Identification of *Mycoplasmales*: Characterization Procedures

THOMAS L. BARBER and JULIUS FABRICANT

Department of Avian Diseases, New York State Veterinary College, Cornell University, Ithaca, New York 14850

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A large collection of avian *Mycoplasma* cultures was used in studies to improve and develop biological and biochemical characterization techniques. Differential patterns among 11 avian serotypes were shown by carbohydrate fermentation, tetrazolium- and methylene blue-reduction reactions, breakdown of arginine, and the formation of film on egg yolk-agar. Some cultures fermented as many as 14 carbohydrates. Polyhydric alcohols and pentoses were among the compounds fermented. An improved procedure for determining methylene blue reduction by *Mycoplasma* was developed. These simple, rapid procedures are reproducible and should be useful in grouping *Mycoplasma* isolates prior to definitive identification by serological or other means.

Studies of *Mycoplasma* have been stimulated by their identification as pathogens of man, cattle, sheep, goats, swine, rodents, and fowl (17). Specific identification of highly pathogenic *Mycoplasma* species is relatively easy. Often the pathogen is limited to one species. However, proving the identity of most *Mycoplasma*, especially those of low or undetermined virulence, is more difficult. Because increasing numbers of *Mycoplasma* are being found in all animal species (19) and in plants (16), the need for their identification is becoming more evident. A recent review (10) listed 35 named species in the genus *Mycoplasma*. It pointed out that some doubt is cast on the concept that these organisms are highly host-specific. Both factors complicate identification of the organisms and indicate the need to improve identification techniques (10–12).

Very few biochemical reactions have been routinely used in identification of *Mycoplasma* (10). The exacting requirements for their growth render most standard bacteriological procedures of little value. A complement of biochemical reactions, like those used in bacterial identification (7), is needed. Approaches to identification have been primarily serological (19, 20, 23). Serological screening of new isolates against all other recognized species is too cumbersome (10). However, preliminary grouping of new isolates by simple biochemical reactions reduces the necessity for comparisons by more complex procedures and aids in detecting mixed cultures. Chemical and serological procedures should be used together, as the independent use of either might not reveal mixed cultures.

This study was to develop or modify biochemical reactions for *Mycoplasma* identification. Simple reproducible procedures which differentiate between *Mycoplasma* species were sought. Such procedures would greatly expedite the orderly classification of *Mycoplasma*.

**MATERIALS AND METHODS**

**Organisms.** More than 300 avian *Mycoplasma* cultures used were previously described (T. L. Barber, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1969). The collection included all known avian *Mycoplasma* serotypes except *M. synoviae* plus some unclassified isolates from ducks. Most cultures were purified by repeated cloning of single colonies, partially characterized and tentatively identified. Serological relationships and suggestions for the reclassification of certain of these avian serotypes are being reported (T. L. Barber and J. Fabricant, Avian Dis., in press).

**Culture media.** The medium for maintenance of stock cultures was Heart Infusion Broth (HIB; Difco) supplemented with 10% serum (11). Bacterial inhibitors were 1,000 units of potassium penicillin G per ml and thallium acetate at a final concentration of 0.1%. The complete liquid growth medium (BS) was dispensed in 2-ml quantities in either glass tubes (13 by 100 mm) with Morton closures or plastic tubes (12 by 75 mm) with caps. These ingredients were used as solid medium by substituting Heart Infusion Agar (HIA; Difco) for HIB and halving the concentration...
of thallium acetate. Plastic, disposable petri dishes (100 by 15 mm) were used for solid media.

Other growth media were prepared as follows (M. M. Sabry, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1968). For basal medium, a slurry was prepared from 500 g of frozen-fat-free rabbit muscle (Pel-Freeze Biologicals, Inc., Rogers, Ark.) and 1 liter of distilled water. The slurry was held overnight at 5 C; it was then heated with stirring for 30 min in a boiling-water bath. The suspension was filtered sequentially through four layers of cheesecloth, coarse filter paper, and fine filter paper. To the filtrate was added 10 g of peptone (Difco), 5 g of sodium chloride, and 10 ml of 10% sodium hydroxide. The suspension was autoclaved and passed through coarse and fine paper filters. Thallium acetate was added to a final concentration 0.1%, and the pH was adjusted to 7.8. The basal medium was filtered to sterilize, held at 37 C overnight, and stored at 5 C.

The complete medium (RYE) consisted of: basal medium, 100 ml; rabbit serum, 10 ml; yeast extract (4), 10 ml; penicillin, 100,000 units; 1% tetrazolium, 1 ml.

Agar medium (RYEP) was prepared as follows. A solution of 2 g of agar (Difco) was added to 34 ml of distilled water. The complete medium was made by mixing 100 ml of basal medium with the agar solution at 50 C.

Test media. (i) Carbohydrate fermentation broth was BS with 1% carbohydrate, 0.002% phenol red with pH adjusted to 7.6 to 7.8. Carbohydrates were prepared as 10% stock solutions and filtered to sterilize, except glyceral which was added directly to the basal medium. All 12 carbohydrates selected for extensive testing were freely soluble except salacin which dissolved with gentle heating. (ii) The methylene blue-reduction medium was biphasic with liquid over a small agar slant. The slant was made by adding 0.12 ml of a 1% stock solution of methylene blue to 100 ml of HIA. Approximately 0.25 ml was allowed to solidify as a slanted butt in a glass tube. The overlay solution was 2 ml of BS medium with 0.024 ml of 1% methylene blue. (iii) For tetratalentum reduction, a 1% stock solution of 2,3,5-triphenyl tetratalentum chloride (General Biochemicals, Chagrin Falls, Ohio) was prepared in distilled water. It was added to both BS and RYE media to final concentrations of 0.01%.

It was also incorporated in viande molie medium and E medium as described elsewhere (1, 12). Cultures were tested in all four media with tetratalentum. (iv) Arginine medium (11) was BS medium with 1% L-arginine monohydrochloride and 0.002% phenol red. The pH was adjusted to 7.0. (v) Film formation on egg yolk-agar (12) was on plates prepared from HIA with 5% swine serum and 10% concentrated egg yolk emulsion (Oxoid Ltd., London, distributed by Colab Lab., Inc., Chicago Heights, Ill.). (vi) Resazurin-reduction medium (15) was prepared by adding 0.004% resazurin (Allied Chemical Corp., New York, N.Y.) to BS medium.

Test procedures. All test media were inoculated with 3- to 4-day-old broth cultures. Carbohydrates, arginine, and tetratalentum were inoculated with 0.1 ml; methylene blue-reduction medium and resazurin were inoculated with 0.2 ml; egg yolk-agar was streaked with 0.01 ml for maximum spread. Inoculated carbohydrate media were held 10 days and other liquid media for 5 days at 37 C. Egg yolk-agar plates were read during 6 days of incubation at 37 C.

Interpretation of tests. (i) A change in color from red to yellow was evidence of carbohydrate fermentation with subsequent acid formation. Visual readings were confirmed at intervals with a pH-meter. (ii) Methylene blue reduction was interpreted as positive when the bluish-green medium became tan or colorless. Although decolorization in the liquid was often transitory, decolorization in the agar butt tended to persist. (iii) When tetratalentum was reduced, the colorless medium became pink or red with or without precipitated tetratalentum salts in the tube. When reduction was rapid, the red sometimes faded to purple. (iv) Breakdown of arginine was indicated by a change of the phenol red from pale reddish-orange to a deep purplish-red. This indicated an alkaline shift from a starting pH of 7.0. (v) Film formation on egg yolk-agar was a crystalline film overlaying the colonies. When viewed at an angle to the light, the film had a faint purplish sheen. The film was usually too dense to be properly viewed with a microscope. When the plate was gently flooded with water, the film easily detached and floated to the surface. In this manner, film formation could be distinguished from dense colony growth.

RESULTS

Carbohydrate fermentation. Forty-five carbohydrates or related compounds (Table 1) were tested with avian Mycoplasma. After extensive preliminary tests, certain carbohydrates were valueless in differential tests for one of these reasons: (i) all fermenting cultures fermented them, (ii) no cultures fermented them, (iii) the carbohydrates became spontaneously acidic, or (iv) variable results were obtained in repeated tests with the same cultures or with different members of a single Mycoplasma serotype (Table 1). As a control, the fermentation medium without carbohydrate was inoculated. In control medium, no cultures caused the phenol red to change to any shade of yellow. Fermenting cultures generally reduced the pH slightly in control medium, whereas nonfermenting strains usually caused a slight pH increase. Uninoculated carbohydrate-containing controls, incubated with every test, were usually satisfactory, but three carbohydrates were discarded because of spontaneous acid formation (Table 1).

Table 2 lists fermentation results for 11 avian Mycoplasma serotypes and unclassified cultures from ducks with the 10 most useful differential carbohydrates. Glycerol and alpha-methyl glucoside appeared potentially useful, but only limited data were obtained. The four serotypes B, E, H, and L did not ferment any carbohydrates. Differential patterns for the fermenting
The use of dextrose fermentation alone has allowed the grouping of Mycoplasma into fermenting and nonfermenting types (2, 9, 14, 25). The value of carbohydrates other than dextrose has been questioned since patterns for all fermenting species have been markedly similar (10, 19, 25). It was generally concluded (17) that most fermenting strains produced acid from dextrose, fructose, mannose, maltose, starch, and glycogen but that some avian strains fermented sucrose and galactose. No Mycoplasma serotypes were reported to ferment lactose, pentoses, or polyhydric alcohols.

In developing differential patterns, certain

| Selected for extensive studies | Reactions identical to dextrose | Not selected for extensive studies and reason | Pattern of fermentation inconsistent in a serotype |
|-------------------------------|--------------------------------|---------------------------------------------|-----------------------------------------------|
| Dextrose                      | Starch                        | Lactose                                    | Hesperidin methyl chalcone                    |
| Mannitol                      | Glycogen                      | Rhamnose                                   | Calcium gluconate                             |
| Mannose                       | Maltose                       | Inulinn                                    | Melezitose                                    |
| Leurolise                     | Dextrin                       | Arabinose                                  | Lyxose                                        |
| Cellobiose                    | Trehalose                     | Melebiol                                   | Amygdalin                                      |
| Salicin                       | Turanose                      | Xyitol                                     |                                               |
| Xylose                        | Gentiobiose                   | Alpha-chloralose                            |                                               |
| Saccharose                    |                                | Sorbose                                    |                                               |
| Sorbitol                      |                                | Fucose                                     |                                               |
| Galactose                     |                                | Deoxyribose                                |                                               |
| Glycerol                      |                                | Inositol                                   |                                               |
| Alpha-methyl glucoside        |                                | Calcium phytate                            |                                               |
|                               |                                | Dulcitol                                   |                                               |
|                               |                                | Adonitol                                   |                                               |
|                               |                                | Raffinose                                  |                                               |
|                               |                                | Esculin                                    |                                               |
|                               |                                | Erythrose                                  |                                               |
|                               |                                | Cellulose                                  |                                               |

serotypes were found. Avian Mycoplasma serotype A (M. gallisepticum) fermented dextrose, mannose, and leurolise but was consistently negative with seven other carbohydrates. This pattern was duplicated only by a few cultures in the complex I, J, K, N, Q, R group. Serotype A, like all fermenting serotypes, also fermented seven other carbohydrates (Table 1) which were not of differential value.

Avian Mycoplasma serotype C-O was unique in fermenting dextrose and saccharose (Table 2). No other avian serotypes consistently fermented saccharose. About one-half of the cultures in this group fermented either mannose or leurolise. Cultures in avian Mycoplasma serotype D-P usually fermented dextrose but were more variable in ability to ferment saccharose.

The only other avian Mycoplasma group tested in adequate numbers was I, J, K, N, Q, R. These cultures (Table 2) all fermented dextrose and most fermented leurolise or mannose. Between 61 and 77% of the cultures fermented mannitol, sorbitol, or cellobiose. Salicin was fermented by 44% of these cultures. Five cultures in an unclassified duck group had a unique pattern in fermenting dextrose, cellobiose, salicin, galactose, and xylose.

Reduction reactions. Four avian Mycoplasma serotypes reduced both tetrazolium red and methylene blue (Table 3). Avian serotype B reduced methylene blue but not tetrazolium; M. anatis reduced tetrazolium but not methylene blue. Resazurin was reduced by representatives of all avian Mycoplasma serotypes except F and H. Resazurin-reduction studies were done with fewer cultures than used for other reactions, so results were not tabulated.

Arginine dihydrolase. Cultures of five avian serotypes (Table 3) split arginine. Four of these, B, E, H, and L, were nonfermenting serotypes. The other serotype positive for the arginine dihydrolase reaction was the complex I, J, K, N, Q, R group which caused reduction reactions and carbohydrate fermentations.

Film on egg yolk-agar. Four of 11 avian Mycoplasma serotypes caused film formation on egg yolk-agar (Table 3). Serotypes positive in this reaction (B, E, L, and M. anatis) did not react identically in any other characteristic.

**DISCUSSION**

The use of dextrose fermentation alone has allowed the grouping of Mycoplasma into fermenting and nonfermenting types (2, 9, 14, 25). The value of carbohydrates other than dextrose has been questioned since patterns for all fermenting species have been markedly similar (10, 19, 25). It was generally concluded (17) that most fermenting strains produced acid from dextrose, fructose, mannose, maltose, starch, and glycogen but that some avian strains fermented sucrose and galactose. No Mycoplasma serotypes were reported to ferment lactose, pentoses, or polyhydric alcohols.

In developing differential patterns, certain
| Avian Mycoplasma serotype | No. of cultures tested | Dextrose | Saccharose | Mannose | Mannitol | Levulose | Sorbitol | Cellobiose | Salicin | Galactose | Xylose |
|--------------------------|------------------------|----------|------------|---------|---------|---------|---------|-----------|---------|-----------|-------|
| A (M. gallisepticum)     | 22                     | +        | −          | +       | −       | +       | −       | −         | −       | −         | −     |
| B (M. gallinarium)       | 16                     |         |            |         |         |         |         | −         | −       | −         | −     |
| C-O                     | 24                     | +        | +          | −       | +       | −       | −       | −         | −       | −         | −     |
| D-P                     | 24                     | +        | V          | (12/24+) | (13/24+) | −       | −       | −         | −       | −         | −     |
| E (M. iners)             | 19                     |         |            |         |         |         |         | −         | −       | −         | −     |
| F                       | 4                      | +        | −          | −       | −       |         |         | −         | −       | −         | −     |
| H (M. meleagridis)       | 4                      |         |            |         |         |         |         | −         | −       | −         | −     |
| L                       | 4                      |         |            |         |         |         |         | −         | −       | −         | −     |
| M. laidlawii var. inocuum| 2                      | +        | −          | −       | −       | −       | −       | −         | −       | −         | −     |
| M. anatis                | 2                      | +        | V          |         | +       | V       |         | +         |         | V         | −     |
| I, J, K, N, Q, R group  | 108                    | +        | −          | (104/108+) | (104/108+) | +       | V       | (66/108+) | (73/108+) | (48/108+) | −     |
| Unclassified duck        | 5                      | +        | −          | (104/108+) | (77/108+) | (104/108+) | (66/108+) | (73/108+) | (48/108+) | +         | +     |

* Symbols: + = all cultures tested were positive for fermentation, − = all cultures tested did not ferment, V = cultures in this serotype were variable in this reaction.

b When less than 100% of cultures were positive or negative, the number positive is given where sufficient numbers were examined.
carbohydrates were discarded after preliminary tests with representatives of avian serotypes (Table 1). It was nevertheless concluded that no carbohydrate should be considered useless for differentiating *Mycoplasma* until it has been tested with representatives from all known serotypes and with unclassified cultures. Hundreds of cultures were tested before any were found to ferment xylose or galactose. In fermentation studies by other workers (8, 26), different procedures and several basal media were used. Both groups regarded a pH change to 6.5, determined either by color or a pH-meter, as a positive reaction. Most cultures in the present study changed the pH of the carbohydrate test medium to 6.5 or lower, but a drop to pH 6.9 was sufficient to change the color of the medium to yellow and was considered positive. This study and other reports (8, 26) indicate that avian *Mycoplasma* serotype A ferments dextrose, mannose, levulose, and the seven other carbohydrates (Table 1) which we regarded to have no differential value. Only one of the latter group, trehalose, was reported (26) not to be fermented by serotype A. Avian *Mycoplasma* serotypes B, E, H, and L did not ferment any of the carbohydrates tested (Table 2), which agrees with previous reports (8, 26).

The fermentation pattern of the complex avian serotype I, J, K, N, Q, R was of great interest. These cultures consistently fermented more carbohydrates than any other serotype. Some members of the group were capable of fermenting dextrose, mannose, levulose, mannitol, sorbitol, cellobiose, salicin (Table 2), and all seven carbohydrates which had no differential value (Table 1). No other avian *Mycoplasma* serotype fermented sorbitol or mannitol. Thus, the fermentation of sorbitol or mannitol immediately marked the isolate as a possible member of the I, J, K, N, Q, R group. Fermentation of different carbohydrates within the I, J, K, N, Q, R group did not correlate to other differential biochemical reactions or to antigenic differences (T. L. Barber and J. Fabricant, Avian Dis., *in press*).

In similar work with cultures from cattle, 8 of 13 bovine *Mycoplasma* serotypes (J. M. Al-Aubaidi, Ph.D. Thesis, Cornell University, Ithaca, N. Y., 1969) fermented dextrose and other carbohydrates. One bovine serotype fermented sorbitol and three fermented xylose. Differential patterns were obtained for most serotypes.

Reduction of tetrathionic compounds by *Mycoplasma* was reported (22) and later used in classification studies (11, 12, 24). This study confirmed reports that certain avian *Mycoplasma* serotypes consistently reduce tetrathionic and that it may be used as a differential characteristic (Table 3). A shortcoming of the test is that more than one medium must be used to demonstrate the phenomenon consistently (11).

Our method for demonstrating methylene blue reduction is an improvement over more cumbersome procedures (5, 14, 18). This technique is rapid, simple, and reproducible. It is an excellent differential reaction because 5 of 11 avian serotypes tested were positive (Table 3). These five serotypes (A, B, L, *M. laidlawii*, and the complex I, J, K, N, Q, R group) were not grouped together on any other single characteristic. Serotypes reducing methylene blue

### Table 3. Characterization of test results with avian *Mycoplasma*  

| Avian *Mycoplasma* serotype | No. of cultures tested | Reduction | Arginine dehydrolase | Film formation on egg yol-agar |
|-----------------------------|------------------------|-----------|----------------------|-------------------------------|
|                             |                        | Tetrazolium | Methylene blue       |                               |
| A (*M. gallisepticum*)      | 20                     | +         | +                    | −                             |
| B (*M. gallinarium*)        | 26                     | −         | +                    | +                             |
| C-O                        | 22                     | −         | +                    | −                             |
| C-P                        | 23                     | +         | +                    | +                             |
| E (*M. iners*)             | 28                     | (9/23+)   | +                    | −                             |
| F                          | 4                      | +         | +                    | −                             |
| H (*M. meleagris*)         | 3                      | −         | −                    | +                             |
| L                          | 11                     | +         | +                    | −                             |
| *M. laidlawii* var. inocuum | 4                      | +         | −                    | +                             |
| *M. anatis*                | 3                      | +         | −                    | +                             |
| I, J, K, N, Q, R group     | 104                    | +         | +                    | −                             |

* Symbols: + = all cultures tested were positive in this reaction, − = all cultures tested were negative in this reaction, V = cultures in this serotype were variable in this reaction.

* When variable results were obtained, the numbers reacting are given in parentheses.
did not necessarily reduce tetrazolium, and the reverse was also true. Both fermenting and nonfermenting serotypes were positive in reduction reactions.

It was also found that 7 of 13 bovine Mycoplasma serotypes were positive for methylene blue reduction by this technique. These seven serotypes, all of which reduced tetrazolium, included both fermenting and nonfermenting organisms (J. M. Al-Aubaidi, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1969).

The reduction of resazurin was of little differential value but was found useful in the metabolic inhibition serological procedure with cultures which were negative in fermentation, arginine dihydrolase, and tetrazolium reduction (T. L. Barber, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1969).

The ability of Mycoplasma to split arginine was first noted in contaminated cell cultures (21). The arginine dihydrolase pathway and its value for identification and classification of these microorganisms were subsequently reported (3). The present study found this reaction to be reliable and consistent in agreement with other reports (2, 6, 11, 13).

The need for additional standard test procedures for identification of Mycoplasma still exists. Results from different laboratories cannot be accurately compared until similar media and procedures are used. Techniques reported in this study are useful in preliminary grouping of avian Mycoplasma. Modification may be necessary before they are applied to Mycoplasma isolated from other species.

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