Mycoplasma spermatophilum, a New Species Isolated from Human Spermatozoa and Cervix

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A mycoplasma isolated from human spermatozoa and a human cervix was shown to be serologically distinct from 98 previously recognized Mycoplasma and Acholeplasma spp. Six mycoplasma colonies were cloned and examined in detail for morphology, growth, and biochemical characteristics; five of these were from sperm samples and one was from a cervix. These strains were closely related and had the following properties: guanine-plus-cytosine content of 32 mol%, requirement for sterol, and anaerobic growth. Glucose was not metabolized, and arginine and urea were not hydrolyzed. Strain AH159 (=NCTC 11720) is the type strain of a new species, Mycoplasma spermatophilum.

Twelve named Mycoplasma and Acholeplasma species have been isolated from the respiratory or genital tracts of humans (6). Mycoplasma buccale, Mycoplasma faucaum, Mycoplasma lipophilum, Mycoplasma orale, Mycoplasma pneumoniae, and Mycoplasma salivarium are found almost exclusively in respiratory tracts. Mycoplasma fermentans has been found infrequently in urogenital tracts, while Mycoplasma primatum, a species commonly present in nonhuman South American primates (15), has on occasion been isolated from humans. Acholeplasma laidlawii, which is also uncommon in humans, has been isolated from many animal species and plants (26).

The more common human genital mycoplasmas are Ureaplasma urealyticum and Mycoplasma hominis, although each of these species has also been found in human respiratory systems. Both of these species have been implicated in certain diseases (e.g., nongonococcal urethritis and pelvic inflammatory disease) (30). The association of ureaplasmas with some other diseases has not been proved as controls are often similarly infected. It is possible that serotyping of ureaplasmas may clarify this position.

Recently, a fastidious mycoplasma was isolated from human genital tracts by using SP4 medium (37). This mycoplasma, Mycoplasma genitalium, was recovered from patients with nongonococcal urethritis (36), but the respiratory tract is probably the primary host site of colonization (3).

Strains 9, 47, 50, 58, AH159T (T = type strain), and Monks were isolated from the urogenital tracts of patients with infertility problems and could not be identified as any of the previously described human species. In this paper I describe the characteristics of these organisms and propose that they are members of a new Mycoplasma species.

MATERIALS AND METHODS

Mycoplasma strains. Mycoplasmas were isolated from the spermatozoa of five patients attending an in vitro fertilization clinic (16) and from the cervix of one woman attending a clinic for infertility (strain Monks). The cervix isolate was obtained from a cervical swab rubbed over the surface of an agar culture medium. Semen and washed sperm were inoculated onto the agar medium and SP4 agar and into glucose, arginine, and urea broth media and SP4 liquid medium (13, 32, 37). Most of the inoculated liquid media were diluted 10-fold; the one exception was urea medium, which was diluted 10-fold to 100. The amount of inoculum depended on the material available from the in vitro fertilization clinic. One colony isolated from each patient was cloned to produce a pure culture; this was done by initially filtering a broth culture through a 220-nm-pore-size membrane filter, culturing the filtrate on solid medium, transferring a single result, and cloning five times (29). The clones were designated strains 9, 47, 50, 58, AH159T, and Monks.


suavi Mayfield (clone B), M. subdolum TB, M. synoviae WVV1853, M. testudinis 01008, and M. verecundum 107.

The following Acholeplasma spp. type strains were used: A. axanthum S743, A. equifetale N93, A. florum L1, A. granularum BTS39, A. hikikou C1, A. laidlawii PG8, A. medicum PG49, A. morum 72-043, A. oculi 19L, and A. parvum H23M.

**Medium and growth conditions.** The culture medium used has been described previously (13, 32, 37). The purified agar used in the growth medium was either Ionagar no. 2 (Oxoid Ltd., Basingstoke, England) or Lab M agar (Lab M, London, England). Depending on their biochemical activities, the mycoplasmas were grown in liquid medium containing 1% (wt/vol) glucose (pH 7.8) or in liquid medium containing 0.5 to 1% (wt/vol) arginine (pH 7.3). The species grown in medium containing arginine were not inhibited by 1% arginine. Agar cultures were incubated at 35 to 37°C either in a humid chamber or under anaerobic conditions in a GasPak system. Liquid cultures were stored at ~70°C in ampoules.

**Growth requirements and characteristics.** The clones were subcultured onto solid medium and incubated under both aerobic and GasPak anaerobic conditions at 35 to 37°C. The levels of susceptibility to methylene blue were investigated by adding 0.002% (wt/vol) methylene blue to the basal solid medium and comparing the growth of inoculated mycoplasmas with the growth of mycoplasmas cultivated on agar medium without methylene blue. Lipolytic activity was tested by inoculating mycoplasmas onto basal medium enriched with 10% egg yolk emulsion (9). The incubated plates were examined after 3, 7, and 14 days for visual evidence of lipoysis (clearing) or film production.

**Absence of reversion.** The clones were subcultured by five passages in both solid and liquid media that contained no microbial inhibitors in order to determine whether the organisms reverted to bacterial forms. Agar culture colonies of each clone were also treated with Dienes stain in order to differentiate mycoplasma colonies from bacterial L forms (33).

**Morphological studies.** Mycoplasma colonies grown on agar were examined microscopically at a magnification of ×100 after 4, 7, and 14 days of incubation. The colonies were transferred to slides and stained with Giemsa stain (17, 18, 19). Liquid cultures were observed by dark-field microscopy, and organisms were stained with Giemsa stain (17). Organisms grown in broth were harvested by centrifugation, and the cell pellet was fixed in 2% (vol/vol) glutaraldehyde and postfixed in 1% (wt/vol) osmium tetroxide for 1 h. Thin sections of these preparations were stained with uranyl acetate and lead citrate and examined by electron microscopy (38).

**Filtration studies.** Cultures (after 24 h of incubation) were diluted 1:10 in liquid medium, and the resulting preparations were filtered and refiltered through a series of membrane filters (Millipore Corp., Bedford, Mass.) with pore diameters of 220, 300, 450, 650, and 800 nm. The number of CFUs per ml in each filtrate was compared with the number in the unfiltered culture dilution.

**Sterol dependence.** Single colonies of strains AH159T, 47, and Monks were seeded onto serum-free solid media supplemented with 0.5% bovine serum albumin, 0.5% glucose, and 10 μg of palmitic acid per ml. Cholesterol, dissolved in Tween 80, was added to give final concentrations of 20, 10, 5, and 1 μg/ml. Plates containing no cholesterol were included (7, 8, 25, 35). The mycoplasmas were also subcultured onto the basal agar medium without serum. When growth occurred on any of the serum-free media, single colonies were passaged three times on similar plates to ensure that growth was not due to a carry-over of serum from the original culture, giving misleading results.

The clones were also tested indirectly for sterol dependence by a paper disk inhibition method (11), using either dried disks that originally contained 0.02 ml of a 1.5% (wt/vol) ethanol solution of digitonin (Sigma Chemical Co., St. Louis, Mo.) or wet disks that contained 0.02 ml of a 20% (wt/vol) aqueous solution of sodium polyethanol sulfonate (Koch-Light Laboratories Ltd., Colnbrook, England) on basal growth medium containing Lab M agar. The widths of zones of growth inhibition were measured in millimeters.

**Biochemical activity.** The six clones were examined for carbohydrate metabolism; for hydrolysis of esculin, arginine (1 and 0.1%), and urea; for reduction of methylene blue, resazurin, tetrazolium, and tellurite; and for phosphatase activity (1, 2, 18, 28, 34, 39).

**Erythrocyte techniques.** The clones were examined for hemolytic activity, hemadsorption, and hemagglutination with guinea pig, human, and sheep erythrocytes (1, 19).

**Polycarylamide gel electrophoresis.** Electrophoresis was carried out as described by Mouches and Bové (21), using a one-dimensional slab. Centrifuged cell suspensions were dissolved in a solution containing 20% glycerol, 5% 2-mercaptoethanol, and 3% sodium dodecyl sulfate in 0.0625 M Tris buffer (pH 6.8). To a 12.5% acrylamide gel 10-, 25-, or 50-μl portions of a cell sample were added. Electrophoresis was performed at room temperature for 1 h at a constant current (20 mA). The gels were stained with Coomassie blue (Sigma).

**DNA base composition.** DNAs were extracted from centrifuged broth culture deposits of strains AH159T, 47, and Monks by using the method of Gross-Bellard et al. (12), and the guanine-plus-cytosine contents were determined from the buoyant densities of the DNAs in cesium chloride by ultracentrifugation (27). DNA extracted from Escherichia coli with a known guanine-plus-cytosine content was included as a control.

**Serological studies.** Antisera were prepared as described by Morton and Roberts (20) and Hill (13). The following three serological methods were used: growth inhibition tests with antiserum-impregnated sterile paper disks (5), metabolism inhibition tests in microtiter plates (14, 23, 24, 31), and immunoperoxidase tests with colonies grown on agar (22). All of the tests were carried out in duplicate. Type strain AH159 and strains 47, 50, and Monks were reacted with antisera prepared against the species listed above. Antisera to the four clones were tested with the previously described Mycoplasma and Acholeplasma species. Antisera to the four clones were reacted with each other to demonstrate their relationships. The strains (antigens) could not be tested with antisera in the metabolism inhibition test because of their lack of biochemical activity.

**RESULTS AND DISCUSSION**

All six strains were visible as very small colonies on primary isolation after 2 to 3 weeks of incubation on agar medium (13, 32) and (for strains 47, 47, and 58) on SP4 medium under anaerobic conditions. The number of colonies was small (5 to 20 colonies). Five strains were isolated from washed sperm samples, but in only two cases were the strains recovered from the semen as well. After several passages the strains grew much more rapidly, but no growth was visible aerobically even after prolonged incubation or after many passages on mycoplasma culture medium. The
colonies (Fig. 1) initially showed little center, but the typical fried egg appearance became more obvious after several passages. Although initially it was difficult to obtain good growth in liquid medium, the titers improved as the organisms became adapted to the medium. No growth was observed on methylene blue agar or medium without serum. No film was produced on egg yolk agar. None of the strains reverted to a bacterial form when the organisms were subcultured on medium without bacterial inhibitors. The colonies rapidly stained with Dienes reagent, confirming that the isolates were true mycoplasmas (members of the Mollicutes) rather than bacterial L forms (33).

No motility was observed when the strains were examined by dark-field microscopy, and helical forms were not observed. Liquid cultures stained with Giemsa stain contained pleomorphic forms characteristic of mycoplasmas. The ultrastructure of three isolates (strains AH159T, 47, and Monks) was typical of mycoplasma morphology when thin sections were viewed by electron microscopy (4). The cells were bounded by a single trilaminar unit membrane and lacked any evidence of cell wall material (Fig. 2).

Filtration of a broth culture of strain AH159T showed that reduced numbers of the $1.4 \times 10^9$ CFU/ml in the original dilution passed through the 300-nm-pore-size membrane ($8 \times 10^7$ CFU/ml) and 220-nm-pore-size membrane ($5 \times 10^6$ CFU/ml).

Strains AH159T, 47, and Monks required cholesterol for growth. They could not be cultured on medium without cholesterol, but they grew and were passaged on medium containing 10 µg of cholesterol per ml. They were susceptible to digitonin and sodium polyanethanol sulfonate, with zones of growth inhibition of 8 and 6 mm, respectively; under these conditions growth was semiconfluent.

The biochemical activities of the six test strains were identical and are summarized in the species description below. In appropriate tests all six strains were hemolytic for guinea pig, human, and sheep erythrocytes but did not hemadsorb or hemagglutinate these cells.

The guanine-plus-cytosine content of this species was determined from its buoyant density in cesium chloride to be 32 mol%. This value is within the range of values for the previously characterized Mycoplasma species.

The serological techniques used revealed similar levels of cross-reactions among the four strains tested. No significant cross-reactions were detected with any of the species listed in Materials and Methods (Table 1).

The six strains had similar electrophoretic protein patterns, and these patterns were distinct from those of M. felinitatum, which also lacks the ability to metabolize glucose and the ability to hydrolyze arginine.

FIG. 2. Electron micrograph of a section of strain AH159T cells stained with uranyl acetate and lead citrate. Bar = 100 nm.
Strain Monks was sent to R. H. Leach (Colindale, United Kingdom) to confirm my findings that it was distinct from \textit{M. feliminutum} (with similar biochemical reactions and anaerobic-bias). Leach found a positive one-way reaction in the immunoperoxidase test with antiserum to \textit{M. feliminutum} received from H. Ern@ (Aarhus, Denmark). Therefore, this antiserum was absorbed against \textit{M. feliminutum} AH159\textsuperscript{T}. There was no reaction with \textit{M. fermentans}, but the level of the reaction with strain Monks remained at 1,000. Therefore, the positive reaction was due to some factor unconnected with \textit{M. feliminutum}. There was no reaction between strain Monks and fluorescein-conjugated \textit{M. fermentans}, and fluorescein-conjugated \textit{M. feliminuturn}.

It can be difficult to demonstrate glucose fermentation or arginine hydrolysis by \textit{M. fermentans}; however, in addition to these properties, the new strains differ from \textit{M. fermentans} in not reducing methylene blue or tellurite, in not producing a film on agar, and in DNA base composition.

Strain AH159\textsuperscript{T} belongs to the family \textit{Mycoplasmataceae} on the basis of its main properties, including absence of cell walls, lack of reversion to bacterial L forms when the organism is grown in antibiotic-free media, penicillin resistance, filterability, and production of typical morphology on agar. It belongs to the genus \textit{Mycoplasma} because it is not a strict anaerobe (in contrast to \textit{Aeroplasma} spp.), because it is nonhelical (in contrast to \textit{Spiroplasma} spp.), because it depends on sterol for growth (in contrast to \textit{Acholeplasma} spp.), and because urease could not be demonstrated (in contrast to \textit{Ureaplasma} spp.).

The six strains belong to the same species as they have identical biological characteristics and exhibited serological and protein pattern similarities. As there were no significant cross-reactions with any of the previously recognized \textit{Mycoplasma} spp. (listed in Materials and Methods), strain AH159\textsuperscript{T} belongs to a new \textit{Mycoplasma} species.

I propose that this new species be named \textit{Mycoplasma spermatophilum} (sper.ma.to.phil'um. Gr. n. sperma, sperm or seed; Gr. adj. phil\textae, loving; M. L. adj. spermatophilum, sperm loving) and that the type strain of this species is strain AH159.

\textit{M. spermatophilum} must be considered rare in humans since although it was not easy to isolate on primary culture, it was recovered from only 1 to 2\% of the samples examined in two surveys (16; unpublished data). Not enough isolates of this species have been recovered to determine their possible role in disease. It is interesting that either eggs did not become fertilized with infected sperm or fertilized eggs did not implant in in vitro fertilization procedures (16; unpublished data).

The main characteristics of \textit{M. spermatophilum} sp. nov. are described below.

\textbf{Morphological and physical characteristics.} Pleomorphic cells bounded by a single unit membrane. Colonies have a typical fried egg appearance after several passages on agar. Organisms are filterable through 220-nm-pore-size membrane filters and are resistant to penicillin.

\textbf{Growth characteristics.} Requires sterol; inhibited by digitonin and sodium polyanethol sulfonate. Optimal growth occurs at 35 to 37°C under anaerobic conditions.

\textbf{Metabolic characteristics.} No carbohydrates are metabolized; arginine, urea, and esculin are not hydrolyzed. Resazurin is reduced (weakly), but methylene blue, tellurite, and tetrazolium are not reduced. Slight production of phosphate.

\textbf{Serological characteristics.} Serologically distinct from all other \textit{Mycoplasma} species.

\textbf{DNA base composition.} The DNA base composition is 32 mol\% guanine plus cytosine.

\textbf{Habitat.} The habitat is human semen and cervixes.

\textbf{Type strain.} The type strain is strain AH159 (= NCTC 11720).

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\section*{REFERENCES}