATCC No. 19884

Negative: M. arthritidis ATCC No. 19611

Arginine hydrolysis:

Positive: M. arthritidis ATCC No. 19611

Negative: M. bovirhinis ATCC No. 19884

Urea hydrolysis:

Positive: (T-strain mycoplasma)

Negative: M. arthritidis ATCC No. 19611

O-F test. The composition of the medium for testing for oxidation or fermentation of glucose was the same as that described above for glucose breakdown except that HIA was substituted for HIB. The complete sterile medium was dispensed aseptically in 1-ml amounts in 15 x 45 mm screw-capped vials. Duplicate test and substrate control vials were inoculated with 0.5 ml of a 24-hr broth culture grown in 10% (v/v) horse serum broth. One test and one substrate control vial were overlaid with 1.5 ml of sterile vaseline and paraffin mixture and screw-capped. The other pair of test and control vials were fitted loosely with screw-caps. The pH values were read by comparison with the set of standards described above, values recorded daily for 1 week, and final readings made at the middle and end of the second week of incubation. Fermentative organisms produced acid in both the aerobic and anaerobic vials, whereas oxidative organisms produced acid in the aerobic vial only.

The agar vial method required less medium and gave results slightly more rapidly than the broth tube method. In later confirmatory runs, therefore, only the agar vial

Table 3. Differential physiologic reactions displayed by glucose negative, arginine positive mycoplasmas.

Mycoplasma species	ATCC No.	Strain	Glucose breakdown	Arginine hydrolysis	Tetrazolium reduction	Tellurite reduction	Methylene blue reduction ^a	Phosphatase	Film and spots	Gelatin hydrolysis	Casein digestion	Serum digestion	Sensitivity to optochin	Hemolysis ^b (sheep cells)	Preferred atmosphere
			ae/an			ae/an			ae/an						
arthritidis	19611	PG6 (Preston)	-	+	-/-	-/-	-/-	+	-	+	-	-	-	a ^{c,d}	Ae
	13988	H606	-	+	-/-	-/-	-/-	+	-	+	-	-	-	a ^{c,d}	Ae
	14124	Jasmin	-	+	-/-	-/-	-/-	+	-	-	-	-	-	a ^{c,d}	Ae
	14152	Campo	-	+	-/-	-/-	-/-	+	-	+	-	-	-	a ^{c,d}	Ae
	23192	PG27 (Campo)	-	+	-/-	-/-	-/-	+	-	+	-	-	-	a ^{c,d}	Ae
gallinarum	19708	PG16 (Fowl)	-	+	+/-	+/-	-/+	-	+	-	-	-	+	a ^{c,r}	Ae
	15319	PG16	-	+	+/-	+/-	-/+	-	+	-	-	-	+	a ^{c,r}	Ae
gateae	23392	CS	-	+	-/+	-/-	-/-	-	-	-	-	-	-	-	Ae
hominis	23114	PG21 (H50)	-	+	-/-	-/-	-/-	-	-	-	-	-	+ ^e	-	Ae
	14027	4387	-	+	-/-	-/-	-/-	-	-	-	-	-	+ ^e	-	Ae
	15056	H34	-	+	-/-	-/-	-/-	-	-	-	-	-	+ ^e	-	Ae

Key: +, positive reaction; +, weak positive reaction; -, negative reaction; a, alpha hemolysis; a', alpha prime hemolysis; r, green ring at outer zone of hemolysis; ae, aerobic; an, anaerobic.

- Reading at 24 hr.
- Reading at 4 days.
- Growth inhibited by test substrate.
- Weak reaction.
- Zone of growth inhibition extended 1 mm from edge of disc.

Table 3. (Continued)

Mycoplasma species	ATCC No.	Strain	Glucose breakdown	Arginine hydrolysis	Tetrasolium reduction	Tellurite reduction	Methylene blue reduction ^a	Phosphatase	Film and spots	Gelatin hydrolysis	Casein digestion	Serum digestion	Sensitivity to optochin	Hemolysis ^b (sheep cells)	Preferred atmosphere
			ae/an			ae/an	ae/an								
inens	19705	PG30 (M)	-	+	-/-	-/-	-/-	-	-	-	-	-	-	-	Ae
	15969	A60Z	-	+	-/-	-/-	-/-	-	-	-	-	-	-	-	Ae
maculosum	19327	PG15 (C27)	-	+	-/+	-/-	-/-	+	+	-	-	-	+	-	Ae
orale, type 1	15539	823B	-	+	-/-	-/-	-/-	-	-	-	-	-	+	-	An
orale, type 2	23204	DC1600	-	+	-/+	+/+	-/-	+	-	-	-	-	+	+	An
pharyngis	15544	Patt	-	+	-/-	o/c	-/-	-	-	-	-	-	+	-	An
salivarium	23064	PG20 (H110)	-	+	-/+	-/-	-/-	-	+	-	-	-	-	-	An
	14277	Buccal 1	-	+	-/-	-/-	-/-	-	+	-	-	-	-	-	An
spumans	19526	PG13 (C48)	-	+	-/-	+/+	-/+	+	-	-	-	-	-	-	Ae
sp. (human)	15497	Navel	-	+	-/+	-/-	-/-	-	-	-	-	-	+	-	An

Table 4. Differential physiologic reactions displayed by glucose negative, arginine negative mycoplasmas.

Mycoplasma species	ATCC No.	Strain	Glucose breakdown	Arginine hydrolysis	Tetrazolium reduction	Tellurite reduction	Methylene blue reduction ^a	Phosphatase	Film and spots	Gelatin hydrolysis	Casein digestion	Serum digestion	Sensitivity to optochin	Hemolysis ^b (sheep cells)	Preferred atmosphere
			ae/an	ae/an	ae/an	ae/an	ae/an	ae/an	ae/an	ae/an	ae/an	ae/an	ae/an	ae/an	ae/an
bovigenitalium	19852	PG11 (B2)	-	-	-/+	-/-	-/+	+	+	-	-	-	-	r r'	As
	14173	PG11	-	-	-/+	-/-	-/+	+	+	-	-	-	-	r r'	As

Key: +, positive reaction; -, negative reaction; α' , alpha prime hemolysis; r, green ring at outer zone of hemolysis; ae, aerobic; an, anaerobic.

a. Reading at 24 hr.

b. Reading at 4 days.

d. Weak reaction.

method was employed to test for glucose breakdown, and in addition, was employed for confirmatory tests for hydrolysis of arginine and urea substrates.

Test-control organisms:

O-F test:

Oxidative: M. pneumoniae ATCC No. 15531

Fermentative: M. bovirhinis ATCC No. 19884

Tetrazolium reduction. Plates for tetrazolium chloride reduction were prepared by adding 20 ml of horse serum, 5 ml of yeast extract stock solution, and 1 ml of stock 2% (w/v) 2, 3, 5-triphenyltetrazolium chloride (Fisher Scientific Co., Fair Lawn, N.J.) solution to 74 ml of HIA. Duplicate plates were inoculated by inverting agar blocks, containing dense colony growth on the test plates and sliding the blocks across the agar surfaces. One plate was then incubated aerobically, the other anaerobically for 2 weeks. The development (usually in 2 to 3 days) of a pink or red color in the area of the block indicated reduction of the substrate to the insoluble formazan and constituted a positive reaction.

Test-control organisms:

Tetrazolium reduction:

Positive aerobically and anaerobically:

M. bovirhinis ATCC No. 19884

Negative aerobically and anaerobically:

M. arthritidis ATCC No. 19611

Tellurite reduction. Plates for the demonstration of tellurite reduction were prepared by adding 20 ml of horse serum, 5 ml of yeast extract stock solution, and 0.5 ml of stock 1% (w/v) potassium tellurite (Fisher Scientific Co.) solution to 74.5 ml of HIA. Duplicate plates were inoculated, each with two inverted agar blocks, one of which showed dense growth, the other of which showed well isolated colonies. One plate was then incubated aerobically, the other anaerobically for 2 weeks. Plates were observed microscopically at frequent intervals for the presence of a true black coloration of colonies, indicative of a positive reaction. Brown or colorless colonies were recorded as negative.

Test-control organisms:

Tellurite reduction:

Positive aerobically and anaerobically:

M. bovirhinis ATCC No. 19884

Negative aerobically and anaerobically:

M. arthritidis ATCC No. 19611

Methylene blue reduction. To test for methylene blue reduction, 0.1 ml of stock 0.1% (w/v) methylene blue chloride (certified biological stain, color index No. 922, National Aniline Division, Allied Chemical and Dye Corp., N.Y.), sterilized by filtration through a 0.45- μ m pore-size Millipore filter, was pipetted into each of two 12x75 mm sterile tubes. Each tube was then inoculated with 2.9 ml of a 24-hr broth culture. One tube was stoppered with a sterile gauze plug; the other was sealed with an overlay of 1.5 ml of sterile vaseline-paraffin mixture. After incubation for 24 hr, tubes were read for reduction. Complete decolorization of the broth indicated a positive reaction, a green color indicated a weak positive reaction, and a blue color indicated a negative reaction.

Test-control organisms:

Methylene blue reduction:

Positive aerobically and anaerobically:

M. bovirhinis ATCC No. 19884

Negative aerobically and anaerobically:

M. arthritidis ATCC No. 19611

Phosphatase. To test for phosphatase activity, plates were prepared by adding 20 ml of horse serum, 5 ml of yeast extract stock solution, and 1 ml of stock 1% (w/v) solution of the sodium salt of phenolphthalein diphosphate (Sigma Chemical Co., St. Louis, Mo.) to 74 ml of HIA. Test plates were inoculated in triplicate with a drop from a 24-hr broth culture. After incubation for 3, 7, and 14 days respectively, plates were tested by flooding the agar surface with 5N NaOH. The appearance of a red color indicated a positive reaction.

Test-control organisms:

Phosphatase:

Positive: M. arthritidis ATCC No. 19611

Negative: M. bovirhinis ATCC No. 19884

Film and spots. The "film and spot" phenomenon, described and illustrated by Edward (7), was detected by inoculating a 24-hr broth culture onto standard growth agar (containing 20% (v/v) horse serum) and incubating for 2 weeks at 37°C. Plates were examined intermittently during incubation for production of the film and spots.

Test-control organisms:

Film and spots:

Positive: M. gallinarum ATCC No. 19708

Negative: M. arthritidis ATCC No. 19611

Gelatin hydrolysis. To test for hydrolysis of gelatin, the medium was prepared by dissolving 12 g of gelatin (Difco) and 2.5 gm of dehydrated HIB in 100 ml of water and adjusting the pH to 7.6 with 5N NaOH. The medium was dispensed in 3.75-ml volumes into screw-capped tubes and autoclaved. Prior to use, 1 ml of sterile horse serum and 0.25 ml of yeast extract stock solution were added to the melted gelatin tubes. The inoculum consisted of an agar block containing dense growth. At weekly intervals during 1 month of incubation, tubes were refrigerated at 4°C for 30 min and read for liquefaction.

Test-control organisms:

Gelatin hydrolysis:

Positive: M. arthritidis ATCC No. 19611

Negative: M. bovirhinis ATCC No. 19884

Casein digestion. Proteolysis of casein was tested by using an overlay technique. The medium was prepared by dissolving 8 g of Skim Milk (Difco) in 80 ml of water, adjusting the pH to 7.6 with 5N NaOH, and autoclaving at 115°C for 10 min. An agar base was prepared by dissolving 2 g of Agar (Difco) in 120 ml of water, adjusting the pH to 7.6 with 5N NaOH, and autoclaving at 121°C for 15 min. The milk solution was then added aseptically to the melted agar and the mixture dispensed in 2-ml volumes into sterile screw-capped tubes. Prior to use, the milk-agar mixture was melted in a boiling water bath and a few drops were pipetted onto a standard growth agar plate containing a band of dense growth. The overlay covered a portion of both the inoculated and uninoculated surface. Plates were read for clearing in the opaque overlay around the line of growth during a 2-week period of incubation.

Test-control organisms:

Casein digestion:

Positive: M. bovirhinis ATCC No. 19884

Negative: M. arthritidis ATCC No. 19611

Serum digestion. The medium for testing serum digestion was prepared by mixing horse serum, HIB, and yeast extract stock solution in a ratio of 15:4:1 respectively. The medium was dispensed in 1.5-ml volumes into 12x75 mm test tubes that were then plugged with cotton and sterilized in a slanted position in flowing steam for 45 min. When cool, tubes were sealed with paraffin-coated sterile corks and stored at 4°C. The tubes were inoculated by sliding an inverted agar block containing dense growth over the slant surface. The tests were examined at frequent intervals

during 1 month of incubation for liquefaction of the top portion of the slant and accumulation of liquid at the bottom portion.

Test-control organisms:

Serum digestion:

Positive: M. bovirhinis ATCC No. 19884

Negative: M. arthritidis ATCC No. 19611

Optochin. Sensitivity to optochin (ethylhydrocuprein hydrochloride) was examined by placing a Taxo P disc (Baltimore Biological Laboratory, Baltimore, Md.) in the center of a plate, which had been seeded with a drop from a 24-hr broth culture diluted to contain 10^4 to 10^5 organisms per ml. The inoculum was allowed to soak into the agar before the disc was put in place. Plates were incubated until colonies were visible, and zones of inhibition were measured. A zone extending approximately 1 mm or more beyond the edge of the disc was recorded as positive inhibition.

Test-control organisms:

Optochin:

Positive: M. pneumoniae ATCC No. 15531

Negative: M. arthritidis ATCC No. 19611

Hemolysis: Hemolytic activity was tested by using a modification of the overlay technique described by Clyde (1). A 1% (w/v) solution of Agar (Difco) dissolved in physiological saline and adjusted to pH 7.4 was dispensed in 4, 5-ml volumes into screw-capped tubes and autoclaved at 121°C for 15 min. Sterile defibrinated sheep blood was centrifuged, and the erythrocytes were collected, washed three times in physiological saline, and resuspended in saline at a final concentration of 50% (v/v). To each tube of melted saline agar 0.5 ml of red blood cell suspension was added and mixed gently. Several drops of the blood cell agar were applied to the surface of a plate containing well isolated colonies. (A 24-hr broth culture diluted to contain approximately 10^4 to 10^5 organisms per ml was a suitable inoculum for obtaining plates of well isolated colonies.) Plates were incubated aerobically and refrigerated for 30 min before each reading. Type of hemolysis was determined by microscopic examination made each day for 4 days. A green zone around the colonies was recorded as alpha hemolysis; a clear area with some nonhemolyzed red cells remaining in the zone around the colony was recorded as alpha prime; and a clear, cell-free zone around the colonies was recorded as beta hemolysis. Some strains showed, in addition, a green "ring" of alpha hemolysis at the outer edge of alpha prime or

beta hemolytic zones; such reactions were recorded as alpha prime, ring or beta, ring.

Test-control organisms:

Hemolysis:

alpha: M. neurolyticum ATCC No. 19988

alpha prime: M. arthritidis ATCC No. 19611

alpha prime with ring: M. gallinarum
ATCC No. 19708

beta: M. pneumoniae ATCC No. 15531

beta with ring: M. bovirhinis ATCC No. 19884

Catalase. Catalase activity was examined by flooding plates containing heavy colony growth (usually present after 24 hr of incubation) with a 30% solution of hydrogen peroxide (Mallinkrodt Chemical Works, New York) and observing the growth for evolution of gas bubbles. Cultures were also tested after incubation for 48 and 72 hr.

Oxidase. Oxidase activity was investigated according to the method described in the Army's technical manual (5) by flooding plates containing moderate to heavy colony growth with a freshly prepared 1% (w/v) aqueous N, N-dimethyl-para-phenylenediamine hydrochloride (Eastman Organic Chemicals, Rochester, N. Y.) solution. The reagent was poured off, and the plates were observed microscopically for the development of red or black colonies.

Deamination of phenylalanine. A modification of the method described by Cowan and Steel (3) was used to test for phenylalanine deamination. HIA was prepared as described for the standard growth medium, except that 0.15 g of D, L-phenylalanine (Eastman Organic Chemicals, Rochester, N. Y.) was added to 75 ml of HIA before autoclaving. Twenty ml of horse serum and 5 ml of yeast extract stock solution were added aseptically prior to pouring the medium into 35 x 10 mm plates. Plates were inoculated in triplicate with a drop from a 24-hr broth culture. After incubation for 3, 7, and 14 days, respectively, plates were tested for the presence of phenylpyruvic acid by flooding the agar surface with 10% (w/v) ferric chloride solution. A positive reaction was indicated by the appearance of a green color in the area of growth.

Oxidation of gluconate. The conversion of gluconate to 2-ketogluconic acid was tested using a modification of the method described by Cowan and Steel (3). A stock solution of 40% (w/v) aqueous potassium gluconate (Fisher Scientific Co.) was prepared, sterilized by filtration, and stored at 4°C. Test broth was prepared by adding 10 ml of horse

serum, 5 ml of yeast extract stock solution, and 10 ml of potassium gluconate solution to 75 ml of HIB. The medium was dispensed aseptically in 5-ml amounts into screw-capped tubes. Duplicate tubes were inoculated with 1 ml of a 24-hr broth culture grown in 10% (v/v) horse serum broth. After incubation for 3, 7, and 14 days, 3 ml of the test broth was tested for the presence of reducing sugars by the addition of 0.5 ml of Benedict's solution, followed by boiling in a water bath for 10 min. The Benedict's solution was prepared by dissolving 17.3 g of sodium citrate and 10 g of anhydrous sodium carbonate in 60 ml of water. A second solution of 1.73 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in 20 ml of water was slowly added to the first solution, and the volume was adjusted to 100 ml with water. A positive control tube containing a small amount of added glucose was included to verify the sensitivity of the reagent. A reddish brown precipitate indicated a positive reaction.

Benzidine test. To test for the presence of cytochrome systems, benzidine test reagent was prepared according to the method of Deibel and Evans (4) as follows: 1 g of benzidine dihydrochloride (Fisher Scientific Co.) was dissolved in 20 ml of glacial acetic acid and 30 ml of water was added to the solution. The solution was heated gently, cooled, and mixed with 50 ml of 95% (v/v) ethanol. The reagent was stored in the dark at 4°C. Tests were performed on 3, 7, and 14-day old plate cultures of the test organism by pipetting 0.5 ml of the test reagent onto the agar surface, followed by 0.5 ml of 5% hydrogen peroxide. The appearance of a blue color constituted a positive test. The sensitivity of the test reagents was demonstrated by adding the reagents to a dilute hemoglobin solution.

RESULTS AND DISCUSSION

Of prime importance for initial biochemical characterization of mycoplasmas are the tests for breakdown of urea, glucose and arginine. This study did not include the T-strain mycoplasmas, which hydrolyze urea; all the included strains were confirmed to be negative for hydrolysis of urea. Thus the mycoplasmas in this study displayed one of the following patterns:

- (1) Glucose positive, arginine negative (Table 1).
- (2) Glucose positive, arginine positive (Table 2).
- (3) Glucose negative, arginine positive (Table 3).
- (4) Glucose negative, arginine negative (Table 4).

Further subdivisions within each of these major groups could be effected by applying the other tests described.

The methods used to test for breakdown of glucose were unable to detect acid production by *M. bovigenitalium*. Although this species has been reported to be glucose negative (8, 16), breakdown of glucose may, under specialized conditions of growth, be detected (R. H. Leach, personal communication). The methods used were readily able to detect acid production by a number of other species that have on occasion been reported as glucose negative, i. e., *M. felis* (2), *M. hyorhinis* (12, 18), *M. granularum* (18), *M. canis* (8), and *M. sp.*, strain PG24 (C21) (9).

There are several acknowledged difficulties in testing for breakdown of glucose and in making valid interpretations of the results. Most of these difficulties could be minimized sufficiently to prevent false negative or false positive readings. For instance, it was important to observe tests for glucose breakdown for a full two weeks before terminating the experiments. Whereas most glucose positive strains yielded a reaction within 2 to 3 days, some few strains displayed weak reactions that were not apparent until the latter part of the incubation period. The opportunity for making a false negative reading was also provided when a second reaction "masked" the production of acid from glucose. An illustration of this difficulty was provided by *M. fermentans*, strain GII. and by *M. sp.*, strain 67-166. Early in the incubation period there was no appreciable drop in the pH of the glucose containing medium for either strain. This was presumably due to the earlier or stronger action of both of these strains on arginine or arginine-like components of the complex medium resulting in alkali production. Only later was it apparent that there had been the required pH drop in the glucose-containing medium and the required pH rise in the arginine-containing medium establishing that both reactions were positive. On the other hand, *M. fermentans*, strain PG18 (G), did not exhibit such a delay in yielding a clear-cut positive glucose reaction, although it too attacked arginine. The possibility of making false positive readings was encountered with other strains. In these instances, media containing serum and phenol red, but lacking the glucose substrate, showed a drop in pH when inoculated with the glucose positive organisms. This was presumably due to breakdown of acid-yielding components of the serum. This effect could be minimized by reducing the serum to the lowest concentration that would yield good growth. As a

further precaution, an organism was required to show a difference of at least 0.5 pH unit in its reaction on glucose-containing medium compared with glucose-lacking medium in order to qualify as positive for glucose breakdown.

The O-F test was valuable for further subdividing the two groups of glucose positive mycoplasmas. Of the glucose positive, arginine negative species, only M. pneumoniae and M. hyorhinis attacked glucose oxidatively; the remaining species attacked glucose fermentatively. (The unexpected oxidative reaction of M. pulmonis, strain Negroni, requires further investigation, possibly with other sublimes of the strain.) Of the glucose positive, arginine positive species, M. sp., strain 67-166, attacked glucose oxidatively, M. fermentans, fermentatively.

Nearly all species that attacked glucose reduced tetrazolium, tellurite, and methylene blue anaerobically. The aerobic reactions, especially for tetrazolium and methylene blue, varied, however, from species to species, and thus were helpful in characterizing individual species. Of the glucose negative species, very few reduced tetrazolium, tellurite, or methylene blue aerobically (in no instance were all three tests positive), and a number of species (notably, M. arthritidis, M. hominis, M. iners, and M. orale, type 1) did not reduce any of these substances either aerobically or anaerobically, although they remained viable.

Fabricant and Freundt (11) reported that the tetrazolium reaction for many mycoplasma strains varied with the composition of the medium employed and varied from culture to culture. In the present study no attempt was made to compare a variety of test media; all 53 strains were tested under identical conditions and tested repeatedly. If a strain was growing optimally, it gave consistent reactions from run to run. For instance, M. granularum and all M. laidlawii strains consistently gave weak positive reactions aerobically and strong positive reactions anaerobically for tetrazolium reduction. Furthermore, different strains of a given species gave similar reactions with only two exceptions, M. pulmonis, strain Negroni (Table 1), and M. salivarium, strain Buccal 1 (Table 3).

The reactions for tellurite reduction appeared to vary from run to run for some strains of mycoplasma. This effect was attributed to partial inhibition by the substrate, since for these strains the number of colonies on test plates was appreciably reduced when compared to control plates lacking tellurite. A very heavy inoculum was required in

such cases to observe the black deposit indicating a reduction reaction in the cores of colonies that survived. The brownish appearance of some colonies may have indicated colonies that died before appreciable reduction had occurred.

The strains of M. pulmonis included in this study illustrated the variations due to the inhibitory effect of tellurite. Strain PG34 (Ash) displayed pronounced reduction of tellurite aerobically and anaerobically accompanied by slight inhibition of growth anaerobically; here the colonies appeared slightly smaller than normal, but the cores were filled with black granules. Strain Negroni was observed to be somewhat more sensitive to tellurite. Aerobically the number of colonies was decreased, but the survivors reduced the substrate and showed black deposits throughout the colonies. Any colonies that survived anaerobically appeared very tiny and dark in color. Strain Kon showed marked inhibition by the substrate; aerobically the few surviving colonies were "ghost-like" in appearance and only slightly darker in color when compared to control plates lacking the substrate. In the majority of test runs, this organism failed to produce colonies when incubated anaerobically; whenever a few colonies managed to survive, they were so distorted and small, that a reading for reduction could not be made at all.

No valid readings could be made for M. pharyngis, which showed marked inhibition by the substrate. Only after repeated testing using a very heavy inoculum could reactions for M. pneumoniae (also very sensitive to tellurite) finally be recorded.

Again it was difficult to get valid readings aerobically and anaerobically on strains that grew poorly under one or the other type of gaseous environment. For example, when incubated aerobically on tellurite medium, the few colonies of M. fermentans that grew appeared brownish in color, suggesting incomplete reduction.

Positive reactions for methylene blue reduction within 24 hr under microaerophilic to anaerobic conditions were reported by Freundt (12) for M. arthritis, bovigenitalium, canis, gallinarum, hyorhinis, pulmonis, neurolyticum, fermentans, and laidlawii. In this laboratory both M. arthritis and M. canis gave negative reactions both aerobically and anaerobically. Methylene blue exerts a toxic effect on most species of mycoplasma; therefore, the ability to reduce the indicator is dependent upon survival of the organism. This in turn is influenced by the age of the culture, number of organisms, and passage history of the strain. These

factors, along with time of reading, may account for differences in results reported here and reported elsewhere in the literature. In this study, reactions were reported after 24 hr of incubation; after further incubation more cultures became positive, but the test did not have as much differential value.

The simplicity of performing and reading the phosphatase test makes it a valuable tool in differentiating mycoplasmas. It could be usefully applied to diagnostic problems on clinical specimens, since it was able to differentiate M. orale, type 2 (phosphatase positive) from M. orale, type 1 and M. salivarium (phosphatase negative). The only other phosphatase positive species of those common to man was M. fermentans.

Seven mycoplasma species produced a consistently strong "film and spot" reaction, i.e., M. felis, pulmonis (except strain Negroni), fermentans, gallinarum, maculosum, salivarium, and bovigenitalium. It should be noted that all strains of M. hyorhinis late in the second week of incubation produced an atypical film-like material on the surface of the agar; the spots could be detected only deep in the agar below the plane of the film. These strains were, therefore, considered negative for the typical film and spot reaction, but more thorough study may later prove them to be positive.

The phenomenon of "film and spot" production is markedly influenced by medium composition as described in detail by Fabricant and Freundt (11). Results in our laboratory were obtained using infusion agar-yeast extract plates containing 20% (v/v) horse serum for all strains tested. Plates must be poured sufficiently thick and maintained in a moist environment to retard drying of the agar surface during the 2-week test period. Readings should not be made after this time, because the incidence of false positive reactions increases.

M. arthritis was the only species included in this study that caused liquefaction of gelatin medium. Other laboratories, using the method of Kohn (15) have detected positive reactions for M. bovis genitalium (12, 14), M. pharyngis (14), M. laidlawii (12), M. canis (12), and M. maculosum (12); many of these reactions were reported as "doubtful liquefaction." The method described in this study gave a positive reaction within 7 days; all other cultures proved negative until tubes were discarded at one month. The negative reaction of M. arthritis, strain Jasmin, is probably due to strain variation within the species.

M. bovirhinis was the only species that demonstrated positive results when tested for digestion of casein and coagulated serum media. Liquefaction of coagulated serum has been reported positive for M. mycoides (12). Casein proteolysis is an easy test to perform and read, and may prove useful for detecting M. bovirhinis among isolates obtained from cattle.

Sensitivity to optochin was recorded simply as positive or negative, since the width of zones of inhibition varied with the concentration of colonies on the plate. The reaction can be obscured entirely by using too heavy an inoculum; therefore, it is helpful to test several dilutions of the culture. With few exceptions, the organisms exhibiting sensitivity always produced zones exceeding 1 mm in width; M. hominis and M. orale type 2 consistently yielded zones extending about 1 mm beyond the edge of the disc. An important feature of this test is that it may be used together with the phosphatase test to distinguish M. pneumoniae from other glucose positive organisms.

The test for hemolytic activity, using a sheep red blood cell overlay did not detect the weak positive reaction reported by Cole et al. (2) for M. gateae tested by another method.

Reactions by an organism were often observed to display a progression of hemolytic types. For example, zones produced by a beta hemolytic strain might appear as alpha zones soon after the addition of the overlay, proceeding to alpha prime, and finally becoming beta by the third or fourth day. Reactions listed in Tables 1 to 4 were recorded at 4 days, although some may have been evident prior to that time. After 4 days, readings were not considered valid, since negative cultures occasionally began to show by then small zones of incomplete hemolysis.

Other blood cells employed during the course of this investigation were horse, human, rabbit, and dog cells. Sheep cells were chosen since they yielded more clear-cut reactions and allowed for more differentiation between strains. Clyde (1) also found sheep cells preferable to other cells. Only isolated colonies away from the edge of the overlay can be read reliably for the type of hemolysis.

The test for oxidation of gluconate and the benzidine test gave negative results for all of the strains. Tests for catalase and oxidase activity and for deamination of phenylalanine also yielded negative results. The three latter tests may prove useful for distinguishing mycoplasmas (all of which

gave negative results) from certain L-phase variants of bacteria (some of which give positive results). On the other hand, the catalase and benzidine test methods were apparently not sensitive enough to detect positive reactions for some mycoplasmas reported by other investigators (12, 19).

A preliminary investigation of several other tests yielded unsatisfactory results. Tests for hydrolysis of starch and of tributyrin, using substrate overlay techniques similar to that described for casein digestion, resulted in false positive reactions due to breakdown of the substrate (starch) by serum enzymes or diffusion of the substrate (tributyrin) into the base medium agar layer. Organisms were also tested for hydrolysis of hippurate, DNA, and RNA, which substrates were incorporated into the base medium. The reagents used to detect the respective end products caused precipitation of the base medium, thus obscuring any positive results which may have been produced by the test organisms.

The test methods described in this study exhibited several degrees of reliability. Tests for glucose, arginine, and urea breakdown, tetrazolium reduction, phosphatase activity, gelatin hydrolysis, and digestion of casein and coagulated serum gave consistent reactions in repeated test runs. Tests for hemolytic activity yielded consistent results except for slight variation in the time of appearance of the recorded results. As mentioned previously, zones of inhibition by optochin were observed to vary with the concentration of the test inoculum. If the inoculum were too dense, it is possible that sensitivity would be entirely obscured, especially with strains exhibiting only slight sensitivity. Strains that produced film and spots were consistent in their reactions each time they were tested. However, in view of the report by Fabricant and Freundt (11), it is conceivable that the medium used in this study did not permit the production of film and spots by some strains. Variations in results when testing for reduction of tellurite and of methylene blue were described previously. As long as results of the latter two tests were carefully interpreted, they were useful as supplementary tools for characterization purposes.

Of the strains investigated in this study, at least 11 species displayed distinctive patterns of biochemical activities. These included M. arthritidis, M. gallinarum, M. gateae, M. maculosum, M. spumans, M. bovirhinis, M. hyorhinis, M. felis, M. fermentans, M. sp. (sheep), and M. bovirgenitalium. Other strains could be subdivided into small groups containing several species with biochemically similar patterns.

It is a generally accepted requirement that new bacterial isolates and strains be characterized as fully as possible biochemically and biologically when studies on such organisms are published. Thus, it would seem to be equally desirable and important that newly isolated or unusual mycoplasma strains be similarly characterized using standardized methods that could readily be duplicated by other laboratories. The methods and results described in this investigation could serve as one framework of reference when characterization of new strains is undertaken.

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