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

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Acholeplasma strains were isolated from the nasopharynx of a horse (strain PN525^T [T = type strain]) and the feces of a rabbit (strain B1). One clone of strain PN525^T 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 PN525^T [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 PN525^T 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 PN525^T (the clone from the horse), and another clone was designated strain B1 (the clone from the rabbit).

Mycoplasma species. Mycoplasma type strains were obtained from the National Collection of Type Cultures, Colindale, England, and from H. Atobe, M. F. Barile, J. M. Bradbury, R. J. Fallon, E. A. Freundt, J. T. Heywood, F. T. W. Jordan, D. E. Jaspar, H. Kirchhoff, R. H. Leach, G. J. McGarrity, D. L. Rose, G. Smith, D. Taylor-Robinson, and J. G. Tully.

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

Mycoplasma agalactiae PG2, *M. alkalescens* D12 (= PG51), *M. alvi* Ilsley, *M. anatis* 1340, *M. anseris* 1219, *M. arginini* G230, *M. arthritis* PG6, *M. bovirhinis* PG11, *M. bovirhinis* PG43, *M. bovis* PG45, *M. bovovulvi* M165/69, *M. buccale* CH-20247, *M. californicum* ST-6, *M. canadense* 275C, *M. canis* PG14, *M. capricolum* California kid, *M. caviae* G122, *M. cavipharyngis* 117C, *M. citelli* RG-2C, *M. cloacale* 383, *M. collis* 58B, *M. columbinasale* 694, *M. columbinum* MMP1, *M. columborale* MMP4, *M. conjunctivae* HRC581, *M. cricetuli* CH, *M. cynos* H831, *M. dispar* 462/2, *M. edwardii* PG24, *M. ellychniae* ELCN-1, *M. equigenitalium* T37, *M. equirhinis* M432/72, *M. fastidiosum* 4822, *M. faucium* DC333, *M. felifaucium* PU, *M. feliminutum* Ben, *M. felis* CO, *M. fermentans* PG18, *M. flocculare* MS42, *M. gallinaceum* DO, *M. gallinarum* PG16, *M. gallisepticum* PG31, *M. gallopavonis* WR1, *M. gateae* CS, *M. genitalium* G-37, *M. glycophilum* 486, *M. hominis* PG21, *M. hyopharyngis* H3-6BF, *M. hyopneumoniae* J, *M. hyorhinis* BTS7, *M. hyosynoviae* S16, *M. iners* PG30, *M. iowae* 695, *M. lactucae* 831-C4, *M. lipofaciens* R171, *M. lipophilum* MaBy, *M. luminosum* PIMN-1, *M. lucivorax* PIPN-2, *M. maculosum* PG15, *M. melaleuca* M1, *M. meleagridis* 17529, *M. moatsii* MK405, *M. mobile* 163K, *M. molare* H542, *M. muris* RIII4, *M. mustelae* MX9, *M. mycoides* subsp. *capri* PG3, *M. mycoides* subsp. *mycoides* PG1, *M. neurolyticum* Type A, *M. opalescens* MH5408, *M. orale* CH-19299, *M. ovipneumoniae* Y98, *M. oxoniensis* 128, *M. pirum* HRC70-159, *M. pneumoniae* FH, *M. primatum* HRC292, *M. pullorum* CKK, *M. pulmonis* PG34 (= ASH), *M. putrefaciens* KS-1, *M. salivarium* PG20, *M. somnolux* PYAN-1, *M. spermatophilum* AH159, *M. spumans* PG13, *M. sualvi* Mayfield (clone B), *M. subdolum* TB, *M. synoviae* WVU1853, *M. testudinis* 01008, and *M. verecundum* 107.

The following *Acholeplasma* spp. type strains were used: *Acholeplasma axanthum* S743, *A. entomophilum* TAC, *A. equifetale* N93, *A. florum* LI, *A. granularum* BTS39, *A. hippikon* C1, *A. laidlawii* PG8, *A. modicum* PG49, *A. morum* 72-043, *A. oculi* 19L, *A. parvum* H23M, and *A. seiffertii* F7.

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

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in medium containing 1% arginine and were not inhibited. *M. genitalium* and *M. synoviae* were grown on SP4 medium (35). Strain PN525^T was isolated and subcultured aerobically on modified Herderscheë medium (12, 22). Strain B1 was isolated aerobically on a medium that has been described previously (2) and was subcultured on modified Herderscheë 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 $\times 100$ 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 $30,000 \times g$ for 45 min, and the resulting cell pellet was fixed in 2% (vol/vol) glutaraldehyde and postfixated 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 PN525^T and B1 were seeded onto serum-free solid media supplemented with 0.5% bovine serum albumin, 0.5% glucose, and 10 μ g of palmitic acid per ml. Cholesterol, dissolved in Tween 80, was added to give concentrations of 20, 10, 5, and 1 μ g/ml. Plates containing no cholesterol were included (6, 7, 26, 34). 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 carryover of serum from the original culture, avoiding misleading results.

The strains were tested indirectly for sterol dependence by a paper disk inhibition method (10), using wet disks containing 0.02 ml of a 20% (wt/vol) aqueous solution of sodium polyanethanol sulfonate (Koch-Light Laboratories Ltd., Coln-

FIG. 1. Colonies of strain PN525^T grown under aerobic conditions for 2 days on an agar medium. Bar = 50 μ m.

brook, England) on basal growth medium containing Lab M agar. The widths of zones of growth inhibition were measured.

Digitonin susceptibility. The test to determine susceptibility to digitonin was performed as described by Tully (33), using a paper disk inhibition assay and Herderscheë solid agar containing 20% serum. *A. laidlawii* and *M. hominis* were used as negative and positive controls, respectively.

Production of pigmented carotenoids. Carotenoid production was measured after 5 days of growth in both Chanock and Herderscheë broth media as described by Razin (25). *A. laidlawii* and *A. axanthum* were used as positive and negative controls, respectively.

Biochemical activity. The strains were examined for the following characteristics: metabolism of arabinose, arbutin, cellobiose, dulcitol, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose; hydrolysis of esculin, arginine (1 and 0.1%), and urea; reduction of methylene blue, resazurin, tetrazolium, and tellurite; and phosphatase activity (1, 3, 17, 28, 32, 37).

Erythrocyte techniques. The strains were examined for hemolytic activity, hemadsorption, and hemagglutination by using chick, guinea pig, and sheep erythrocytes (1, 18).

Polyacrylamide gel electrophoresis. Electrophoresis was carried out as described by Mouches and Bové (20), 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 0.00625 M Tris buffer (pH 6.8). To a 12.5% acrylamide gel, 10- or 25- μ l 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 PN525^T by using the method of Gross-Bellard et al. (11), and the guanine-plus-

FIG. 2. Electron micrograph of a section of a strain PN525^T cell stained with uranyl acetate and lead citrate. Bar = 200 nm.

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 PN525^T and B1 were reacted with each other to demonstrate the relationship of these organisms.

RESULTS AND DISCUSSION

Colonies of strains PN525^T 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). 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 PN525^T reduced the viable count from 3×10^8 CFU/ml in the original dilution to 2×10^5 CFU/ml in the filtrate after passage through a membrane with a pore size of 220 nm.

Strains PN525^T 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 PN525^T 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 PN525^T 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 PN525^T 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.

TABLE 1. Serological tests comparing strains PN525^T and B1 with previously described species

Antigen	Growth inhibition zone width (mm) with antiserum to:			Metabolic inhibition titer with antiserum to:			Immunoperoxidase titer with antiserum to:		
	Strain PN525 ^T	Strain B1	Previously described species	Strain PN525 ^T	Strain B1	Previously described species	Strain PN525 ^T	Strain B1	Previously described species
PN525 ^T	3	5	0	8,000	64,000	<100	4,000	>16,000	<100
B1	3	5	0	2,048	32,000	<100	4,000	>16,000	<100 ^a
All previously described species	0	0		<100	<100		<100	<100	

^a Strain B1 antigen produced a one-way cross-reaction with *M. verecundum* antiserum in the immunoperoxidase test.

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 *Anaeroplasm* 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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