Mycoplasma phocidae sp. nov., Isolated from Harbor Seals (*Phoca vitulina* L.)

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In 1979 and 1980, more than 400 harbor seals (*Phoca vitulina*) along the New England coast of the United States died of epizootic pneumonia that was attributed to an influenza virus. Six mycoplasma isolates that were recovered from the respiratory tracts of affected seals were investigated and were found to be serologically identical and distinct from previously described species. These isolates required serum for growth, did not possess a cell wall, and did not hydrolyze urea. Arginine was hydrolyzed, glucose was not fermented, film and spots were observed on horse serum agar, phosphatase was produced, tetrazolium was not reduced, and serum and casein were not digested. The guanine-plus-cytosine content of the DNA was 27.8 mol%. We propose the name *Mycoplasma phocidae* for these isolates. The type strain of *M. phocidae* is strain 105 (= ATCC 33657).

In 1979 and 1980, an epizootic of pneumonia killed at least 445 harbor seals along the New England seaboard of the northeastern United States (2, 8, 10, 14). An influenza virus designated A/Seal/Mass/1/80 was isolated from the lungs of these seals. Specimens from affected seals were submitted to laboratories in both Guelph, Ontario, Canada, and Boston, Mass., and similar *Mycoplasma* strains were isolated from the lungs, tracheae, bronchi, and heart muscles. These isolates did not react with 55 antisera against previously described *Mycoplasma* species in immunofluorescence tests and were therefore believed to represent a new species (14). In this paper we describe the characteristics of the isolates and propose that they should be classified as a new species in the genus *Mycoplasma*, *Mycoplasma phocidae*; the type strain of this species is strain 105 (= ATCC 33657).

MATERIALS AND METHODS

Seals. Between December 1979 and March 1980 more than 400 harbor seals, most of them immature, died along the New England coast of acute pneumonia associated with influenza virus A/Seal/Mass/1/80(H7N7). Lung tissue samples from eight animals that died were submitted for mycoplasma culture in Guelph, and lung, trachea, bronchus, and heart muscle samples from other animals that were sacrificed were examined in Boston.

Mycoplasma strains. Strains 84 and 105^T (T = type strain) were isolated in Boston from lung and trachea samples from seals that died, and strains M4359, M4360, M4361, and M4362 were isolated in Guelph from the pneumonic lungs of necropsied seals. The reference strains listed in Table 1 were obtained from J. G. Tully, National Institute of Allergy and Infectious Diseases, Frederick, Md., E. A. Freundt, WHO/FAO International Reference Centre for Animal Mycoplasmas, Aarhus, Denmark, and R. H. Leach, National Collection of Type Cultures, Colindale, United Kingdom. Cultures of and antisera to *Mycoplasma phocacerebrale* 1049^T and *Mycoplasma phocarhinis* 852^T, two new species that were isolated from seals, were kindly supplied by H. Kirchhoff, Hannover, Germany.

Media, cultivation, and purification. Specimens were cul-

tured by using mycoplasma broth and agar (12, 16) containing horse serum or swine serum (at Guelph) and GIBCO horse blood brucella agar (at Boston). Cultures were incubated at 37°C in air containing 7% CO₂ and also anaerobically (Gaspak CO₂ + H₂). For ureaplasma cultures we used mycoplasma broth adjusted to pH 6.0 and Shepard A3 agar containing 0.015% manganese sulfate (17). Ureaplasma agar plates were incubated anaerobically as described above. The isolates were plate cloned four times and then passed through a membrane filter (average pore diameter, 450 nm).

Characterization studies. Morphology was examined by using light microscopy, dark-field microscopy, and electron microscopy. Ultrathin sections of organisms that were grown for 48 h in an agar overlay were prepared for electron microscopy by previously described methods (4). Tests for absence of reversion were performed by subculturing the preparations seven times in broth that did not contain bacterial inhibitors and subculturing at each passage onto blood agar plates after 24 h of incubation. Filterability was tested by using membrane filters with pore diameters of 450 and 220 nm. The sterol requirement was tested by determining susceptibility to 1.5% digitonin and growing the organisms in medium that did not contain serum (7). The guanine-plus-cytosine (G+C) contents of strain 105^T and M4359 DNAs were determined for us by J. Flossdorf, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany (6). Other biochemical and biological properties were tested by using the methods described by Aluotto et al. (1).

Serological tests. Antisera to strains 105^T (Boston) and M4359 (Guelph) were prepared in rabbits and were used in the direct and indirect immunofluorescence tests (5, 15) and in the growth inhibition test (3) against the reference strains listed in Table 1. Antisera for reference strains were prepared in Guelph or were obtained from the National Institute of Allergy and Infectious Diseases, Frederick, Md., the WHO/FAO International Reference Centre for Animal Mycoplasmas, Aarhus, Denmark, or R. Del Giudice.

RESULTS AND DISCUSSION

Strains 84, 105^T, M4359, M4360, M4361, and M4362 grew well on mycoplasma media containing either horse or swine serum or on horse blood agar and produced typical fried egg colonies. Film and spots were observed on horse serum

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TABLE 1. *Mycoplasma* and *Acholeplasma* strains and antisera used in comparative serological tests with the seal mycoplasma

Strain	Source(s) of antiserum ^a
<i>Mycoplasma agalactiae</i> PG2 ^T	Aarhus, NIAID
<i>M. alkalescens</i> D12 ^T	NIAID, VLS
<i>M. alvi</i> Ilsey ^T	NIAID
<i>M. anatis</i> 1340	Aarhus, NIAID
<i>M. anseris</i> 1219 ^T	NIAID
<i>M. arginini</i> G230 ^T	NIAID
<i>M. arthritis</i> PG6 ^T	NIAID
<i>M. bovigenitalium</i> PG11 ^T	NIAID, VLS
<i>M. bovirhinis</i> NCTC 10118	VLS
<i>M. bovis</i> Donetta ^T	NIAID, VLS
<i>M. bovovuli</i> M165/69	NIAID
<i>M. buccale</i> CH20247 ^T	NIAID
<i>M. californicum</i> ST-6 ^T	NIAID
<i>M. canadense</i> 275C ^T	NIAID, VLS
<i>M. canis</i> PG14	NIAID
<i>M. capricolum</i> California Kid ^T	NIAID
<i>M. cloacale</i> 383 ^T	NIAID
<i>M. columbinasale</i> 694 ^T	NIAID
<i>M. columbinum</i> MMP-1 ^T	NIAID
<i>M. conjunctivae</i> HRC581	NIAID
<i>M. cynos</i> H831	NIAID
<i>M. dispar</i> 462/2	NIAID
<i>M. edwardii</i> PG15	NIAID
<i>M. equirhinis</i> M432/72 ^T	NIAID
<i>M. faucium</i> DC333 ^T	NIAID
<i>M. felifaucium</i> PU ^T	NIAID
<i>M. fermentans</i> PG18 ^T	NIAID
<i>M. flocculare</i> Ms42 ^T	NIAID
<i>M. gallinarum</i> PG16 ^T	NIAID, VLS
<i>M. gallisepticum</i> S6	VLS
<i>M. gateae</i> CS ^T	NIAID
<i>M. hominis</i> PG21 ^T	NIAID
<i>M. hyopharyngis</i> H3-6B ^T	NIAID
<i>M. hyopneumoniae</i> J ^T	NIAID
<i>M. hyorhinis</i> Mh31	VLS
<i>M. hyosynoviae</i> S16 ^T	NIAID, VLS
<i>M. iners</i> PG30 ^T	NIAID
<i>M. iowae</i> 695 ^T	NIAID, VLS
<i>M. lipofaciens</i> R171 ^T	NIAID
<i>M. lipophilum</i> MaBy ^T	NIAID
<i>M. maculosum</i> PG15 ^T	NIAID
<i>M. meleagridis</i> 17259 ^T	NIAID, VLS
<i>M. moatsii</i> MK405 ^T	NIAID
<i>M. molar</i> H542	NIAID
<i>M. muris</i> RIII4 ^T	NIAID
<i>M. mycoides</i> subsp. <i>mycoides</i> Y3343	Aarhus
<i>M. neurolyticum</i> Type A	NIAID
<i>M. opalescens</i> MH5408 ^T	Aarhus, NIAID
<i>M. orale</i> CH19299 ^T	NIAID
<i>M. phocacerebrale</i> 1049 ^T	NIAID, HK
<i>M. phocarhinis</i> 852 ^T	NIAID, HK
<i>M. pirum</i> 70-159 ^T	NIAID
<i>M. primum</i> HRC292 ^T	NIAID
<i>M. pulmonis</i> PG34	Aarhus
<i>M. putrefaciens</i> KS-1	Aarhus
<i>M. salivarium</i> PG20 ^T	Aarhus, NIAID
<i>M. spermatophilum</i> AH159 ^T	NIAID
<i>M. spumans</i> PG13 ^T	NIAID
<i>M. suis</i> Mayfield B ^T	NIAID
<i>M. subdolum</i> TB ^T	NIAID
<i>M. verecundum</i> 107 ^T	NIAID
<i>Mycoplasma</i> sp. bovine Leach group 7	
strain NCTC 10133	VLS
<i>Mycoplasma</i> sp. bovine strain California calf	NIAID
<i>Mycoplasma</i> sp. ovine genital strain 3306	NIAID

Continued

TABLE 1—Continued

Strain	Source(s) of antiserum ^a
<i>Mycoplasma</i> sp. primate throat strain HRC291	NIAID
<i>Mycoplasma</i> sp. goat leg abscess strain 145	NIAID
<i>Mycoplasma</i> sp. horse uterus strain M2851	NIAID
<i>Mycoplasma</i> sp. caprine strain GM13	NIAID
<i>Mycoplasma</i> sp. canine strain HRC689	NIAID
<i>Acholeplasma laidlawii</i> NCTC 10116	VLS
<i>A. axanthum</i> NCTC 10138	VLS
<i>A. granularum</i> MG39	VLS
<i>A. oculi</i> 19L	NIAID
<i>A. modicum</i> NCTC 10134	VLS

^a Abbreviations: Aarhus, E. A. Freundt, WHO/FAO International Reference Centre for Animal Mycoplasmas, Aarhus, Denmark; NIAID, J. G. Tully, National Institute of Allergy and Infectious Diseases, Frederick, Md.; NCTC, R. H. Leach, National Collection of Type Cultures, Colindale, England; HK, H. Kirchhoff, Hannover, Germany; VLS, Veterinary Laboratory Services, Guelph, Canada.

agar. Growth appeared to be better on medium containing swine serum, and growth was enhanced by carbon dioxide enrichment or in an anaerobic environment. *Ureaplasma* sp. was not isolated. Morphological examination by phase-contrast microscopy revealed mostly coccoid forms with some pleomorphism and filaments that were 0.1 to 0.4 µm in diameter, but no helical forms were observed. Electron microscopy of ultrathin sections of colonies on agar revealed pleomorphic, round, and coccoid forms that possessed a three-layer membrane and no evidence of a cell wall (Fig. 1).

There was no evidence of reversion to bacterial forms after seven passages in medium that did not contain bacterial inhibitors. The organisms passed freely through filters with pore diameters of 450 nm but were retained by filters with 220-nm pore diameters. All of the strains were susceptible to 1.5% digitonin (zone of inhibition, 8 mm), and there was no growth on medium that lacked serum. The average G+C content of the DNA was 27.8 mol% for strains 105^T and M4359.

The results of other biochemical and biological tests are given in the species description below.

Strains 105^T, M4359, M4360, M4361, and M4362 were identical to each other, and there were no cross-reactions with *M. phocacerebrale* or *M. phocarhinis*. There was no serological evidence of a relationship with any other nonfermenting or arginine-hydrolyzing members of the genus *Mycoplasma*. Thus, we concluded that these strains represent a new species of the genus *Mycoplasma* in the class *Mollicutes* as defined by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (13). We propose that this organism should be assigned to the family *Mycoplasmataceae* and the genus *Mycoplasma* on the basis of its requirement for sterol and its lack of urea utilization, and we suggest the name *Mycoplasma phocidae*; strain 105 is the type strain.

The isolation of mycoplasmas from the epizootic of pneumonia in seals in 1979 and 1980 has been described previously (8, 10, 14). The necrotizing bronchitis and bronchiolitis and hemorrhagic alveolitis observed at necropsy were considered to be due to the influenza virus. Environmental factors and population density may have been contributing factors. The contribution of the mycoplasmas to the syndrome is not clear. Attempts to infect grey seals and harp seals with mycoplasma strain M4359 and/or the influenza

FIG. 1. Electron micrographs of ultrathin sections of seal mycoplasma strain 105^T, showing the trilaminar structure of the membrane and the complete absence of a cell wall. Bars = 0.5 μ m.

virus have been described previously (9). No respiratory disease was observed, and no mycoplasma was recovered from any of the seals, although an unrelated mycoplasma was isolated from the mouths, eyes, lungs, and noses of all of the experimental seals.

Recently, Giebel et al. described *M. phocarhinis* and *M. phocacerebrale*, which were isolated from seals suffering from an epizootic of respiratory disease in the North Sea (11). These species are not related serologically to *M. phocidae*. In 1974 Smith et al. described the isolation of six mycoplasma strains from sea lions in an investigation of abortions, but these strains were never characterized (18).

Description of *Mycoplasma phocidae* sp. nov. *Mycoplasma phocidae* (pho'cid.ae. L. n. *phoca*, seal; *phocidae*, of a seal).

Thin sections reveal mainly coccoid forms with a clearly defined three-layer membrane and no cell wall. Colonies on agar have a typical fried egg appearance. The organism grows at 37°C microaerophilically or anaerobically. Cells pass through filters having pore diameters of 450 nm but are retained by filters having pore diameters of 220 nm. The organism is resistant to penicillin and thallium acetate, and there is no reversion to bacterial forms in medium that lacks these substances.

Cultures are inhibited by digitonin and require serum for growth. Arginine is hydrolyzed, but glucose and urea are not. Film and spots are produced on horse serum agar. Phosphatase is produced. Tetrazolium is not reduced. Serum and casein are not digested. Alpha-hemolysis occurs on bovine blood agar. The organism hemagglutinates but does not hemadsorb on sheep, guinea pig, chicken, or seal erythrocytes.

The G+C content of the DNA is 27.8 mol%.

The species is serologically distinct from other *Mycoplasma* species.

The habitat is the respiratory tracts of harbor seals. The organism appeared to be avirulent following experimental inoculation of grey or harp seals (10), but it may contribute to respiratory disease of harbor seals in association with other infections or environmental factors.

The type strain is strain 105 (= ATCC 33657).

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