

Physical Properties of Human *Mycoplasma* Species

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Received for publication 11 March 1966

ABSTRACT

KIM, KWANG S. (University of North Carolina, Chapel Hill), WALLACE A. CLYDE, JR., AND FLOYD W. DENNY. Physical properties of human mycoplasma species. *J. Bacteriol.* 92:214-219. 1966.—Studies were made of the comparative morphology and stability of five *Mycoplasma* species of human origin (*M. hominis* type 1, *M. salivarium*, *M. fermentans*, *M. pneumoniae*, *M. pharyngis*). Broth-cultivated organisms were examined by electron microscopy to determine their relative appearance after uniform processing, including fixation-drying with formaldehyde vapor. *M. pneumoniae* was characterized by the occurrence of 250- to 300- μ spheres in clusters, and *M. pharyngis* by the appearance of filaments 120 μ by 1.5 to 8 μ ; the remaining species revealed a variety of structures, including spheres, rings, and short filaments. To complement these findings, the effect of physical stresses on viability of the mycoplasmas was measured by exposing the organisms to heat (in saline), osmotic variations (in sucrose), and sonic oscillation and repetitive freeze-thawing (in culture medium). *M. pneumoniae* was most resistant to heat, vibration, and freeze-thawing; *M. pharyngis* was most sensitive to heat and vibration, but was least affected by osmotic changes. The remaining organisms assumed intermediate positions. The type-related variations in relative morphology and stability suggest differing physical attributes of the mycoplasmas studied, supporting taxonomic differentiation of the five species based on metabolic and immunological criteria.

The metabolic and immunological characteristics of diverse *Mycoplasma* species have been studied by numerous investigators, and are compiled in *Bergey's Manual* for those species classified through 1957. Less attention has been given to physical properties of organisms in the genus *Mycoplasma*; data concerning morphology, the characteristic studied in greatest detail, have been conflicting and difficult to collate (3, 7, 9). In this study, two newly identified mycoplasmas from man (*M. pneumoniae* and *M. pharyngis*) were employed, along with three types previously classified (*M. hominis* type 1, *M. salivarium*, and *M. fermentans*), in an evaluation of the relative morphology of the species. Additionally, comparison of the effect of physical stresses on viability of the organisms has been made to seek characteristics which might supplement metabolic-immunological differentiation of the five species. The findings support the current taxonomic separation of these five human mycoplasmas into distinct species, provide stability data relevant to the conduct of laboratory studies of the organisms, and suggest criteria which may be useful in the classification of other mycoplasmas.

MATERIALS AND METHODS

Medium. The formula used for growth of *M. pneumoniae* by Chanock, Hayflick, and Barile (2), as described by Hayflick (8), was employed throughout these studies. The medium consisted of Difco PPLO broth or agar base, 20% unheated horse serum, and 10% aqueous extract of bakers' yeast (6), with 1,000 units of penicillin G per ml.

***Mycoplasma* strains.** Prototype strains of five *Mycoplasma* species which have been isolated from man were employed: *M. salivarium* (strain PG-20, Edward) and *M. fermentans* (strain PG-18, Edward) were obtained from R. M. Chanock; *M. hominis* type 1 was supplied by the American Type Culture Collection (no. 14-027); *M. pneumoniae* (strain Mac, Eaton) was originally received from C. Liu; *M. pharyngis* (W. A. Clyde, Jr., Federation Proc. 23:579, 1964), strain Patt (4; American Type Culture Collection no. 15544), was originally isolated in this laboratory. [This *Mycoplasma* species is microaerophilic, non-hemolytic, nonglycolytic, and a common inhabitant of the normal human oropharynx (4, 5). The name *Mycoplasma pharyngis* was proposed by us at the suggestion of E. A. Freundt. *Mycoplasma orale* is the name independently proposed by Taylor-Robinson et al. (15) for a similar organism. Studies of these isolates in our laboratory and that of Taylor-Robinson indicate that the strains are serologically indis-

tinguishable (16; Clyde, *unpublished data*). The earliest known isolation of this species serotype, which has been identified in our laboratory (strain 823), was made from HeLa cell cultures in 1961 by Hayflick (8; *personal communication*).] An additional strain of *M. hominis* type 1 (DC-63, a respiratory-tract isolate) was supplied by M. A. Mufson, and strain CH 19299 of *M. orale* (*M. pharyngis*) from R. M. Chanock was also studied. As these two strains provided results identical to those described for the species prototypes, no differentiation has been made in the data reported below.

Determination of viability. Culture samples to be tested were diluted in 1-log increments with broth medium. From each dilution, three 0.01-ml drops were placed on slightly dried agar plates which were then incubated at 37 C in humidified air with 5% CO₂ for 3 to 7 days to permit colony development. The 0.01-ml drops spread to a circle approximately 1.5 cm in diameter, which filled the field at 14 times magnification of a colony microscope fitted with an ocular grid. The average number of colonies from the three determinations for each specimen was then corrected by the dilution and sampling factors to provide the number of colony-forming units (CFU) per milliliter. As determined from 20 replicate tests with *M. pneumoniae*, this method had a standard deviation of 20 colonies when counts fell in the range 10 to 100 per 0.01 ml. With the consideration of 2 standard deviations from the mean, a random variation exceeding a half-log would occur only once in 20 tests.

Electron microscopy. Broth cultures to be examined were centrifuged at 15,000 × *g* for 30 min at 4 C, and the buttons were suspended in phosphate-buffered saline (0.01 M, pH 7.2) to produce a 5- to 10-fold concentration of the initial culture volume. Drops of these suspensions were placed on blocks of 2% nutrient agar which were exposed to 40% formaldehyde vapor for 8 to 14 hr at 4 C to provide fixation-drying. The blocks were coated with 0.75% collodion in amyl acetate, and the films were transferred to grids. After being shadowed with chromium at a 15° angle, the specimens were examined and photographed in an Akashi Transcope TRS-501.

RESULTS

Morphological observations. The morphology of the *Mycoplasmataceae* has been a controversial subject, conflicting observations by different workers apparently having occurred through the influence of technical variations (9). In the present experiments, five *Mycoplasma* species were examined to seek *relative* differences which might exist among organisms. The specimens employed were all collected from log-phase broth cultures, and processing for electron microscopy was performed with the use of identical conditions for all species.

Representative electron photomicrographs from three to five studies of each strain are reproduced in Fig. 1-5. A variety of forms were encountered which have been seen by others,

including spherical bodies, rings, rods, and irregular filaments. *M. hominis* type 1 (Fig. 1) revealed rounded bodies measuring 600 mμ in diameter, and rodlike forms 180 mμ thick by 0.8 to 2 μ in length. Elements from the *M. salivarium* cultures (Fig. 2) were somewhat smaller, with spheres averaging 300 mμ in diameter and filaments 0.6 to 1 μ long. *M. fermentans* (Fig. 3) was distinguished by the occurrence of twisted, augulated filaments up to 1.5 μ in length with beadlike structures about 120 mμ across; spherical bodies 600 mμ in diameter were also seen.

M. pneumoniae sediments were more difficult to visualize because of the clumping which occurs with cultivation of this species in liquid media. The single spherical elements contained in clusters of this organism were 250 to 300 mμ in diameter, as shown in Fig. 4. Notably, rodlike or filamentous shapes found in samples of the other species were not seen in any of the *M. pneumoniae* preparations examined with the techniques described. *M. pharyngis* also differed from the other species by consistently demonstrating long branching filamentous forms and stellate structures (Fig. 5). These filaments averaged 1.5 to 4 μ long, occasionally reaching a length of 8 μ, and were about 120 mμ wide.

The centrifugation, formaldehyde vapor fixation, and drying of the samples studied probably produced some distortion of the organisms' actual morphology. Since the same forces were applied equally to all species, however, the consistent differences encountered suggest that differences in physical characteristics exist among the organisms. Further definition of the physical properties of the five *Mycoplasma* species under consideration was sought by examination of the relative stability of the organisms to thermal, mechanical, and osmotic stresses.

Thermal stability. The effect of thermal factors on the survival of the organisms was examined by defining the speed with which the mycoplasmas were inactivated under conditions in which growth would not occur. Log-phase broth cultures were centrifuged at 30,000 × *g* for 30 min at 4 C. The buttons obtained were washed once with iced normal saline, and were then suspended in saline (pH 7.0) to the original culture volume. Portions of the suspensions were placed at 4, 37, or 56 C, and samples were removed for colony counting at intervals ranging from 30 sec to 4 days. Curves of the data were constructed, from which the half-life of each species at each temperature could be estimated. The results are shown in Table 1, and represent average values obtained from three to five replicate experiments with each organism. All strains were rapidly killed by exposure

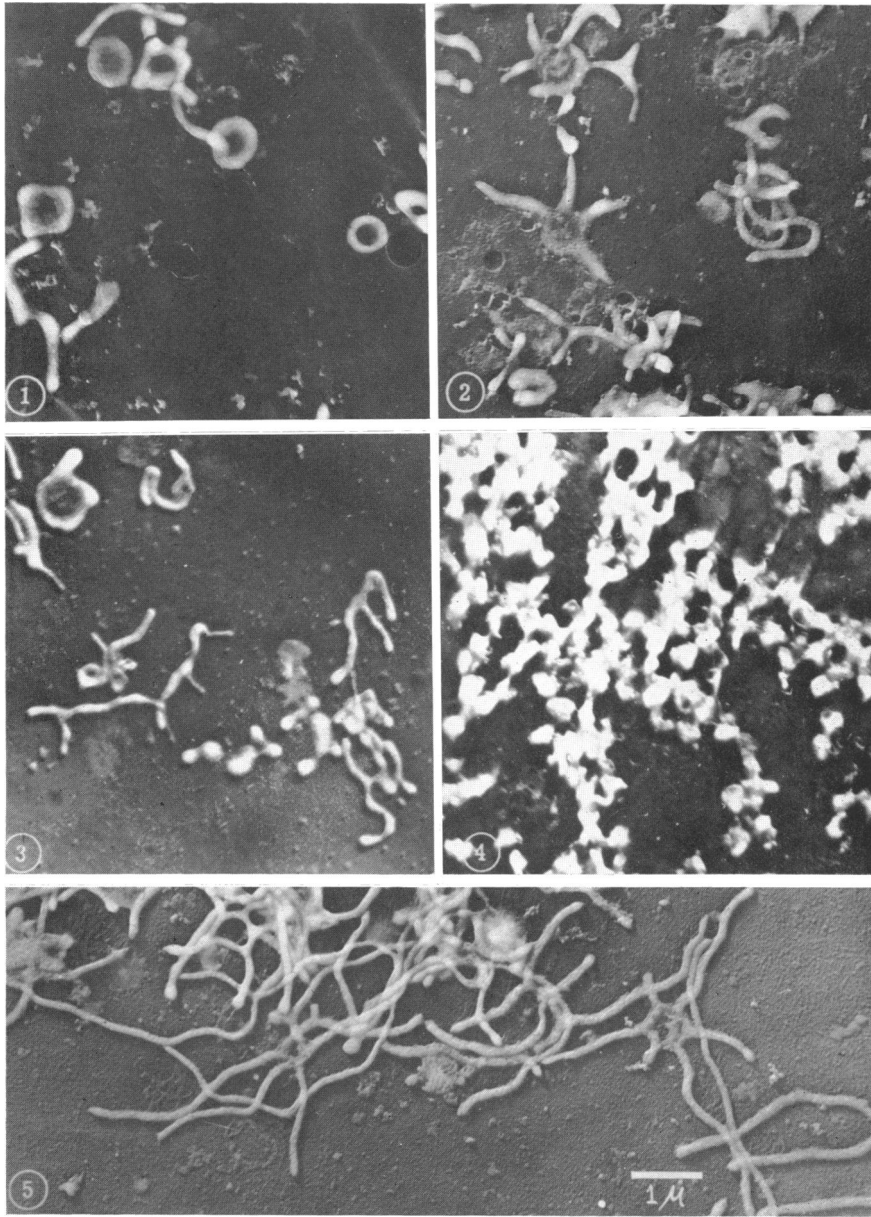


FIG. 1-5. Electron photomicrographs of five human *Mycoplasma* species from log-phase broth cultures after formaldehyde vapor fixation-drying, collodion replication, and chromium shadowing: Fig. 1, *M. hominis* type 1; Fig. 2, *M. salivarium*; Fig. 3, *M. fermentans*; Fig. 4, *M. pneumoniae*; Fig. 5, *M. pharyngis*.

to 56 C, 50% mortality occurring within 32 sec. Differences in thermolability were best appreciated at 4 C, where *M. pharyngis* was inactivated rapidly, *M. Pneumoniae* was most resistant, and the remaining three species had intermediate half-life values of 1.3 to 3.4 hr. Similar but less marked species differences were measured at 37 C. The speed with which saline-suspended

mycoplasmas were inactivated was thus species-dependent and was directly proportional to the temperature employed.

Mechanical stability. The effect of mechanical stress on the five *Mycoplasma* species was examined by measuring survival of the organisms after periods of exposure to sonic vibration and after repetitive freeze-thaw cycles. Log-phase

TABLE 1. Effect of temperature on *Mycoplasma* viability

Species ^a	Mean half-life ^b		
	4 C	37 C	56 C
<i>M. hominis</i> type I	3.4 hr	22 min	32 sec
<i>M. fermentans</i>	1.3 hr	15 min	8 sec
<i>M. salivarium</i>	2.4 hr	11 min	32 sec
<i>M. pneumoniae</i>	37 hr	5 hr	8 sec
<i>M. pharyngis</i>	26 min	9 min	9 sec

^a Organisms suspended in 0.85% NaCl, pH 7.0.

^b Extrapolated from curves depicting results of three to five replicate experiments with each species.

broth cultures of the organisms were exposed to 9-kc vibrations in a Raytheon sonic oscillator operated at maximal plate voltage, the chamber being maintained at 4 C during the procedure. Samples were removed at 10-min intervals for 30 min, and colony counts were determined in reference to untreated portions of the cultures held at 4 C. Three replicate experiments were performed; the results of a representative test are depicted in Fig. 6. As in the thermal stress experiments, *M. pharyngis* was most sensitive to sonic vibration, having an extrapolated half-life of 2.4 min, compared with values of 4.5 min for *M. fermentans* and approximately 6 min for the remaining species. *M. pneumoniae* differed from the other *Mycoplasma* species by demonstrating a rising count over the first 10 min of vibration, which shorter-term experiments (not depicted) revealed to be maximal within the first 60 sec. The slight initial rise in *M. hominis* counts was within the experimental error, and was not reproducible.

Stability differences among the species were also sought by employing repetitive cycles of freezing and thawing. Broth cultures were quick-frozen in 95% ethyl alcohol at -65 C and thawed rapidly in running tap water. Colony counts were performed after 1 through 5, 10, 15, and 20 cycles, providing the data shown in Table 2. The rate of inactivation was similar for all species, a decline of 90 to 99% in viability occurring by the twentieth freeze-thaw cycle; however, 10⁵ to 10⁶ CFU/ml, depending upon the initial colony counts, remained in the samples after 20 cycles.

Osmotic stability. Solutions of varying osmotic value were prepared by dissolving reagent-grade NaCl or sucrose in glass-distilled water. Log-phase broth cultures of the organisms were centrifuged 15,000 × g for 30 min at 4 C, and the pellets were suspended to the original volume in one each of the following: glass-distilled water, 0.85% NaCl, and 0.25, 0.5, 1, 2, 3, 4, and 5 molal

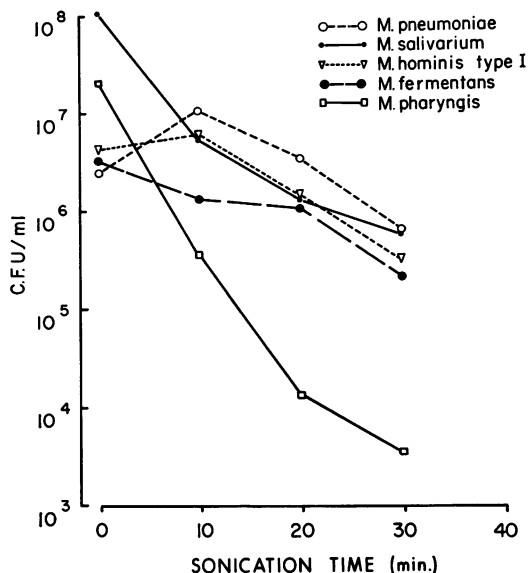


FIG. 6. Effect of sonic vibration on *Mycoplasma* viability. Portions of log-phase cultures were exposed to 9 kc, and samples were removed for viability counts at the intervals indicated. From these curves, the half-life of each species was estimated (see text).

TABLE 2. Effect of repetitive freeze-thawing on *Mycoplasma* viability

Freeze-thaw cycles ^a	Viability counts (CFU/ml, log ₁₀)				
	<i>M. hominis</i> I	<i>M. fermentans</i>	<i>M. salivarium</i>	<i>M. pneumoniae</i>	<i>M. pharyngis</i>
0	6.5	7.9	8.2	7.2	8.2
1	6.4	7.7	8.2	6.9	8.0
2	6.2	7.5	8.0	7.0	7.8
3	6.0	7.2	8.0	6.9	7.7
4	5.8	7.6	7.8	7.1	7.8
5	5.9	7.2	7.8	7.0	7.6
10	5.2	6.7	6.9	6.6	7.3
15	5.0	6.6	6.1	6.1	7.1
20	4.6	6.7	6.0	6.0	6.8

^a Freezing at -65 C, thawing in tap water = 1 cycle.

sucrose. Samples of each suspension for colony counting were removed at once and after 2 hr of incubation at 37 C. The net decrease in colony count for each species in each vehicle is shown in Fig. 7. Generally, there was no effect on viability produced immediately: colony counts for a given species at zero-time in all vehicles were within the experimental error of the method. After 2 hr, varying degrees of reduction in viability had occurred, depending upon the species and the vehicle. Inactivation of all species was maximal in distilled

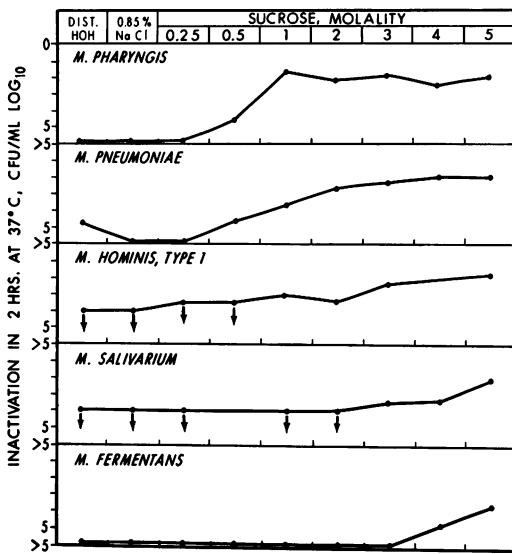


FIG. 7. Effect of osmotic variations on *Mycoplasma* viability. Organisms from log-phase cultures were sedimented and suspended in the indicated vehicles. Viability counts were performed immediately and after 2 hr of incubation at 37 C; the points represent values measurable by the technique employed.

water, 0.85% saline, and 0.25 molal sucrose, consisting of a net decrease in colony counts of 10^5 or more CFU/ml. Increased survival was noted with increasing sucrose molality, best preservation of the organisms occurring under the most hypertonic conditions. *M. pharyngis* was stabilized as well by 1 molal as by 5 molal sucrose. *M. fermentans* proved the most fragile of the species in these experiments, and was poorly preserved even in 5 molal sucrose. It should be noted that a decline of 99% in viability occurred in most species after 2 hr, even in 5 molal sucrose; this degree of loss was equivalent to that produced by incubation of the organisms at 37 C under conditions in which growth could not occur, as shown above.

DISCUSSION

The results described indicate that there were differences in the relative morphology and reaction to physical stresses of five *Mycoplasma* species from man. *M. pneumoniae*, which demonstrated aggregates of spherical bodies in broth culture sediments, was found to be the most resistant of the species to thermal and mechanical stresses; *M. pharyngis* which was characterized by a delicate filamentous morphology, was the most sensitive of the species to the same forces. These two *Mycoplasma* species represented the extremes, and *M. hominis* type 1, *M. salivarium*, and *M. fermentans* assumed an intermediate position with less

notable differences among the three types. It should be emphasized that the experiments reported were designed to seek relative differences in physical properties of the mycoplasmas, and that they do not define the basis of the variations which were measured.

Since the *Mycoplasmataceae* are assumed to be plastic structures, virtually any techniques used in preparing specimens for electron microscopy could produce marked morphological changes. The appearance of the organisms described in the current experiments is not intended as a definition of actual morphology, therefore, but as an indication that the species behave differently when exposed to the same technical manipulations. Visualization of mycoplasmas by conventional light microscopy is difficult because of the organisms' minute size; however, several workers have reported observations on viable broth cultures by use of phase-contrast optical systems. Freundt (7) described mycelial characteristics of various species, and suggested that the types could be divided into three groups on the basis of filament length. Examples of similar differences are provided by the present studies. The filamentous morphology of *Mycoplasma* has recently been confirmed by Razin and Cosenza (12), whose phase-contrast photomicrographs reveal many similarities to the organisms depicted in Fig. 1-5.

The work of various investigators on thermal stability of diverse mycoplasmas suggests that species-related differences exist. These studies are difficult to compare or relate to the present results because of technical variations summarized by Freundt (7), including culture age, growth phase, medium composition, and pH effects. Control of these factors for comparative purposes revealed the marked stability differences among the five human species shown in Table 1.

The increased survival of the organisms produced by suspension in hypertonic sucrose solutions occurred in a pattern which differed from that determined for thermal or mechanical stability. Thus, *M. pharyngis*, which was relatively thermolabile, was preserved as well in 1 molal as in 5 molal sucrose, whereas *M. fermentans*, which had intermediate thermolability, was poorly preserved even in the 5 molal solution. Smith and Sasaki (13) previously reported increased survival of an unclassified human *Mycoplasma* and a poultry isolate in hypertonic suspension. These authors speculated that preservation occurred by dehydration of the organisms, or that toxic factors were inhibited under these conditions. If dehydration is the explanation, the current experiments suggest a species differential in the facility with which this occurs, possibly on the basis of varying membrane permeability. In contrast, the five

species examined were similar in regard to inactivation produced by exposure to isotonic or hypotonic conditions. The experimental conditions used may have been inadequate to demonstrate differences in this case, as species-related variations in hypotonic lysis are indicated for the mycoplasma tested by Butler and Knight (1) and Razin (11).

The *Mycoplasma* species studied can be distinguished by certain of their biological properties (5; *Bergey's Manual*): *M. fermentans* and *M. pneumoniae* are both glycolytic; *M. pneumoniae* hemolyzes mammalian erythrocytes through production of a peroxide (14); *M. pharyngis*, *M. salivarium*, and *M. fermentans* grow poorly under aerobic conditions. These species also have distinct immunological properties (4, 10, 15, 16). The present report provides evidence of differential physical properties, which supplement and extend characterization of the organisms on the basis of metabolic and immunological features. The observations support the current classification of the seven human *Mycoplasma* strains studied into five distinct species. Additionally, the stability data provide technical implications concerning studies on the human *Mycoplasma* in the laboratory.

ACKNOWLEDGMENTS

This investigation was conducted under sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, and was supported by the U.S. Army Medical Research and Development Command, Department of the Army. K. S. Kim was an Infectious Diseases Trainee under Public Health Service training grant AI-45-04 from the National Institute of Allergy and Infectious Diseases. W. A. Clyde, Jr., was a Career Development Awardee under Public Health Service grant K3-AI-9676 from the National Institute of Allergy and Infectious Diseases.

The assistance of W. R. Bibb with the electron photomicrography is gratefully acknowledged.

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