Mycoplasma somnilux sp. nov., Mycoplasma luminosum sp. nov., and Mycoplasma lucivorax sp. nov., New Sterol-Requiring Mollicutes from Firefly Beetles (Coleoptera: Lampyridae)

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Strain PYAN-1T (T = type strain), which was isolated from a pupal gut of the firefly beetle Pyractonema angulata, and strains PIMN-1T and PIPN-2T, which were isolated from guts of adult Photinus marginalis and Photinus pyralis fireflies, respectively, were demonstrated to be sterol-requiring mollicutes. Cells of the three strains were shown by electron and dark-field microscopy to be small, pleomorphic, nonhelical, nonmotile bodies surrounded by single membranes. No evidence of a cell wall was observed, and the organisms were not susceptible to 500 U of penicillin per ml. The three strains grew rapidly in SP-4 broth medium. Strains PIMN-1T and PIPN-2T grew in medium supplemented with bovine serum fraction, but strain PYAN-1T did not.

All three strains grew on solid media when the cultures were incubated aerobically, but only strains PYAN-1T and PIPN-2T formed colonies when anaerobic conditions were employed. The three strains catabolized glucose but hydrolyzed neither arginine nor urea. All of the strains grew at temperatures of 18 to 32°C; strains PYAN-1T and PIMN-1T also grew at 10°C. The optimal temperature for growth for strains PYAN-1T and PIPN-2T was 30°C; strain PIMN-1T grew equally well at 30 or 32°C. None of the three strains grew at 37°C. The genome sizes of strains PYAN-1T, PIMN-1T, and PIPN-2T were about 527 (478 to 589), 570 (480 to 630), and 762 (635 to 871) megadaltons, respectively. The guanine-plus-cytosine contents of the DNAs of the strains, as determined by buoyant density, thermal denaturation, and high-performance liquid chromatography methods, were as follows: 27.4, 30.2, and 27.5 mol%, respectively, for strain PYAN-1T; 28.8, 31.2, and 29.2 mol%, respectively, for strain PIMN-1T; and 28.4, 30.4, and 31.2 mol%, respectively, for strain PIPN-2T. All three strains were serologically unrelated to the type strains of previously established Mycoplasma species (including Mycoplasma ellychniae), to each other, and to 15 other unclassified sterol-requiring strains isolated from animals, plants, and insects. Along with M. ellychniae, these three organisms represent an unusual cluster of strains belonging to the genus Mycoplasma isolated from insects. Strain PYAN-1 (= ATCC 49194) is the type strain of Mycoplasma somnilux sp. nov.; strain PIMN-1 (= ATCC 49195) is the type strain of Mycoplasma luminosum sp. nov.; and strain PIPN-2 (= ATCC 49196) is the type strain of Mycoplasma lucivorax sp. nov.

The possibility that, like acholeplasmas, sterol-requiring Mycoplasma species may be specifically associated with insect-plant interactions was suspected for some time, but was only recently established. In an extensive study (24), 30 mollicute strains from the guts or hemolymphs of insects were characterized. Five of these strains were found to be sterol-requiring, nonhelical mollicutes. We recently described the taxonomic characteristics of one of these organisms, strain ELCN-1, which was isolated from the hemolymph of the firefly beetle Ellychnia corrusca, and proposed that it be named Mycoplasma ellychniae (22). This species was the first species to be clearly shown to be specifically associated with insects. A number of putative species in the genus Mycoplasma have been cultivated from plant surfaces, where they may have been deposited by insects (20). In accompanying papers two of these plant-derived strains are characterized and named Mycoplasma melaleucae and Mycoplasma lactucae (17, 23).

We describe here the characteristics of three new distinct mollicutes that were isolated from firefly beetles and propose that they be given species status within the genus Mycoplasma.

MATERIALS AND METHODS

Mycoplasma strains. The techniques used for primary isolation of strains PYAN-1T (T = type strain), PIMN-1T, and PIPN-2T from the guts of firefly beetles have been described previously (14, 24). Preliminary identification of these strains as nonhelical sterol-requiring mollicutes and serological evidence of their possible unique taxonomic status were reported previously (24). All of the strains were cloned by conventional filtration techniques (18).

Culture media and cultivation techniques. All isolations of strains were made in SM-1 or M1D medium (25) at 30°C, and after three to five broth passages the isolates were lyophilized. Ampoules of the cultures were later revived and passaged twice before cloning in SP-4 broth (25) at 30°C. Formulations of SP-4 medium contained 500 U of penicillin

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G per ml. Triply cloned strains were chosen and were used as candidates for subsequent characterization. Other culture media used included the Edward formulation of conventional 20% horse serum mycoplasma broth (8), serum-fracton broth supplemented with 1% bovine serum fraction (19), and serum-free media supplemented with fatty acid mixtures (15, 19). Solid formulations of these media were made by adjusting the final concentration of Noble agar (Difco Laboratories, Detroit, Mich.) in the heated media to 0.8%. Cultures on solid media were incubated at 30°C either aerobically, in the presence of 5% carbon dioxide (GasPak system; BBL Microbiology Systems, Cockeysville, Md.), or anaerobically (hydrogen GasPak system).

The temperature requirements for growth of the strains were determined by preparing a series of 10-fold dilutions of the organisms in SP-4 broth. One series of each diluted mycoplasma was incubated at each of six temperatures (10, 18, 25, 30, 32, and 37°C). The relative number of organisms was estimated by recording the highest dilution in which growth occurred, as evidenced by a color change (red to yellow) in the phenol red indicator in the medium or by turbidity or both. Results were recorded after incubation for 3 weeks. Early passages of each strain were also grown in SP-4 liquid medium devoid of antibiotics for at least five consecutive passages. After each subculture, the strains were plated onto conventional blood agar and incubated aerobically at 37°C. After 2 to 10 days, the plates were examined for evidence of reversion, which, if observed, would presumably have been manifest as bacterial colonies.

Morphological studies. Cells of the strains from SP-4 broth cultures in logarithmic phase were examined by dark-field microscopy, using a magnification of ×1,250. For electron microscopy, cells were grown in about 10 ml of broth and pelleted by centrifugation. They were then fixed for 2 h in 3% glutaraldehyde, postfixed in 1% osmium tetroxide for 1 h, dehydrated in acetone, embedded in Epon-araldite, sectioned, and stained with 1% aqueous uranyl acetate and Reynold lead citrate (22).

Sterol requirement. Sterol requirements for growth of the strains were determined as previously described (16), except that cultures were incubated at optimum growth temperatures (30°C) and protein assays were performed with a kit obtained from Bio-Rad Laboratories, Richmond, Calif.

Tests for biological and biochemical properties. The procedures used to demonstrate carbohydrate fermentation (1), arginine and urea hydrolysis (1), filtration characteristics (18) in SP-4 broth, and the film and spot reaction (9), as well as the hemadsorption assay procedure (10), have been described previously.

Serological tests. Antiserum to each strain was raised in rabbits. Hyperimmune antiserum to previously established *Mycoplasma* species and to 15 other unclassified mycoplasmas (see reference 21 for a list of species) were derived from the reference collection of the Mycoplasma Section of the National Institute of Allergy and Infectious Diseases at Frederick, Md. These antisera and strains PYAN-1T, PIPN-2T, and PIMN-1T were reacted in a standard disk growth inhibition test (7) on SP-4 agar, with aerobic incubation at 30°C. In addition, direct immunofluorescence tests (11) were performed on colonies on SP-4 agar medium by using fluorescein-conjugated antiserum to the mycoplasmas listed previously (22). The inhibition discs with the three insect-derived strains were also performed with antiserum to the 11 previously recognized *Acholeplasma* species (see list in reference 22).

Genomic analysis. The techniques used for extraction and purification of chromosomal DNA from molicutes have been described previously (3). Genome sizes were determined by DNA renaturation kinetics, as described previously (2). The guanine-plus-cytosine contents of purified DNAs from the three strains were determined by the buoyant density, melting temperature, and high-performance liquid chromatography methods (4). Purified DNA from *Spiroplasma citri* (genome size, about 1,000 megadaltons [MDa]; base composition, 26 mol% guanine plus cytosine) was used as a control in these determinations.

RESULTS AND DISCUSSION

Cultural and morphological properties. All three strains grew well in SP-4 broth and formed colonies on agar media prepared with that formulation. Multiplication also occurred in conventional mycoplasma media containing horse serum (Edward formulation). Two of the strains, strains PIMN-1T and PIPN-2T, grew on mycoplasma broth base supplemented with bovine serum fraction. Growth of all strains occurred over a temperature range from 18 to 32°C; strains PIMN-1T and PIPN-2T also grew at 10°C; and the optimum temperature for growth was 30°C, although strain PIMN-1T grew equally well at 32°C. Growth was not apparent in broth media when strains were incubated at 37°C for 3 weeks. Colonies of all three strains on horse serum and SP-4 agar media usually exhibited typical fried-egg morphology (Fig. 1A, C, and D), although strain PYAN-1T produced a more granular type of colonies on SP-4 agar (Fig. 1B). Strain PIMN-1T formed colonies on agar only when plates were incubated aerobically or in an atmosphere containing 5% carbon dioxide. While the other two strains (strains PYAN-1T and PIPN-2T) produced growth on agar under both aerobic and anaerobic conditions, plates incubated anaerobically always had larger and more numerous colonies.

When logarithmic-phase cultures of all three strains were examined by dark-field microscopy, we observed numerous pleomorphic coccoidal or subcoccoidal cells and short, branching, nonmotile, nonhelical filaments. Cells of the organisms sedimented from broth cultures and examined by electron microscopy appeared to be typically mycoplasmal, consisting largely of pleomorphic coccoidal cells (Fig. 2). Most cells were about 200 to 300 nm in diameter and were surrounded by a single membrane. No evidence of a cell wall was observed.

Sterol requirement. The responses of the strains to additions of cholesterol to a serum-free base medium are shown in Table 1. Insignificant or very small amounts of growth occurred in base broth alone, but enhanced growth was observed when cholesterol concentrations of 5 to 20 μg/ml were present. Growth responses to these levels of cholesterol have been observed with other sterol-requiring moli)cutes.

Biochemical and biological properties. All of the strains fermented glucose with the production of acid and a concomitant decline in the pH of the culture medium. Arginine and urea were not hydrolyzed. Strains PIMN-1T and PIPN-2T showed positive film and spot reactions, and colonies of strain PIMN-1T on solid medium hemadsorbed guinea pig erythrocytes. The remaining responses to the film and spot and hemadsorption tests were negative. The results of passage of cells of the three strains through a graded series of membrane filters are summarized in Table 2.

Serological tests. Growth inhibition and plate immunofluorescent tests, which were performed with antiserum or conjugates prepared to known mycoplasmas and acholeplasmas,
indicated that none of the strains was related to previously established species in the two genera or to 15 other unclassified, nonhelical, sterol-requiring mollicutes that represent putative mollicute species.

**Genome size and DNA base composition.** DNA renaturation kinetics indicated that the average genome size of strain PYAN-1T was 527 MDa (range, 478 to 589 MDa). The average genome size of strain PIMN-1T was 570 MDa (range, 480 to 630 MDa), and the average genome size of strain PIPN-2T was 762 MDa (range, 635 to 871 MDa). Values for the base composition (guanine-plus-cytosine content) of the DNA of strain PYAN-1T were 27.4, 30.2, and 27.5 mol% as determined by the buoyant density, melting temperature, and high-pressure liquid chromatography techniques, respectively. The values determined for strain PIMN-1T were 28.8, 31.2, and 29.2 mol%, respectively, and the values determined for strain PIPN-2T were 28.4, 30.4, and 31.2 mol%, respectively.

**Habitat.** All three strains described here were isolated from guts of firefly beetles. Strain PYAN-1T was derived from a pupa of *Pyraconema angulata*; strain PIMN-1T was isolated from an adult *Photinus marginalis*; and strain PIPN-2T came from an adult *Photinus pyralis*. The isolation of these three *Mycoplasma* species from the gut flora of insects differs from the isolation of the type strain of the recently described organism *M. elychniae* (22), which was found only in the hemolymph of its host beetle, *E. corroscus*. Whether any of the three strains is pathogenic for its firefly host or for other insects has not been determined.

Other mollicutes (acholeplasmas and spiroplasmas) occur much more frequently than *Mycoplasma* species in insects (5, 6, 12, 20, 21, 26). Although two other distinct, nonhelical, sterol-requiring mollicutes have been isolated from plant sources and are proposed as new *Mycoplasma* species in accompanying papers (17, 23), the concentration of four distinct *Mycoplasma* species in members of the family Lampyridae is remarkable.

The properties described here for strains PYAN-1T,
FIG. 2. Electron micrographs of sectioned and stained cell pellets of strains PYAN-1^T (A), PIMN-1^T (B), and PIPN-2^T (C). Sections were stained with 2% aqueous ureil acetate and Reynold lead citrate. The arrows indicate the unit membrane. Bar = 100 nm.

PIMN-1^T, and PIPN-2^T fulfill the criteria (13) for species descriptions of members of the class Mollicutes. A cell wall is absent, and the cells are filterable, fail to revert to walled bacteria when they are grown in antibiotic-free media, are resistant to penicillin, and produce typical colonies on solid media. The growth requirement for sterol or serum, in conjunction with the lack of helicity, places these organisms in the order Mycoplasmatales and the family Mycoplastaceae. The inability of the strains to hydrolyze urea mandates assignment to the genus Mycoplasma. Finally, the lack of serological relatedness of these strains to other Mycoplasma species and to other unclassified strains that could represent putative species in the genus demonstrates that they represent previously unrecognized species. We propose the following names: Mycoplasma somnilux for strain PYAN-1^T, Mycoplasma luminosum for strain PIMN-1^T, and Mycoplasma lucivorax for strain PIPN-2^T.

The taxonomic descriptions below summarize the properties of the three strains.

Mycoplasma somnilux sp. nov. Mycoplasma somnilux (som.ni'lux. L. masc. n. somnus sleep; L. fem. n. lux, light; N.L. neut. n. somnilux, sleeping light, referring to the quiescent pupal stage of the host from which the organism was isolated, which precedes the luminescent adult stage).

Cells are nonhelical, lack true cell walls, and are either pleomorphic coccoidal or subcoccoidal (diameter, 200 to 300 nm) or occur as short, branched or unbranched filaments. Nonmotile.

Colonies on solid medium containing 0.8% Noble agar usually have a fried-egg appearance.

Chemoorganotroph. Acid is produced from glucose. Arginine and urea are not hydrolyzed. Film and spot reaction negative. Does not hemadsorb guinea pig erythrocytes.

Cholesterol or serum is required for growth.

The temperature range for growth is 10 to 32°C; optimum growth occurs at 30°C.

Serologically distinct from other Mycoplasma species. Isolated from a pupal gut of the firefly beetle Pyractonema angulata. Pathogenicity for insects has not been determined.

The genome size averages 527 MDa. The guanine-plus-cytosine content of the DNA is 27.4 ± 1 mol%, as determined by the buoyant density method.

The type strain is strain PYAN-1 (= ATCC 49194).

Mycoplasma luminosum sp. nov. Mycoplasma luminosum (lu.min.o'sum. L. neut. adj. luminosum, full of light; N.L. neut. adj., luminosum, emitting light, referring to the luminescence of the adult host from which the organism was isolated). Cells are nonhelical, lack true cell walls, and are either pleomorphic coccoidal or subcoccoidal (diameter, 200

**TABLE 1. Growth responses of three insect-derived mollicutes to cholesterol**

<table>
<thead>
<tr>
<th>Supplement(s) added to serum-free base medium</th>
<th>Cholesterol concn (µg/ml)</th>
<th>Ampt of protein (mg/100 ml)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse serum (20%)</td>
<td>Control</td>
<td>3.0</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>6.24</td>
</tr>
<tr>
<td>Albumin (1%), Tween 80 (0.01%), and palmitic acid (10 µg/ml)</td>
<td></td>
<td>8.00</td>
</tr>
</tbody>
</table>

^a The assay was performed with cultures incubated at 30°C.

^b IG. Insufficient growth.

**TABLE 2. Passage of insect-derived mollicute strains through membrane filters of various porosities**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative no. of organisms (no. of color-changing units per ml) in filtrates after broth culture passage through membrane filters having the following pore sizes^b:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No filtration</td>
</tr>
<tr>
<td>PYAN-1^T</td>
<td>10^8</td>
</tr>
<tr>
<td>PIMN-1^T</td>
<td>10^9</td>
</tr>
<tr>
<td>PIPN-2^T</td>
<td>10^8</td>
</tr>
</tbody>
</table>

^b Broth passage was performed in mycoplasma media containing 20% horse serum at 30°C.

^c Neg. No color change.
to 300 nm) or occur as short, branched or unbranched filaments. Nonmotile.

Colonies on solid medium containing 0.8% Noble agar usually have a fried-egg appearance.

Chemoorganotroph. Acid is produced from glucose. Arginine and urea are not hydrolyzed. Film and spot reaction positive. Agar colonies hemadsorb guinea pig erythrocytes.

Cholesterol or serum is required for growth. The temperature range for growth is 10 to 32°C; optimum growth occurs at 30°C.

Serologically distinct from other Mycoplasma species. Isolated from a gut of the firefly beetle Photinus marginata. Pathogenicity for insects has not been determined.

The genome size averages 570 MDa. The cytosine content of the DNA is 28.8 mol%, as determined by the buoyant density method.

The type strain is strain PIMN-1 (= ATCC 49195).

*Mycoplasma lucivorax* sp. nov. *Mycoplasma lucivorax* (luc.i’vor.ax. L. fem. n. lux, light; L. neut. adj. vorax, glutinous, devouring; N.L. neut. adj. lucrivorax, light devouring, referring to the predacious habit of the host insect, which preys on other luminescent firefly species). Cells are nonhelical, lack true cell walls, and are either pleomorphic coccolial or subcoccolial (diameter, 200 to 300 nm) or occur as short, branched or unbranched filaments. Nonmotile.

Colonies on solid medium containing 0.8% Noble agar usually have a fried-egg appearance.

Chemoorganotroph. Acid is produced from glucose. Arginine and urea are not hydrolyzed. Film and spot reaction positive. Does not hemadsorb guinea pig erythrocytes.

Cholesterol or serum is required for growth. The temperature range for growth is 10 to 32°C; optimum growth occurs at 30°C.

Serologically distinct from other Mycoplasma species. Isolated from a gut of the firefly beetle Photinus pyralis. Pathogenicity for insects has not been determined.

The genome size averages 762 MDa. The guanine-plus-cytosine content of the DNA is 27.4 ± 1 mol%, as determined by the buoyant density method.

The type strain is strain PINP-2 (= ATCC 49196).

LITERATURE CITED