**Acholeplasma multilocale** sp. nov., Isolated from a Horse and a Rabbit

AURIOL C. HILL,¹ ANNA A. POLAK-VOGELZANG,² AND ALEJANDRO F. ANGULO²*

Toxology Unit, Medical Research Council Laboratories, Carshalton, Surrey SM5 4EF, United Kingdom, and National Institute of Public Health and Environment Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands²

Acholeplasma strains were isolated from the nasopharynx of a horse (strain PN525⁷ [T = type strain]) and the feces of a rabbit (strain B1). One clone of strain PN525⁷ and one clone of strain B1 were examined in detail. These clones were indistinguishable from each other and were serologically distinct from the previously described *Acholeplasma* and *Mycoplasma* spp. The strains had the following properties: guanine-plus-cytosine content of 31 mol%; sterol was not required for growth, which occurred under both aerobic and anaerobic conditions; glucose was metabolized; and arginine was hydrolyzed. Strain PN525 (= NCTC 11723) is the type strain of a new species, *Acholeplasma multilocale*.

Mycoplasma colonies were isolated, on a medium used for detection of *Mollicutes*, from a nasopharyngeal swab taken from a horse. After cloning and preliminary investigation, the isolate was found to consist of a mixture of *Acholeplasma oculi* and another *Mollicutes* species (strain PN525⁷ [T = type strain]) which could not be further identified. Another strain (strain B1) was isolated from rabbit feces and was identified as an *Acholeplasma* sp. (2). This strain was cloned from a mixture which contained *Acholeplasma laidlawii*. The farm where the horse was kept was 30 km from the breeding unit where the rabbits were kept, and neither the animals nor their caretakers came in contact. In this study the properties of strains PN525⁷ and B1 were further investigated, and these organisms were serologically compared with previously described species.

**MATERIALS AND METHODS**

**Mycoplasma strains.** Mycoplasmas were isolated from the nasopharynx of a horse kept on a farm and from the feces of a rabbit kept at a breeding unit. Small pieces of agar that were obtained from the primary inoculated agar plates and contained single colonies were streaked onto fresh agar plates. After incubation for 4 days, this process was repeated, and after incubation of the resulting plates for 4 days, single colonies were inoculated into broth. After 3 days of incubation, each broth culture was filtered through membrane filters (pore size, 220 nm; (Sartorius, Gottingen, Germany). The resulting filtrate was diluted 10-fold, and each dilution was inoculated onto agar plates to obtain single colonies. This cloning procedure was repeated once. One clone was designated strain PN525⁷ (the clone from the horse), and another clone was designated strain B1 (the clone from the rabbit).


The following *Mycoplasma* spp. type strains were used:


**Medium and growth conditions.** The culture medium which we used has been described previously (13, 30). The purified agar used in the growth medium was 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 grew

* Corresponding author.
in medium containing 1% arginine and were not inhibited. *M. genitalium* and *M. synoviae* were grown on SP4 medium (35). Strain PN525T was isolated and subcultured aerobically on modified Herdersche medium (12, 22). Strain B1 was isolated aerobically on a medium that has been described previously (2) and was subcultured on modified Herdersche medium. Agar cultures were incubated at 35 to 37°C either in a humid chamber or under anaerobic conditions by using a GasPak system (B.D. UK Ltd., Cowley, Oxford, England). Liquid cultures were stored at −70°C in ampoules.

**Growth requirements and characteristics.** The strains were subcultured onto solid medium and were incubated under both aerobic and GasPak anaerobic conditions at 35 to 37°C. The levels of susceptibility to methylene blue were determined 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 that were cultivated on agar medium without methylene blue. Lipolytic activity was tested by inoculating mycoplasmas onto basal medium enriched with 10% egg yolk emulsion (8). The incubated plates were then examined after 3, 7, and 14 days for visual evidence of lipolysis (clearing) or film production.

**Absence of reversion.** The strains were subcultured for five passages by using 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 (31).

**Morphological studies.** Mycoplasma colonies grown on agar were examined microscopically at a magnification of ×100 after 2, 7, and 14 days of incubation. The colonies were transferred to slides and stained with Giemsa stain (9, 16). Liquid cultures were observed by using dark-field microscopy, and organisms were stained with Giemsa stain (16). Organisms grown in broth were harvested by centrifugation at 30,000 × g for 45 min, and the resulting 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 embedded in epoxy resin were stained with uranyl acetate and lead citrate and were examined by using electron microscopy (36).

**Filtration studies.** Cultures (after 24 h of incubation) were diluted 1:10 in liquid medium and then filtered through a series of membrane filters (Millipore Corp., Bedford, Mass.) with pore diameters of 220, 300, 450, 650, and 800 nm. The number of colony-forming units per milliliter in each filtrate was determined by plating the preparation onto agar and was compared with the number of colony-forming units per milliliter in the unfiltered culture dilution.

**Sterol dependence.** Single colonies of strains PN525T and B1 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 a solution containing 20% glycerol, 5% 2-mercaptoethanol, and 3% sodium dodecyl sulfate in 0.00625 M Tris buffer (pH 6.8). To a 12.5% acrylamide gel, 10- or 25-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 Chemical Co., St. Louis, Mo.).

**DNA base composition.** DNA was extracted from centrifuged broth culture deposits of strain PN525T by using the method of Gross-Bellard et al. (11), and the guanine-plus-adenine content was determined by using a modification of the method of Gross-Bellard et al. (11) and the guanine-plus-
cytosine contents were determined from the buoyant density of the DNA in cesium chloride by ultracentrifugation (27). DNA extracted from Escherichia coli, which had a known guanine-plus-cytosine content, was included as a control.

**Serological studies.** Antisera were prepared as described by Morton and Roberts (19), Hill (13), and Polak-Vogelzang et al. (21). 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, 29), and immunoperoxidase tests with colonies grown on agar (15, 21). All of the tests were performed in duplicate. Type strain PN525 and strain B1 were reacted with antisera prepared against the species listed above, and antisera to these two strains were tested with the previously described Mycoplasma and Acholeplasma species. Antisera to strains PN525T and B1 were reacted with each other to demonstrate the relationship of these organisms.

**RESULTS AND DISCUSSION**

Colonies of strains PN525T and B1 were visible in subcultures after 4 days of incubation under aerobic and anaerobic conditions. The colonies (Fig. 1) had a typical fried-egg appearance. Growth was not inhibited by methylene blue or on medium without serum. A film was produced on egg yolk agar, but no lipolytic activity was observed. Neither of the strains reverted to a bacterial form when it was subcultured on medium without bacterial inhibitors. The colonies rapidly stained with Dienes reagent, confirming that the isolates were true mycoplasmas (Mollicutes) rather than bacterial L-forms (31).

No motility was observed in fluid media when the strains were examined by using dark-field microscopy, and helical forms were not seen. Preparations from liquid cultures stained with Giemsa stain contained pleomorphic forms characteristic of mycoplasmas. The ultrastructure of the organisms was typical of mycoplasma morphology when thin sections were examined by using electron microscopy (4).

Preparations from liquid cultures stained with uranyl acetate and lead citrate were studied by transmission electron microscopy. Fig. 2. Electron micrograph of a section of a strain PN525T cell stained with uranyl acetate and lead citrate. Bar = 200 nm.

The cells were bounded by a single trilaminar unit membrane and lacked any evidence of a cell wall (Fig. 2). Filtration of a broth culture of strain PN525T reduced the viable count from $3 \times 10^8$ CFU/ml in the original dilution to $2 \times 10^5$ CFU/ml in the filtrate after passage through a membrane with a pore size of 220 nm.

Strains PN525T and B1 did not require cholesterol for growth as they could be cultured on medium without cholesterol. They were not susceptible to sodium polyanethol sulfonate. The growth of strain PN525T was inhibited by digitonin to 5 mm from the disk edge, while A. laidlawii was inhibited to 3 mm around the disk edge; the inhibition of M. hominis reached more than 10 mm from the edge.

The production of carotenoids by strain PN525T was two to three times higher than that by A. axanthum but only 50 to 75% of that by A. laidlawii.

The biochemical activities of the strains PN525T and B1 were identical and are summarized in the species description below. In appropriate tests both strains were hemolytic for all three types of erythrocytes tested, but did not hemadsorb these cells. Both strains hemagglutinated the cells which we tested.

The two strains produced similar protein electrophoretic patterns.
The guanine-plus-cytosine content of strain PN525\(^T\) DNA was determined from its buoyant density in cesium chloride to be 31 mol\%. This value is within the range of values for previously characterized *Acholeplasma* species.

The serological techniques which we used revealed similar levels of cross-reactivity between strains PN525\(^T\) and B1 (Table 1). No significant cross-reactions were detected with any of the species listed in Materials and Methods, except for a one-way cross-reaction with *M. verecundum* antiserum in the immunoperoxidase test. However, *M. verecundum* belongs to a different genus than strains PN525\(^T\) and B1.

Strains PN525\(^T\) and B1 belong to the family *Acholeplasmataceae* because of their main characteristics, including absence of cell walls, lack of reversion to bacterial forms when the organisms are grown in antibiotic-free media, penicillin resistance, filterability, and production of typical morphology on agar. They belong to the genus *Acholeplasma* because they are not strict anaerobes (in contrast to *Anaeroplasma* spp.), are non-helical (in contrast to *Spiroplasma* spp.), and do not depend on sterol for growth (in contrast to *Mycoplasma* spp.) and because the presence of urease activity could not be demonstrated (in contrast to *Ureaplasma* spp.).

Strains PN525\(^T\) and B1 belong to the same species as they have identical biological characteristics and produce identical serological and protein patterns. As they are distinct from all previously described *Acholeplasma* spp. (see above), strains PN525\(^T\) and B1 belong to a new *Acholeplasma* species.

**Description of Acholeplasma multilocale sp. nov. Acholeplasma multilocale** (mul.ti.lo.ca'le. M.L. gen. multilocale, referring to more than one location).

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

**Growth characteristics.** Does not require sterol. Optimal growth occurs at 35 to 37°C under aerobic or anaerobic conditions. A film is produced on egg yolk agar.

**Metabolic characteristics.** Glucose, fructose, maltose, mannose, and sucrose are metabolized; arginine is hydrolyzed; resazurin, methylene blue, and tellurite are reduced.

**DNA base composition.** The DNA base composition is 31 mol\% guanine-plus-cytosine.

**Habitat.** Isolated from a horse nasopharynx and rabbit feces.

**Type strain.** The type strain is strain PN525 (= NCTC 11723).

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### REFERENCES


