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Characterization of *Mycoplasma pulmonis* Variants Isolated from Rabbits

I. Identification and Properties of Isolates[1]

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Mycoplasma showing at least two colony types were isolated from the nares and oropharynx of New Zealand white rabbits. Two strains were purified by single-colony passages and characterized. Morphology by phase-contrast and electron microscopy was typical of *Mycoplasmataceae*. Both grew anaerobically as well as aerobically, caused hemolysis of guinea pig, sheep, and horse red blood cells, and fermented glucose. These characteristics are shared by members of the species *M. pulmonis*, commonly isolated from the respiratory tracts of laboratory rats and mice. By use of the growth-inhibition test and agar-gel double-diffusion tests, the two strains were found to be serologically related to each other and to *M. pulmonis* ATCC 14267 but not to other representative *Mycoplasma* species from man and animals.

Mycoplasma species have been found frequently in small laboratory animals. Rats and mice have been shown to harbor *M. pulmonis* (8, 13) as well as other species of *Mycoplasma*. In 1961, Lemcke was able to show evidence for linking *M. pulmonis* to disease in these animals. In essence, she demonstrated that complement-fixing antibody titers rise with the degree of bronchiectasis in rats infected with *Mycoplasma* (10). The finding that some *Mycoplasma* species parasitize animals, producing overt disease only when animals are experimentally inoculated with infected tissue or when the host is abnormally stressed, has complicated many an experiment requiring laboratory animals (8). Thus, the possible existence and disease potential of such organisms in normal animals must constantly be kept in mind by any investigator wishing to use animals experimentally. This communication describes the isolation of variants of *M. pulmonis* from the oropharynx of New Zealand White rabbits. The isolation of *M. pulmonis* has not previously been reported from rabbits.

[1] Some of the data in this paper were presented in a preliminary communication: Bacteriol. Proc., p. 48, 1966. This paper is part of a thesis submitted by B. J. D. in partial fulfillment of the requirements for the M.S. degree in Preventive Medicine, University of Washington, Seattle.

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MATERIALS AND METHODS

Culture media. Agar medium consisted of 70% basal medium 2% soy peptone (Sheffield Chemical Co., Norwich, N.Y.), 0.5% NaCl, 1% Noble special agar in water, adjusted to *p*H 8 with NaOH prior to autoclaving) to which was added 20% unheated horse serum (Grand Island Biological Co., Grand Island, N.Y.) and 10% fresh yeast extract (7).

Broth medium consisted of the same formula without agar in the basal medium, and with 1% glucose and 0.04% phenol red indicator.

Diphasic medium was made by layering broth over hardened agar.

Tetrazolium medium included 0.5% 2,3,5-triphenyltetrazolium chloride (tetrazolium red; Calbiochem, Los Angeles, Calif.) at 5 ml per 100 ml of broth without phenol red.

Dialysate broth was made by dialyzing 200 ml of 10% soy peptone and 100 ml of 5% yeast extract (Yeastolate Difco) against 4 liters of glass-distilled water for 2 to 3 days at 4 C. To the dialysate NaCl was then added to a concentration of 0.5% and phenol red to a concentration of 0.04%; the *p*H was adjusted to 7.0 to 7.5. This solution was dispensed and autoclaved. At the time of inoculation, horse serum and glucose (separately autoclaved) were added to a concentration of 20 and 0.1%, respectively. This dialysate broth medium is a modification of that previously described (6) as a means of minimizing medium contamination of *Mycoplasma* concentrates.

In all of the above media, when antibiotics were desired, crystalline penicillin was added to a concentration of 100 units per ml, or a combination of peni-

cillin and thallium acetate, to a concentration of 0.033% was employed.

Antigen preparation. Dialysate broth was employed for growing organisms to be used as antigens; 20% agamma calf serum (ag CaS; Hyland Laboratories, Los Angeles, Calif.) was used in media to grow organisms for immunizing rabbits; and 20% agamma horse serum (ag HoS; Hyland Laboratories) was used to grow antigens for serological testing. Cultures were incubated at 37 C and agitated with a magnetic spin bar slowly rotating within the flask. Cultures were harvested when a faint turbidity and color change indicating acid production could be detected in the medium. The cultures were then centrifuged at 13,200 $\times$ *g* for 1 hr, and the pellet was washed twice with 0.85% phosphate-buffered saline (PBS). Final pellets were resuspended in an amount of diluent (PBS or distilled water) equal to one-hundredth of the original volume of culture and stored at -70 C until use. Antigens for gel-diffusion tests were resuspended in distilled water and freeze-thawed two or three times prior to testing. This method was found to be more efficient for solubilizing antigens than was sonic treatment for 5 to 8 min.

Antisera production. New Zealand white rabbits were used to produce all antisera. The antigen consisted of organisms grown in dialysate broth employing agamma calf serum and resuspended after centrifugation in PBS as a 100 times concentrate. The immunization schedule, modified from that of Lemcke (11), was as follows: 2 ml of antigen emulsified in 2 ml of Freund's incomplete adjuvant (Difco) was given intramuscularly. This was followed, after a 3-week interval, by a series of four intravenous injections of aqueous antigen given at 3-day intervals in progressively increasing doses (0.125, 0.25, 0.50, and 1.0 ml). Cardiac blood samples were taken prior to immunization, 5 to 7 days after the last inoculation, and weekly thereafter for at least 1 month. The first serum obtained after the immunization series was found most satisfactory for the serological tests performed and was used throughout. Preimmunization serum samples were tested and found negative for growth-inhibiting antibodies against either of the two rabbit *Mycoplasma* strains.

Antigens and antisera for strains other than the rabbit *Mycoplasma* were similarly prepared (6).

Biological tests. Hemolysis tests were performed by incubating plate cultures 4 to 7 days old with an overlay of agar containing 1% washed red blood cells (RBC). Plates were examined after 4 hr and after 20 hr for the presence of hemolytic zones.

RBC adsorption tests were done with fresh concentrates of organisms which had been washed once with a glucose-potassium-sodium-phosphate solution, GKNP (0.1% glucose, 0.04% KCl, 000.9% $Na_2HPO_4 \cdot 7H_2O$, 0.006% KH_2PO_4, 0.8% NaCl), mixed with 0.1% guinea pig RBC in 1 $\times$ GKNP, centrifuged slightly, and examined under phase-contrast for evidence of attachment of *Mycoplasma* to RBC.

Numbers of viable colony-forming units (CFU) in a culture were determined by making 10-fold dilutions GKNP with 2% horse serum added (GKNP-2HoS) and plating 0.01-ml drops of each dilution on agar plates. Counts were made after 7 days of incubation in a moist atmosphere.

Growth-inhibition tests. The procedure for growth-inhibition tests was modified from that described by Clyde (2). Agar plates were poured, covered, and left overnight at room temperature to allow the desired amount of evaporation. Inoculations of *Mycoplasma* were made from pretitered 10 $\times$ concentrates in fresh broth medium which were stored at -70 C and were diluted on thawing with GKNP-2HoS to give a concentration of organisms known to show obvious and dependable inhibition zones with homologous antisera. After seeding, the plates were allowed to dry at room temperature for about 2 hr. A dry agar surface was essential for an accurate test. Sterile filter-paper discs (6 mm in diameter) were saturated with undiluted serum and placed on the agar surface. Plates were incubated in a moist atmosphere at 37 C and were examined after 4 and 7 days with a stereoscopic microscope. Zones of inhibition were measured from disc edge to beginning of growth. A few abortive colonies usually extended beyond the edge of the zone as measured. In tests employing species to be tested for cross-related neutralizing antibody, two or three 10-fold serial dilutions of fresh broth cultures were used.

Agar-gel double-diffusion tests. Immuno-Plates (Ouchterlony) Patterns A and E from Hyland Laboratories were used to demonstrate antigen-antibody precipitation reactions. Wells were always filled only once, and all wells were filled as close to the same time as possible. Plates were incubated overnight at 37 C, and readings were made 18 to 24 hr after inoculation; subsequent observations were made for 1 week thereafter.

Light microscopy. Colonies growing on 1 to 2 mm thick agar were examined by bright-field and anoptral phase contrast (Reichert) at 125 times magnification. Dialysate broth cultures were observed at 1,250 times magnification with anoptral phase-contrast.

Electron microscopy. Cultures were centrifuged at 164 $\times$ *g* for 15 min, and the deposits were resuspended in Veronal-acetate buffer (*p*H 6.1) containing 40% sucrose and 0.8% osmium tetroxide, and were fixed for 1 hr at room temperature. A field of low gravitation was employed to minimize distortion of organisms possible in high gravitational fields. Under the circumstances described, only "spherules" (fluid medium colonies) and clumps of organisms would sediment. After fixation, the cells were recentrifuged and washed once in buffer; the pellets of cells were gently resuspended in a small amount of molten, 2% agar (Difco bacteriological) and set in cylindrical molds. The agar pieces were sequentially dehydrated, infiltrated, and embedded in Epon 812 according to the method of Luft (12). The infiltration time in the resin-propylene oxide mixture was extended to 5 hr.

Ultrathin sections were cut, stained for 2 hr in a 3% aqueous solution of uranyl acetate and examined with an RCA EMU-3G electron microscope.

RESULTS

The first *Mycoplasma* isolate from a rabbit was from the oropharynx of a New Zealand white

used as a control in a *M. pneumoniae* (Eaton agent) immunization experiment. The organism produced dark "spherules" (fluid medium colonies) in diphastic media, and about 15 colonies were cultivated on agar. (Primary isolations were made in media with antibiotics.) After this isolation, 15 to 20 of the departmental laboratory animals were surveyed four times; nose, throat, conjunctival, and vaginal swabs were taken. Primary diphasic and agar plate cultures were obtained from the nares and oropharynx of three additional rabbits. Two or more colony types could be consistently isolated from each of these rabbits for the following 2 months, at which time isolation attempts had to be terminated. The isolation of *Mycoplasma* could not be associated with disease in these rabbits; two of the animals appeared healthy and two had "snuffles," as did about 50% of the rabbits in the animal quarters at that time.

Two colony types (referred to throughout as 63 and 47) were chosen for characterization, purified by four single-colony passages, and tested for stability by several passages through antibiotic-free media. By use of a dissecting microscope, the colonies of both could be detected on agar media as early as 2 days after inoculation, thereafter colony size increased for more than 1 month or until the agar medium could no longer support growth. After 4 days of incubation, both showed the typical "fried-egg" appearance of *Mycoplasmataceae*. With continued incubation, the colonies of 63 became so dark and granular that the indented centers were obscured. Frequently, colonies were observed with darker colored radial striations; occasionally, the dark color was observed to start in a small section of a colony and gradually spread throughout (Fig. 1). The colonies of 47 remained clear and relatively agranular (Fig. 2). Colonies could be detected macroscopically after about 1 week of incubation, and the 63 colonies were white. The colony morphology of either strain did not change throughout 10 serial passages in antibiotic-free media.

Phase-contrast microscopy of dialysate broth cultures showed a morphology characteristic of *Mycoplasma* (4), that is, pleomorphism, many forms having smaller size than most bacteria, a predominance of filamentous and budding forms during the growth phase, mainly swollen round forms during the death phase, and a tendency for clumping (more pronounced with 47 cultures). Electron micrographs (Fig. 3 and 4) of dialysate broth cultures (with or without antibiotics) revealed that the organisms lacked a cell wall, possessed a triple-layered membrane, frequently contained a membrane-lined vacuole, and displayed filamentous and budding forms. The structure of these organisms appears identical to that of other *M. pulmonis* strains prepared in a similar manner (1, 5), and is apparently typical of the *Mycoplasmataceae* (3).

The two organisms were alike in other biological properties: both produced acid in media containing glucose; both reduced tetrazolium; both grew anaerobically as well as aerobically; and both produced β-hemolysis around colonies when incubated with agar overlays containing 1% RBC of guinea pig, horse, or sheep origin. Both produced β-hemolysis, but neither yielded colonies when concentrates were inoculated onto (sheep) blood-agar plates, and both lacked the ability to adsorb to guinea pig RBC (Table 1). Growth curves for the two types were plotted by use of stationary dialysate broth cultures. Growth was found to be as good with agamma calf serum as a supplement as with agamma horse serum. Maximal plate counts were found when faint turbidity and slight acid production could be noted in the medium (Fig. 5).

To discover the relationship of the rabbit

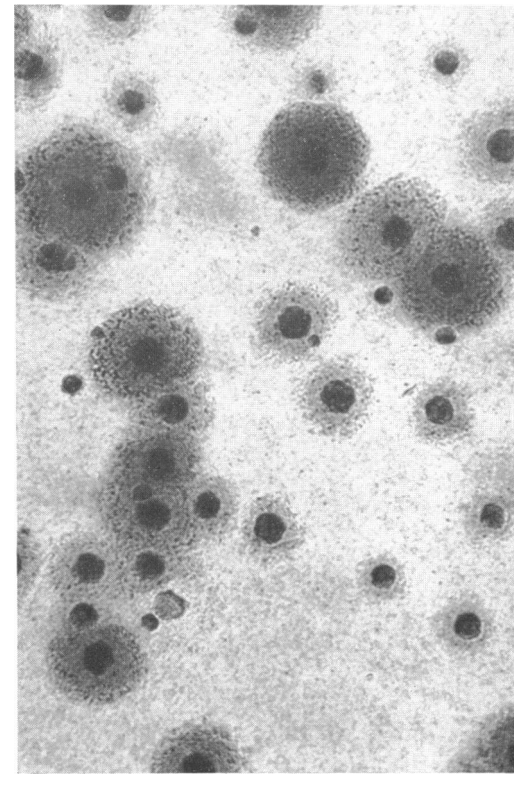

FIG. 1. *Colony morphology of Mycoplasma pulmonis strain 63. Note granularity and pigmentation of colonies (bright-field, × 125, 10-day incubation).*

Mycoplasma to known species, growth-inhibition tests and agar-gel double-diffusion tests were performed with representative strains and antisera prepared for them (Table 2). All strains tested were found to produce at least one precipitin line with homologous antiserum (6). Growth of all acid-producing strains tested was found to be inhibited by homologous antiserum. The growth-inhibition test was reported by Clyde to be species-specific (2), and indeed this did appear to be true. Growth of the two rabbit *Mycoplasma* strains was inhibited by antiserum for either strain or *M. pulmonis* ATCC 14267 but not by antiserum for the other glucose-utilizing species. The neutralization was totally reciprocal and specific, since antisera to 47 or 63 inhibited growth of 47, 63, or ATCC 14267 (Table 3). In addition, colony morphology of ATCC 14267 was identical to that of 47. On agar-gel double-diffusion plates, 63 and 47 were found to share antigens with each other and ATCC 14267 but with no other strain.

Figure 6 represents the precipitation lines which formed on Immuno-Plates when ATCC 14267, 47, and 63 antisera were placed in center wells and unheated 100 times concentrates ("raw" antigens) were placed in peripheral wells. In each of the three systems pictured, two or more common precipitation lines appeared, indicating the presence of at least two shared antigens for the three strains. Anti-14267 gave three lines with 63 and 47 "raw" antigens and four lines with homologous "raw" antigen. It appeared that the "raw" antigen and antiserum for 14267 were less active than those for the rabbit strains. Anti-47 showed four antigenic components shared by 47, 14267, and 63, although two of these were not identical as judged by the spur formation at one juncture with 14267 and one with 63. Anti-47 gave two additional precipitation lines (close to the antigen well) against homologous "raw" antigen. Anti-63 showed that 63 had two common antigens with 14267 and at least four with 47, but gave six lines with homologous "raw" antigen. The specific antigens again reacted close to the antigen well. Rabbit strain 63 appeared to possess an additional specific antigen which gave precipitation reactions close to the third line from the antigen well. This component may explain the spur formation at the juncture of this line when 63 "raw" was reacted with anti-47. In addition, it appeared that 47 possessed a component possibly related to the complex of 63 antigens which reacted close to the antigen well when 63 antigen was tested against homologous antiserum.

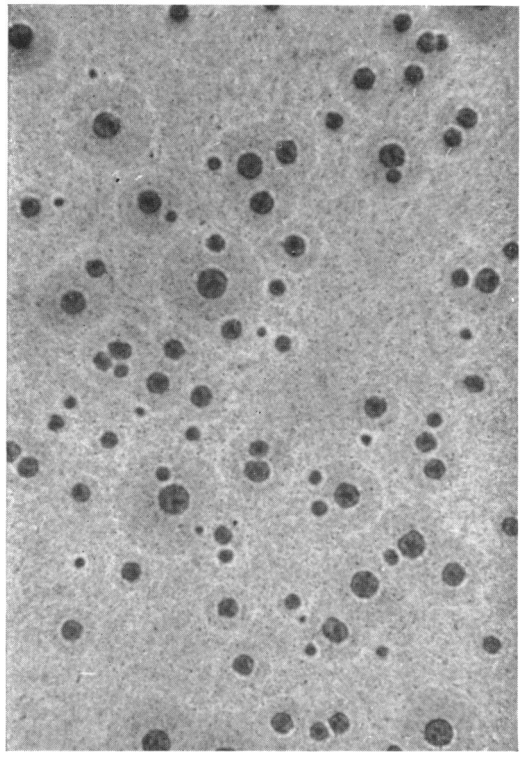

FIG. 2. *Colony morphology of Mycoplasma pulmonis strain 47. The peripheral zone of the classic "fried egg" colony is barely visible by bright-field* (× *125*).

In Fig. 7, the "raw" antigens were in the center; antisera were in peripheral wells. "Raw" 14267 made two lines against anti-63 and anti-47. "Raw" 14267 did make one specific precipitation line with homologous antiserum. "Raw" 47 made four common lines with anti-63 and anti-14267 (two close to the antigen well). In this pattern, anti-47 made more precipitation lines against 14267 than 14267 did against homologous antiserum. It appeared that 47 and 14267 shared a component not possessed by 63 and that 47 possessed one specific antigen which reacted close to the antigen well. "Raw" 63 made three "lines of identity" with homologous antiserum and anti-47. Two of these may have been shared by 14267. A component common to the three strains reacted around the antigen well, but 63 did appear to have a specific antigenic component reactive in this area also.

In summary, it appeared that: (i) antigen and antiserum potency was greatest for strain 47, and least for 14267; (ii) strains 14267, 47, and 63 shared two or more precipitating antigens; and (iii) each of the three strains possessed one or more specific precipitating antigens.

Further serological studies with the antigens of *M. pulmonis* variants are reported in the following paper.

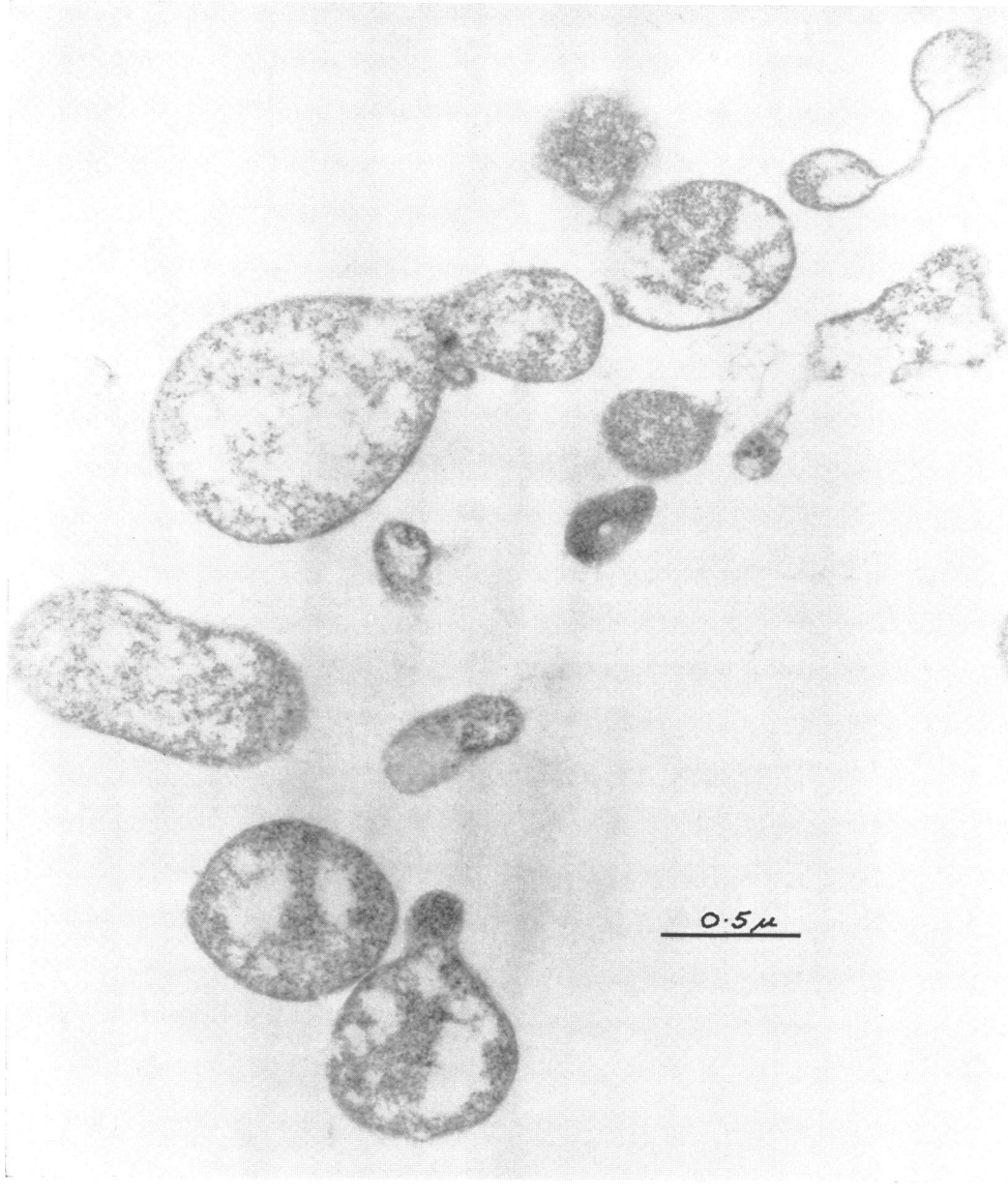

FIG. 3. *Ultrastructure of Mycoplasma pulmonis strain 47. Organisms show triple-layered cell membrane, clear areas suggestive of nuclear areas, and ribosome-like granules in the cytoplasm. Budding and filamentous forms are represented.* × *40,000.*

DISCUSSION

Since the growth-inhibition test appears to be the most valuable test for *Mycoplasma* species identification (i.e., it must measure antibody responses to antigen(s) common to a group of related, but not necessarily identical organisms), the fact that antisera for *M. pulmonis* ATCC 14267 and the two rabbit *Mycoplasma* share neutralizing antibodies is sufficient to place them in the same species. The gel-diffusion results and comparison of biological properties with those described by Freundt (4) and Tully (13) for *M.*

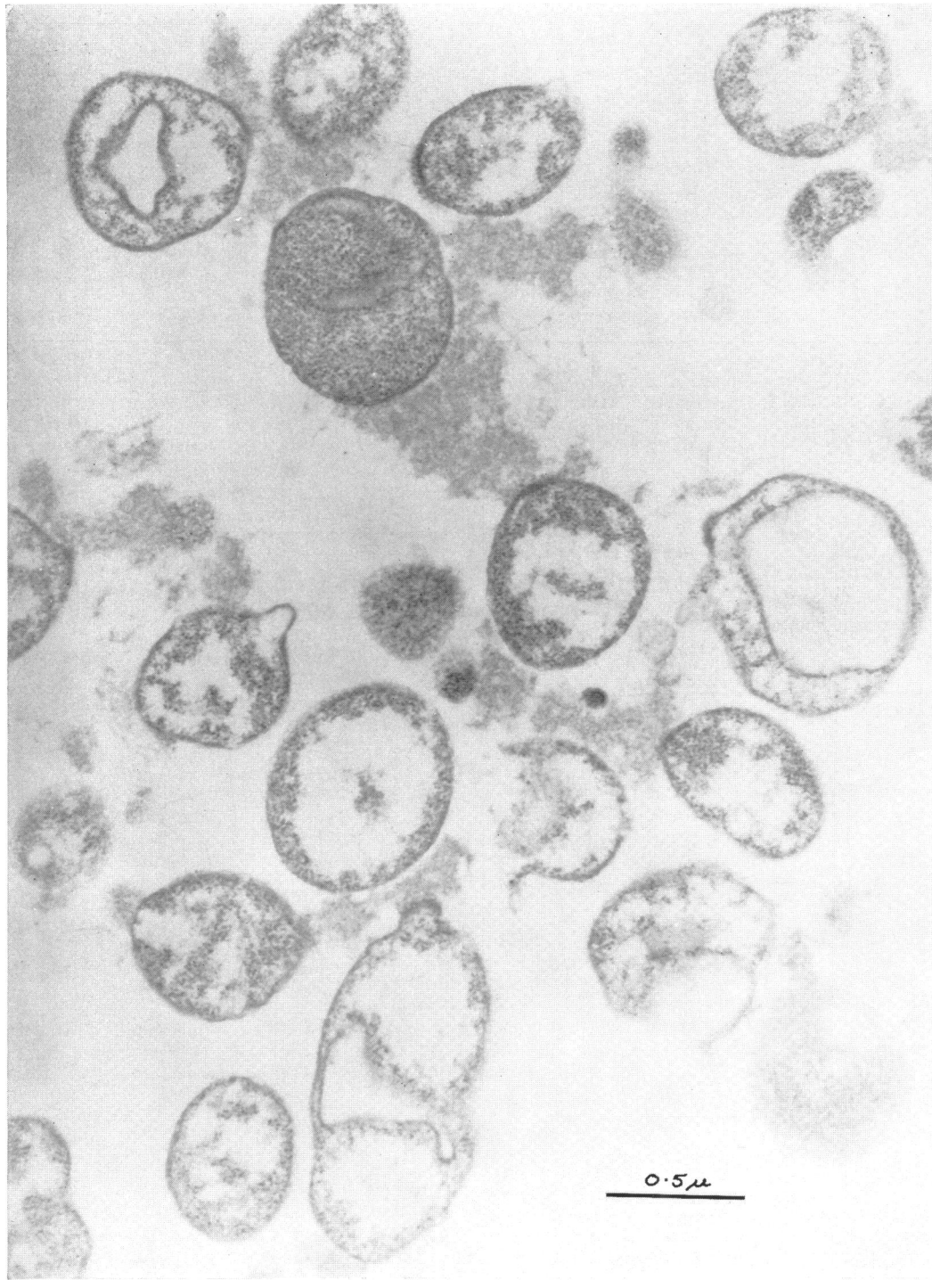

FIG. 4. *Ultrastructure of Mycoplasma pulmonis strain 47. In this plane of the section, several forms show membrane-lined "vacuoles". Budding forms are also present* (× 40,000). (*The morphology of strain 63 was identical to that of 47.*)

TABLE 1. *Some cultural and biological characteristics of Mycoplasma pulmonis strains*

Strain	Colony appearance with stereoscope (on agar medium with horse serum)	Growth in		Acid production in broth with glucose	Hemolysis of RBC			Methylene blue reduction	Tetrazolium reduction	Adsorption of GP[a] RBC
		O_2	N_2, CO_2		GP[a]	Sheep	Horse			
Ash (PG34) (L5) suggested prototype[b]	Transparent, typical indented centers	+	+	+	β	β	α	+	?	?
880 and Negroni[c]	Black, granular, radiations			+	?	+	+	?	?	?
Rabbit 63	Black, granular, radiations	+	+	+	β	β	β	?	+	−
Rabbit 47	Transparent, typical indented centers	+	+	+	β	β	β	?	+	−

[a] GP = guinea pig.

[b] Prototype strain suggested by Tully (13) and found serologically related to strains: Kon, M1, M50 (PG-22), Nelson A. Data from reference 13.

[c] Two *Mycoplasma* strains isolated via cell cultures and serologically related to Ash. Data from reference 9.

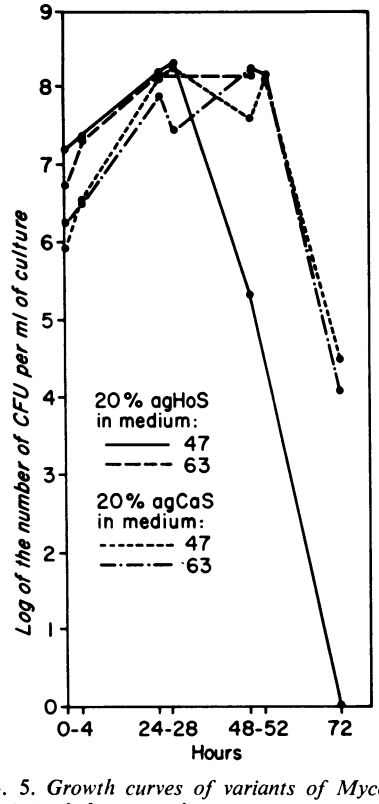

FIG. 5. *Growth curves of variants of Mycoplasma pulmonis in dialysate medium.*

TABLE 2. *Mycoplasma species tested for serological relationship to rabbit strains 63 and 47*

Strain	Obtained from
M. pneumoniae (AP 164)[a]	Seattle isolate
M. fermentans (PG 18)[a]	R. M. Chanock
M. fermentans (K 10)[a]	W. Murphy
M. orale Type II (CH 20247)	R. M. Chanock
M. salivarium	W. Clyde
M. pharyngis (Orale I) (Patt)	W. Clyde
M. hominis Type I (ATCC 14027)	American Type Culture Collection
583[a]	H. Flamm
M. laidlaw A[a]	W. Clyde
M. laidlaw B[a]	W. Clyde
M. gallisepticum (ATCC 15302)[a]	American Type Culture Collection
M. arthritidis (Hominis Type II) (ATCC 14152)	American Type Culture Collection
M. arthritidis (ATCC 14124)	American Type Culture Collection
M. pulmonis (ATCC 14267)[a]	American Type Culture Collection

[a] Utilize glucose.

pulmonis strains help to substantiate the above conclusion.

It would now be of value to compare serologically other known murine strains of *Mycoplasma* with those from rabbits. It would also be interesting to see how closely related the rabbit strains are to the Negroni and 880 strains of *M. pulmonis* isolated from leukemia cell cultures (9), since the reported colony morphology of these organisms is strikingly similar to the morphology of colonies of 63. In the following paper, we will

TABLE 3. *Serological relationship of rabbit Mycoplasma to M. pulmonis (ATCC 14267)*

Antigen	Antisera					
	14267		47		63	
	Growth inhibition[a]	Precipitation lines[b]	Growth inhibition	Precipitation lines	Growth inhibition	Precipitation lines
M. pulmonis (ATCC 14267).....	+	3–4	+	2–4	+	2
Rabbit 47.....	+	3–5	+	6	+	4–5
Rabbit 63.....	+	3–3	+	4	+	5–6

[a] Growth inhibition zones all over 2 mm.
[b] Immuno-Plate Patterns A and E.

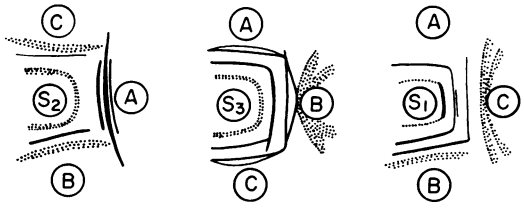

FIG. 6. *Diagrammatic representation of precipitin lines obtained when immune sera (center wells) were tested against "raw" antigens prepared from three strains of Mycoplasma pulmonis. S_1, antisera to 63 strain; S_2, antisera to 14267 strain; S_3, antisera to 47 strain; A, 14267 antigen; B, 47 antigen; C, 63 antigen.*

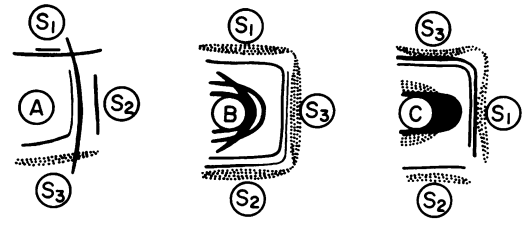

FIG. 7. *Diagrammatic representation of precipitin lines obtained when "raw" antigens (center wells) prepared from three strains of Mycoplasma pulmonis were tested against immune sera. S_1, antisera to 63 strain; S_2, antisera to 14267 strain; S_3, antisera to 47 strain; A, 14267 antigen; B, 47 antigen; C, 63 antigen.*

present a subtyping scheme used for the rabbit *Mycoplasma*, which may well prove to be a great aid in the serological classification of all *M. pulmonis* strains.

Since *M. pulmonis* has long been linked with a chronic respiratory disease in rats and mice, it would surely be of value to learn the relation, if any, to a similar troublesome disease in rabbits, commonly called "snuffles." The fact that *M. pulmonis* could be isolated repeatedly from the same rabbits would indicate that rabbits can at least harbor *M. pulmonis*.

The source of the *Mycoplasma* is unknown. It is possible that the rabbits may have been infected by aerosol or from contact with rats or mice in the vivarium where they were stored or at the rabbit farm where they were raised.

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