Heterogeneity Among Strains of *Mycoplasma granularum* and Identification of *Mycoplasma hyosynoviae*, sp. n.

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Twelve filtrable, pleomorphic organisms isolated from swine joints and respiratory tracts had typical colonial and microscopic characteristics of mycoplasmas. They resisted penicillin and did not revert to cell wall-producing bacterial forms in media devoid of bacterial inhibitors. The morphological and growth characteristics of these mycoplasmas were similar to those described previously for *Mycoplasma granularum*. However, a new name, *M. hyosynoviae*, is proposed for them since they differed biologically, serologically, and electrophoretically from the prototype strain of *M. granularum*. *M. hyosynoviae* required sterols, was stimulated by gastric mucin, and metabolized arginine; however, it did not metabolize urea, ferment glucose, or reduce tetrazolium. The organism produced "film and spots" on horse serum-supplemented medium and produced alpha hemolysis of guinea pig and sheep erythrocytes; however, it did not digest serum, produce phosphatase, or hemadsorb guinea pig or swine erythrocytes. *M. hyosynoviae* was distinguished from three other swine mycoplasmas, *M. granularum*, *M. hyorhinis*, and *M. laidlawii*, by means of acrylamide gel electrophoresis, growth inhibition, metabolic inhibition, and immunodiffusion techniques. It was also serologically and electrophoretically distinct from 13 additional non-swine mycoplasmas which require sterols and metabolize arginine.

*Mycoplasma granularum*, a cause of polyarthritis in 3- to 6-month old swine, was initially identified by its characteristic granular growth and pellicle formation in broth medium, microscopic morphology in Giemsa-stained culture sediment, and enhanced growth response to gastric mucin (21). These criteria were used along with a number of other biological and pathogenic characteristics to distinguish *M. granularum* from *M. hyorhinis*, another common swine mycoplasma. Both organisms were further compared and characterized serologically by immunodiffusion (6), growth inhibition (6), indirect immunofluorescence (25), and complement fixation (15).

Recently, Tully and Razin showed that the prototype culture of *M. granularum*, strain 39, and three other strains of the organism grew in serum-free medium, produced carotenoid pigments, and fermented glucose (26). In addition, these strains were shown to be serologically and electrophoretically similar to *M. laidlawii*.

Comparative studies in this laboratory revealed that many mycoplasmas isolated from arthritic swine joints and tentatively identified as *M. granularum* were biochemically, serologically, and electrophoretically unlike the prototype (strain 39) of that species. The purpose of this report is to characterize these mycoplasmas and to compare them with known mycoplasmas which possess similar biological properties.

**MATERIALS AND METHODS**

The term AUSR (arginine utilizing-sterol requiring) was used during this investigation as a designation for swine mycoplasmas which were tentatively identified as *M. granularum* but which differed from the prototype (strain 39) of that species. Information regarding the source and identification of mycoplasmas which were used in this study is presented in Table 1. All mycoplasmas were cloned three times by selection of single colonies or by three terminal dilutions in broth media.

**Media and culture procedures.** A fresh beef heart infusion-turkey serum medium described previously was used for routine growth of mycoplasmas (18, 20). Fresh beef hearts were trimmed of most of the fat, ground, and infused for 16 to 18 hr at 4°C in deionized water (500 g/liter). The infusion was heated 30 min at 93 to 95°C in a boiling-water bath, clarified with gauze and a Whatman GF/A or 93AH glass-filter pad, and supplemented with 0.5% NaCl, 0.5% Mucin Bacteriological (Difco), and 0.2% Hemoglobin (Difco). Approximately 0.5% Celite (Analytical Filteraid, Johns-Manville) was added to facilitate
clarification of this medium and all other media supplemented with mucin. The pH level was adjusted to 7.8 with NaOH, and the mixture was stirred 1 hr at room temperature. It was again heated at 93 to 95°C for 3 min and clarified with a Whatman GF/A or 934AH glass-filter pad. Before sterilization, the infusion was supplemented with 1% peptone or a peptone mixture similar to that used in FM-1 medium (0.3% casamino acids, 0.3% neopeptone, 0.1% casitone, and 0.1% tryptone) by Frey et al. (8). The complete medium (BHI-TS) contained 85% infusion and 15% heated (56°C for 30 min) turkey serum.

A second medium (D-TS) consisted of 85% PPL0 broth, 0.5% mucin, and 15% heated turkey serum.

Sterol dependence was determined in a serum-free medium (DY), composed of 90% PPL0 broth and 10% fresh yeast extract (26), and Edward medium supplemented with 0.01% Tween 80, 0.1% albumen, and three different levels of cholesterol (27).

Biochemical activity of the various mycoplasmas was determined in Hayflick medium (2,9). This medium (DY-HS) contained 70% PPL0 broth, 20% unheated horse serum, 10% fresh yeast extract, 0.002% phenol red, and substrates of 1% glucose, 1% arginine, and 0.1% urea. The pH level of the medium was adjusted to 7.8, 7.2, or 7.0, respectively when glucose, arginine, or urea was added.

Glucose fermentation was determined by an acid shift in pH, and arginine and urea metabolism were indicated by an alkaline shift in pH. Inoculated media without added substrates and uninoculated, substrate containing media were used as controls to evaluate both the acid and alkaline reactions.

Tetrazolium reduction was determined in BHI-TS medium containing 0.005% 2,3,5-triphenyl-2H-tetrazolium chloride (19).

Serum digestion and phosphatase production were determined with the media and methods described by Alutto et al. (1).

Solid medium (BHI-TS) for cloning, hemadsorption, hemolysis, and growth inhibition tests contained 85% fresh beef heart infusion, 1% Noble agar, 1% peptone (Difco), and 15% sterile heated turkey serum. "Film and spots" formation was determined on solid Hayflick medium (DY-HS) composed of 70% PPL0 agar (Difco), 20%, unheated horse serum, and 10% fresh yeast extract.

Aerobic incubation of cultures on agar medium was done in a high-humidity incubator (National Appliance and Manufacturing Co.). A disposable anaerobic
system (Gaspak, BBL) was used for anaerobic incubation of agar cultures.

**Morphology.** Giemsa-stained (20) and wet-mount sediments from cultures grown for 24 hr in D-TS medium were examined by light microscopy and phase-contrast microscopy, respectively.

**Hemadsorption and hemolysis.** Hemadsorption was performed according to the procedure described by Del Giudice and Pavia (Bacteriol. Proc., p. 71, 1964). Hemolysin production was determined by a concentrated culture technique of Cole et al. (5) and an overlay technique of Clyde (3).

**Soluble antigen preparation.** Mycoplasmal cells grown in BHI-TS were washed twice with 0.25 M NaCl and suspended in 1% sodium deoxycholate (DOC) in 0.05 M phosphate buffer (pH 8.5, 1 g of packed cells per 25 ml of DOC solution; reference 28). The mixture was allowed to stand at room temperature overnight, and the following day the pH was adjusted to 7.5 with 0.1 N HCl. Deoxyribonuclease (10 µg per ml) was added, and the extract was incubated for 80 min at room temperature. Insoluble material was removed by centrifugation and discarded. Supernatant liquid was dialyzed against six changes of distilled water at 4°C and lyophilized.

**Antiserum production.** Antisera were produced in rabbits with three subcutaneous injections (5, 10, and 20 mg at 5-day intervals) of DOC extract in complete Freund adjuvant. In some cases an additional booster injection was given. Antisera against *M. hyorhinis*, designated RAB II and W75B, were kindly supplied by R. W. Moore (Texas A & M Univ., College Station, Tex.).

**Serological procedures.** Growth inhibition was performed by the method of Clyde (4) by using solid BHI-TS medium. A metabolic inhibition test similar to that described by Purcell et al. (16) was used for arginine-metabolizing mycoplasmas. The medium consisted of DY-HS adjusted to pH 7.2 and supplemented with 1% arginine, 0.003% phenol red, and 0.5% Mucin Bacteriological. Unheated guinea pig serum (6%) was added to enhance the metabolic-inhibiting activity of antisera against these organisms. Metabolic-inhibiting antibody against *M. hyorhinis*, *M. granularum*, and *M. laidlawii* was measured in DY-HS adjusted to pH 7.8 and supplemented with 0.002% phenol red and 1% glucose (24). Guinea pig serum was not used in metabolic inhibition tests with these three organisms.

**Immunodiffusion** was done in 0.5% Ionagar no. 2 (Oxoid) in 0.85% NaCl. Test materials consisted of DOC extracts of mycoplasmas and rabbit antisera against these extracts absorbed with 10 mg of lyophilized BHI-TS per ml to remove antibody against medium components.

**Polyacrylamide gel electrophoresis.** Mycoplasmal cell proteins were electrophoresed in polyacrylamide gel by the method of Razin and Rottem (17), except that electrophoresis was done in a Buchler Polyanalyst at 3 mA per tube for 3 hr.

**RESULTS**

Twelve strains of AUSR mycoplasmas isolated from swine synovial fluid, nasal secretions, and tonsils grew readily in mucin-supplemented BHI-TS, D-TS, and DY-HS media. They grew poorly when the mucin was omitted. Turbidity developed throughout the medium during the first 12 to 18 hr, followed by an apparent granular aggregation of the growth which settled to the bottom of the tube. A waxy pellicle formed on the surface of the medium within 48 hr of incubation. All strains grew aerobically and anaerobically, produced typical "fried egg" colonies approximately 220 µm in diameter and developed film and spots within 3 to 4 days on solid BHI-TS medium. Film and spots also developed within 14 days of incubation on solid DY-HS medium without mucin (7 days at 37°C and then 7 days at 20°C). *M. granularum*, 39 and "Friend;" *M. hyorhinis*, SK76 and 7; and *M. laidlawii* 14192 did not form a pellicle on fluid medium or film and spots on solid medium.

Light microscopic examination of Giemsa-stained culture sediment and phase-contrast microscopic examination of wet mounts of sediment from the 12 AUSR cultures revealed small, pleomorphic, coccolid to filamentous organisms. Amorphous round or elongate bodies of bluish precipitate, approximately 5 µm in diameter, were frequently observed in Giemsa-stained sediment from older cultures. These bodies were not observed in similar preparations of *M. granularum* strain 39. All AUSR cultures were gram-negative.

The prototype AUSR strain (S16) passed through a 0.2-µm cellulose acetate filter. All 12 strains grew in the presence of 1,000 units of penicillin per ml and thallous acetate (1:4,000) and did not revert to a cell wall-forming bacterial state after over 10 consecutive subcultures in fluid media devoid of bacteriostatic agents.

None of the AUSR strains produced visible growth in serum-free medium (DY), nor was growth seen in subcultures to serum containing BHI-TS after five passages in the DY medium. Strain S16 did not produce visible growth in Edward medium supplemented with 0.01% Tween 80, 0.1% albumen, and 1, 5, or 10 µg of cholesterol per ml. Growth was not detected in subcultures to serum containing BHI-TS after five passages in these media. *M. granularum* strain 39 produced visible growth for five passages in both DY and Edward media.

All 12 AUSR strains metabolized arginine, but none metabolized urea, fermented glucose aerobically, or reduced tetrazolium aerobically. *M. granularum* strains 39 and Friend and *M. hyorhinis* strains SK76 and 7 fermented glucose and reduced tetrazolium aerobically but did not utilize arginine or urea. Addition of 0.5% mucin to the DY-HS medium resulted in improved growth of AUSR mycoplasmas; however, it
interfered with glucose fermentation by *M. hyorhinis* and *M. granularum*.

The prototype AUSR strain (S16) did not produce phosphatase or digest serum.

None of the 12 AUSR strains hemadsorbed swine or guinea pig erythrocytes. All strains of AUSR mycoplasmas produced alpha hemolysis of sheep and guinea pig erythrocytes, whereas *M. hyorhinis* and *M. granularum* produced beta hemolysis of these erythrocytes.

Antisera against strains S16 and S149 produced 2- to 5-mm zones of inhibition of all AUSR strains in the disc growth inhibition test. No inhibition resulted when antiserum against *M. laidlawii* B, *M. granularum* 39, and *M. hyorhinis* SK76 were used. Antisera against AUSR strains S16 or S149 did not inhibit *M. laidlawii* B, *M. granularum* 39 and Friend, *M. hyorhinis* SK76 and 7, or any of 13 additional arginine-metabolizing mycoplasmas (*M. arthritidis*, *M. arginini*, *M. gallinarum*, *M. meleagridis*, *M. iners*, *M. hominis*, *M. orale* type 1, *M. orale* type 2, *M. salivarium*, *M. fermentans*, *M. spumans*, *M. maculosum*, and *M. gatueae*).

Metabolic inhibition tests revealed that all AUSR strains were inhibited by antiserum to strain S16 at dilutions 1/1,280 to 1/10,240. *M. granularum* 39 and *M. hyorhinis* SK76 antisera did not inhibit the AUSR strains nor were these organisms inhibited by antiserum to strain S16 (Table 2). Metabolic-inhibiting antibody against strain S16 was not detected in antisera against *M. hyoarthrinosa*. None of 13 additional established species of arginine-metabolizing mycoplasmas was inhibited by antiserum against strain S16.

Immunodiffusion revealed that strain S16 was serologically distinct from *M. granularum* 39 and *M. hyorhinis* SK76 (Fig. 1).

Polyacrylamide gel electrophoresis of cell proteins revealed that all AUSR strains had virtually identical patterns and that they were electrophoretically dissimilar to *M. hyorhinis*, *M. granularum*, and *M. laidlawii* (Fig. 2, 3). They were also electrophoretically dissimilar to the 13 non-swine, arginine-utilizing mycoplasmas listed in Table 1.

**DISCUSSION**

The morphological and biological characteristics of 12 isolates of a filtrable organism from swine joints and respiratory tracts were consistent with those of the genus *Mycoplasma* (7). Although these organisms possessed many characteristics described for *M. granularum*, the results presented here demonstrate that they differed biologically, serologically, and electrophoretically from two established strains (strain 39 and Friend) of that organism.

AUSR mycoplasmas metabolized arginine and required serum for growth, but did not ferment glucose. As reported by Tully and Razin (26), *M. granularum* fermented glucose, but did not utilize arginine or require serum for growth. *M. granularum* 39 and Friend rapidly reduced tetrazolium, whereas AUSR mycoplasmas did not. The two groups of mycoplasmas were serologically distinct, but strains within each group were closely related to each other. The close similarity of AUSR strains was also demonstrated by electrophoresis in polyacrylamide gel. *M. granularum*, *M. laidlawii*, and *M. hyorhinis* were all electrophoretically distinct from the AUSR strains and from each other. In addition, the AUSR strains were serologically and electrophoretically distinct from 13 other arginine-metabolizing mycoplasmas.

A new species, *M. hyosynoviae*, is proposed for...
M. granularum was initially identified by its characteristic granular growth and pellicle formation in fluid medium, amorphous precipitates surrounded by coccoid mycoplasmal cells in Giemsa-stained culture sediments, and enhanced growth response to gastric mucin (21, 22). The organism did not reduce tetrazolium. Although these characteristics are consistent with those observed for M. hyosynoviae, they are not consistent with those observed for the prototype of M. granularum. M. granularum strain 39 did not form a pellicle, it was not stimulated by mucin, and amorphous precipitates were not observed in Giemsa-stained sediment from broth cultures of the organism. It fermented glucose and reduced tetrazolium.

Glucose fermentation by both M. granularum and M. hyorhinis was more consistent and easier to interpret when mucin was omitted from the test medium. Previous work regarding glucose metabolism by these two species was done in medium supplemented with mucin (22).

It seems likely that most mycoplasma isolates of swine origin identified as M. granularum in the past were identical to M. hyosynoviae. This contention is supported by the fact that two strains belonging to a "granular" group of swine mycoplasmas (18), Jt 14 and Jt 19, were identified as M. hyosynoviae in this work. In addition, all of 36 isolates from field cases of arthritis in swine attributed to M. granularum during the past 3 years were non-tetrazolium-reducing mycoplasmas with morphological and growth characteristics identical to those of M. hyosynoviae (unpublished data). Furthermore, mycoplasmas resembling M. granularum strain 39 have never been reported from arthritic swine joints. Strain 39 was isolated from swine nasal secretions (26), and Friend was recovered from a murine leukemia cell line (25). Since neither strain was associated with actual lesions, the pathogenicity of M. granularum is undetermined.

At least five different mycoplasmas may be isolated from the respiratory tract of swine. M. hyopneumoniae, a common cause of chronic pneumonia in swine, is extremely fastidious and will not grow in most conventional mycoplasma media (11, 12). M. hyopneumoniae has been shown to ferment glucose (22) and to differ electrophoretically and serologically from M. hyosynoviae (Ross and Switzer, unpublished data). A more complete comparison of these two species will be possible when metabolic and growth inhibition tests have been adapted for use with M. hyopneumoniae. The important differential biological characteristics of the other four species, M. hyorhinis, M. granularum, M. hyosynoviae, and M. laidlawii, are presented in Table 3. Al-

Fig. 2. Electrophoretic patterns of cell proteins from M. hyosynoviae Jt 19 and S16 and M. granularum 39 and "Friend."

Fig. 3. Electrophoretic patterns of cell proteins from M. hyorhinis 7, M. hyosynoviae S16, M. granularum 39, and M. laidlawii 14192.

this new swine mycoplasma to reflect the species "hyos" and location "synovia" of the organism in the diseased host. Strain S16 of M. hyosynoviae was selected as the prototype culture and deposited in the American Type Culture Collection as accession no. 25591.
Table 3. Biological characteristics of four swine mycoplasmas

<table>
<thead>
<tr>
<th>Mycoplasma sp.</th>
<th>Aerobic</th>
<th>Production of spots and film</th>
<th>Reduces tetrazolium</th>
<th>Growth enhanced by mucin</th>
<th>Requires serum</th>
<th>Hemolysis</th>
<th>Substrate used</th>
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<tbody>
<tr>
<td><em>M. hyorhinis</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Glucose</td>
</tr>
<tr>
<td><em>M. hyosynoviae</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Arginine</td>
</tr>
<tr>
<td><em>M. granularum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Glucose</td>
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<tr>
<td><em>M. laidlawii</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Glucose</td>
</tr>
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</table>

though *M. granularum* and *M. laidlawii* can not be easily differentiated biologically, they can be distinguished serologically (10, 26).

Moore et al. isolated a mycoplasma from arthritic swine joints which was characterized by granular growth in fluid medium, an enhanced growth response to gastric mucin, and a cytopathic effect in swine kidney cell cultures (14). They named the organism *M. hyoarthrinosa* and reported that it was serologically distinct from *M. granularum* and *M. hyorhinis*. Unfortunately, *M. hyoarthrinosa* cultures were not available from the original author or the ATCC. Therefore, a critical comparison of that organism with *M. hyosynoviae* could not be made. However, results obtained with *M. hyoarthrinosa* antisera in metabolic inhibition tests indicated no relationship to *M. hyosynoviae*.

We were unable to obtain *M. hyogentialium*, (13) another mycoplasma reported from swine, and it has never been deposited in the ATCC.

Dinter et al. reported that six swine mycoplasma strains, designated B1, B2, B3, B4, B5 and B6, belonged to two serotypes which were distinct from *M. granularum* and *M. hyorhinis* (6). Taylor-Robinson and Dinter found that B1, B2, and B5 were biochemically and serologically similar to *M. gallinarum* and that B4 and B6 were closely related to *M. laidlawii* and *M. iners*, respectively (23). Although none of the strains of Dinter were compared directly with *M. hyosynoviae*, it is doubtful that they are identical to that organism since it was serologically unrelated to *M. iners*, *M. laidlawii*, and *M. gallinarum*. Strain B3 of Dinter metabolized glucose but not arabinose (23), whereas the reverse was true of *M. hyosynoviae*.

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LITERATURE CITED


