Acholeplasma parvum, a New Species from Horses

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Four glucose-nonfermenting *Acholeplasma* strains were isolated from oral cavities of horses and a horse vagina. The biological and serological properties of these isolates were distinct from those of the eight currently recognized *Acholeplasma* species. These strains were regarded as belonging to a new species, which was given the name *Acholeplasma parvum*. Strain H23M was designated the type strain of *A. parvum*, and a culture of this strain has been deposited in the American Type Culture Collection as strain ATCC 29892.

Previously, we reported (14, 24) the isolation of four strains of *Acholeplasma* from horses; these strains differed from the previously described species of *Acholeplasma* by lacking the ability to ferment glucose. This study was undertaken to determine whether these strains constitute a new species.

MATERIALS AND METHODS

Bacterial strains. Strains H7M, H15M, and H23M^T (type strain) were isolated from the oral cavities of healthy horses, and strain H23V was isolated from the vagina of a healthy horse. Each strain was purified by picking growth from a well-isolated single colony, inoculating this growth into broth, and, after 4 days of incubation, filtering the resulting broth culture through a 450-nm filter; the filtrate was plated onto a solid medium to obtain isolated colonies. This procedure was repeated at least three times (21). The following type strains of *Acholeplasma* and *Mycoplasma* species were used for comparative purposes: *Acholeplasma laidlawii* PG8 (= ATCC 23206), *Mycoplasma arthritidis* PG6 (= ATCC 19611), and *Mycoplasma bovis genitalium* PG11 (= ATCC 19852), which were obtained from D. G. ff. Edward, Wellcome Research Laboratories, Beckenham, Kent, England; *Acholeplasma granularum* BTS39 (= ATCC 19168), obtained from W. P. Switzer, Iowa State University Veterinary Medical Research Institute, Ames, Iowa; *Acholeplasma axanthum* S743 (= ATCC 25176) and *Acholeplasma morum* 72-043 (= ATCC 33211) obtained from J. G. Tully, National Institute of Allergy and Infectious Diseases, Bethesda, Md.; *Acholeplasma modicum* PG49 (= ATCC 29102), obtained from R. H. Leach, Mycoplasma Reference Laboratory, Central Public Health Laboratory, London, England; *Acholeplasma oculi* 19L (= ATCC 27350), obtained from J. J. Callis, Plum Island Animal Disease Center, Long Island, N.Y.; and *Acholeplasma equifetale* C112 (= ATCC 29724) and *Acholeplasma hippikon* C1 (= ATCC 29725), obtained from H. Kirchhoff, Institute für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Hannover, West Germany.

Media. The *acholeplasmas* were cultivated in a medium containing seven parts of PPLO broth without

crystal violet (Difco Laboratories, Detroit, Mich.), two parts of unheated horse serum, and one part of 25% (wt/wt) fresh yeast extract; this medium was supplemented with 1% Phytone (BBL Microbiology Systems, Cockeysville, Md.) and 1% penicillin G (100,000 U/ml). A serum-free medium (the medium described above but without serum) was also used. Solid medium was prepared by adding 1.2% granulated agar (catalog no. 11849; BBL) to the liquid medium.

Morphological studies. Agar plates were incubated at both 30 and 37°C under aerobic conditions; 5-day-old colonies were observed with a stereomicroscope.

For electron microscopy, cells collected by centrifugation from 24-h-old serum-free broth cultures were fixed in a mixture containing 5% glutaraldehyde and 1% paraformaldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead nitrate and were examined with a Hitachi HU-12 electron microscope.

Reversion experiments. The strains were subcultured five times in liquid medium without penicillin, and each culture was inoculated onto solid medium without bacterial inhibitors.

Filtration studies. Filtration tests were performed with 24-h-old cultures of strains H7M and H23M^T grown in the serum-free medium. Filterability was determined by using a Swinny hypodermic adapter and membrane filters (Millipore Corp., Bedford, Mass.) with average pore diameters of 800, 450, 220, and 100 nm.

Sterol requirement. To determine the sterol requirements of the strains, the method of Razin and Tully (17) was used, except that growth was determined on solid medium by observing the development of colonies. To the serum-free basal medium bovine serum albumin (5 mg/ml), palmitic acid (10 µg/ml), and cholesterol (0, 1, 5, or 20 µg/ml) were added. Susceptibility to 1.5% digitonin and 20% sodium polyanethol sulfonate was determined by the paper disk inhibition method described by Freundt et al. (7).

Growth conditions. Growth at 22, 37, and 45°C under aerobic or anaerobic (5% CO₂ in nitrogen gas) conditions was examined every other day for 10 days.

Biochemical tests. Biochemical tests were performed in both serum-supplemented and serum-free media.

Tests for utilization of glucose, salicin, arginine, and urea were performed in medium containing 1% test substrate and 0.0017% phenol red. Esculin hydrolysis was determined by the method of Williams and Witter (25). Tests for reduction of 2,3,5-triphenyltetrazolium chloride (0.05%), methylene blue (0.0001%), and tellurite (0.05%) were performed by the method of Aluotto et al. (3). Hemolysis was tested by the overlay method, using horse, sheep, and guinea pig erythrocytes (3).

Hexokinase activity. Hexokinase activity was determined in frozen and thawed cell suspensions by the method of Cirillo and Razin (4, 26), as follows. Cells in logarithmic phase were harvested from 1,000 ml of a broth culture by centrifugation, washed twice in 0.25 M NaCl, and resuspended in the same solution at a concentration of approximately 10 mg of cell protein per ml. Protein concentrations were measured by the method of Lowry, using bovine serum albumin as the standard. Each reaction mixture contained 0.25 μ mol of D-[U-¹⁴C]glucose (0.06 μ Ci/ml; about 10^5 cpm) 0.4 μ mol of adenosine triphosphate, 0.03 μ mol of MgCl₂, 0.8 μ mol of NaF, 8.0 μ mol of glycyl-glycine buffer (pH 7.5), and 0.4 to 0.6 mg of cell protein in a final volume of 0.25 ml. This mixture was incubated in a tube at 37°C, and the reaction was stopped after 30, 60, and 90 min by adding 1 ml of ice-cold, deionized water, followed by centrifugation. The supernatants were adsorbed to a column (lower diameter, 6 mm; upper diameter, 8.5 mm; length, 780 mm) of Bio-Rad AG1-X2 anion exchange resin (Cl⁻ form; 50 to 100 mesh), free D-[U-¹⁴C]glucose was washed from the column with 25 ml of deionized water, and the labeled products were eluted with 6 ml of 1.0 M LiCl. The eluate was mixed with scintillation liquor [333 ml of Triton X-100, 666 ml of toluene, 5.5 g of 2,5-diphenyl-oxazole, 125 mg of dimethyl-1,4-bis-2-(5-phenyloxazolyl)benzene] and counted with a Packard Tri-Carb liquid scintillation spectrometer. Radioactivity values were determined by correcting for the values obtained for parallel mixtures lacking adenosine triphosphate at zero time. Results were expressed as nanomoles of glucose phosphorylated per milligram of cell protein per minute. Hexokinase activities were also determined for *A. laidlawii* PG8^T, *A. granularum* BTS39^T, *M. arthritisdis* PG6^T, and *M. bovigentialium* PG11^T, which were used as positive and negative controls.

Carotenoid pigments. The abilities of the organisms to synthesize carotenoid pigments were tested by using the procedure of Razin and Cleverdon (15). The organisms were grown in 500 ml of the serum-free liquid medium described above supplemented with 0.5% sodium acetate. Carotenoid pigments were extracted from whole cells with 5 ml of boiling ethanol. The amounts of carotenoid pigments present were expressed as absorbance at 438 nm per milligram of cell protein.

Peptone requirement. Portions (0.1 ml; 10^4 colony-forming units [CFU] per ml) of a 48-h-old culture in serum-free medium of each isolate were inoculated onto peptone media. These peptone media contained 6.8 g of NaCl, 0.4 g of KCl, 0.2 g of MgSO₄ · 7H₂O, 0.1 g of Na₂HPO₄ · 12H₂O, 6.0 g of tris(hydroxymethyl)aminomethane, 2.5 mg of nicotinic acid, 2.5 mg of riboflavin, 12 g of Bacto-Agar (Difco), 20-g portions of various kinds of peptone (Phytone [BBL], Soytone [Difco], Bacto-Peptone [Difco], Difco Proteose Pep-

tone no. 3, or tryptone [Difco]), and 1,000 ml of distilled water, and the pH was adjusted to 7.8 with HCl. The plates were incubated at 37°C for 2 weeks, and the number of CFU on each medium was determined.

Polyacrylamide gel electrophoresis of cell proteins. The polyacrylamide gel electrophoresis procedure described by Razin and Rottem (16) for determining cell proteins was used. Cell proteins were dissolved in phenol-acetic acid-water (2:1:0.5, wt/vol/vol) and were run in polyacrylamide gels containing 5 M urea and 35% acetic acid.

Determination of deoxyribonucleic acid base composition. Deoxyribonucleic acid was prepared by the method of Marmur (11), which was modified to include phenol extraction steps in place of the chloroform-isomyl alcohol deproteinization (19). The guanine-plus-cytosine content of the deoxyribonucleic acid was determined by the method of Marmur and Doty (12), using an automatic recording spectrophotometer (Komatsu Electronics, Tokyo, Japan).

Serological techniques. Antisera against all of the type strains listed above except *A. morum* 72-043^T were prepared in rabbits by a previously described procedure (27); antiserum to *A. morum* 72-043^T, was kindly supplied by J. G. Tully. The growth inhibition test was performed with filter paper disks by the method of Clyde (5). Each antigen was diluted 1:100 and 1:1,000 (containing about 10^5 and 10^4 CFU/ml, respectively) in broth and then tested against undiluted antiserum. The indirect immunofluorescence technique with unfixed colonies was performed as described by Rosendal and Black (18).

RESULTS

Morphology and cultural characteristics. Colonies of strain H23M^T on media with and without horse serum after 5 days at 30 and 37°C are shown in Fig. 1. Colonies on serum-supplemented and serum-free media at 30°C exhibited a "fried-egg" shape, although colonies on serum-free medium were usually smaller; the colonies on media incubated at 37°C were of the rough type and had no peripheral zone. Strain H23M^T produced only slight turbidity in liquid medium. When they were examined by electron microscopy, cells from a 24-h-old broth culture of strain H23M^T exhibited coccobacillary forms (Fig. 2). The cells were bounded by a single, triple-layered plasma membrane. A serum-free broth culture of strain H23M^T containing 7×10^7 CFU/ml was passed through filters with pore diameters of 800, 450, 220, and 100 nm, yielding 2×10^7 , 5×10^6 , 3.5×10^3 , and 0 CFU/ml, respectively. Similar results were obtained with strain H7M. When each strain was subcultured serially five times on a medium without bacterial inhibitors, there was no colonial or cultural evidence of reversion to bacterial forms.

Sterol requirement. All of the isolates grew on the basal medium, and they grew almost equally well on all of the media tested. They did not exhibit any growth response to increasing cho-

FIG. 1. Colonies of strain H23M^T on agar after 5 days. (A) Colonies on agar containing 20% horse serum at 30°C. (B) Colonies on serum-free agar at 30°C. (C) Colonies on agar containing 20% horse serum at 37°C. (D) Colonies on serum-free agar at 37°C. Bar = 200 μ m.

lesterol concentrations in the medium. All of the isolates were resistant to 1.5% digitonin and 20% sodium polyanethol sulfonate.

Growth conditions. On media with and without serum, all of the isolates grew almost as rapidly at 22 as at 37°C. They failed to grow at 45°C. All of the isolates grew under both aerobic and anaerobic conditions.

Biochemical properties. None of the four strains fermented carbohydrates (including glucose, salicin, and esculin), hydrolyzed arginine or urea, or reduced tetrazolium chloride or methylene blue. All four strains reduced potassium tellurite and weakly hemolyzed horse, sheep, and guinea pig erythrocytes. After 14

days of incubation in the medium containing glucose, the pH did not differ by more than 0.5 U from the pH of the inoculated control in medium without glucose. In this test, the growth of the strains was confirmed by plate counts, and usually 10^7 to 10^8 CFU/ml was present in a 5-day-old culture. No strain produced any appreciable pH change in the glucose-containing medium when the inoculum was increased 100-fold or when the organism was serially subcultured up to 15 times in the glucose-containing medium. The type strains of eight currently recognized *Acholeplasma* species fermented glucose in the same test medium. In addition, the utilization of glucose was examined by determining hexokinase activity, which demonstrates the presence of the glycolytic pathway. The type strains of two glucose-fermenting *Acholeplasma* species, *A. laidlawii* and *A. granularum*, showed high hexokinase activity and phosphorylation of glucose, whereas the glucose-nonfermenting *M. arthritidis* and *M. bovis genitalium* strains tested had only low activities; the hexokinase activity of strain H23M^T was almost the same as the activities of the latter group (Table 1 and Fig. 3). Our results indicated that strain H23M^T does not ferment glucose.

Carotenoid pigments. Strain H23M^T and the type strains of *A. axanthum*, *A. modicum*, *A. equifetale*, and *A. hippikon* failed to produce carotenoid pigments, as determined by the method described above. The optical densities at 438 nm of the carotenoid pigments of *A. laidlawii*, *A. granularum*, and *A. oculi* were 57.3×10^{-3} , 36.9×10^{-3} , and 6.8×10^{-3} per mg of protein, respectively.

FIG. 2. Electron micrograph of a thin section of strain H23M^T cells grown on serum-free medium for 24 h at 37°C. Bar = 100 nm.

TABLE 1. Hexokinase activities of frozen and thawed cell preparations of selected members of the *Mycoplasmatales*

Strain	Reaction time at 37°C (min)	Amt of glucose phosphorylated		
		nmol/mg of protein	nmol/mg of protein per min	%
<i>A. laidlawii</i> PG8 ^T	30	538.5	17.95	80.0
	60	555.5	9.26	82.6
	90	521.6	5.80	77.6
<i>A. granularum</i> BTS39 ^T	30	425.2	14.17	60.0
	60	491.8	8.20	69.4
	90	504.8	5.61	71.2
<i>A. parvum</i> H23M ^T	30	2.7	0.09	0.7
	60	4.5	0.08	1.2
	90	5.2	0.06	1.3
<i>M. arthritidis</i> PG6 ^T	30	1.3	0.043	0.3
	60	1.9	0.034	0.5
	90	5.9	0.062	1.5
<i>M. bovis genitalium</i> PG11 ^T	30	1.7	0.057	0.3
	60	5.7	0.095	1.2
	90	6.6	0.073	1.3

Peptone requirement. The isolates grew best in Phytone- and Soytone-containing media, although the *Acholeplasma* type strains grew equally well in all of the peptone-containing media tested. With strain H23M^T, the number of CFU obtained per milliliter from cultures grown in the Phytone- and Soytone-containing media were usually 3 logs higher than the values obtained with the other peptone-containing media.

FIG. 3. Rates of phosphorylation of glucose by frozen and thawed cell preparations. Symbols: ■, *A. laidlawii* PG8^T; □, *A. granularum* BTS39^T; ●, *A. parvum* H23M^T; △, *M. arthritidis* PG6^T; ×, *M. bovis genitalium* PG11^T.

Electrophoretic patterns of cell proteins. The electrophoretic pattern of the cell proteins of strain H23M^T differed from the patterns of the type strains of *Acholeplasma* species (Fig. 4). Furthermore, the patterns of the type strains of the *Acholeplasma* species were distinct from one another.

Deoxyribonucleic acid base composition. The guanine-plus-cytosine content of the deoxyribonucleic acid of strain H23M^T was 29.1 mol%, as determined by the thermal melting point method, whereas the values obtained for *A. laidlawii* PG8^T and *A. granularum* BTS39^T were 30.4 and 29.8 mol%, respectively.

Serological properties. The four strains isolated from horses and the type strains of the *Acholeplasma* species were examined for antigenic relationships by reciprocal growth inhibition tests. None of the isolates showed a zone of growth inhibition around disks of antisera against the type strains of *A. laidlawii*, *A. granularum*, *A. axanthum*, *A. modicum*, *A. oculi*, *A. equifetale*, *A. hippikon*, and *A. morum*, and antiserum against strain H23M^T inhibited only the four new isolates. However, there was slight inhibition of both *A. modicum* and *A. morum* by *A. hippikon* antiserum and of *A. equifetale* by both *A. modicum* and *A. oculi* antisera. In each of these instances, the zone of inhibition was less than one-half the size of the zone produced by the homologous antiserum. Strain H23M^T also differed from the type strains of the *Acholeplasma* species when it was tested by the indirect immunofluorescence method (Table 2).

FIG. 4. Electrophoretic patterns of *Acholeplasma* cell proteins. Lane A, *A. laidlawii* PG8^T; lane B, *A. granularum* BTS39^T; lane C, *A. modicum* PG49^T; lane D, *A. axanthum* S743^T; lane E, *A. oculi* 19L^T; lane F, *A. equifetale* C112^T; lane G, *A. hippikon* C1^T; lane H, *A. parvum* H23M^T.

DISCUSSION

The isolates from horses were identified as members of the order *Mycoplasmatales* on the basis of morphology and passage through filters with a pore size of 220 nm. These isolates were placed in the family *Acholeplasmataceae* because of their growth in media without cholesterol and their resistance to digitonin. Growth of the isolates at 22°C is also consistent with membership in *Acholeplasmataceae*. Moreover, fried-egg colonial morphology was obtained by incubation at 30°C but not by incubation at 37°C, suggesting that the temperature for optimum growth is close to 30°C. The probable host of these isolates has not been identified. Because other *Acholeplasma* species have been isolated from various hosts (1, 2, 8–10, 22, 24), it is not reasonable at present to conclude that the horse is the specific habitat of the isolates described above. With respect to biological properties, the new isolates are clearly distinct from the eight currently recognized species of *Acholeplasma* (*A. laidlawii*, *A. granularum*, *A. axanthum*, *A. modicum*, *A. oculi*, *A. equifetale*, *A. hippikon*,

and *A. morum*). The new isolates did not ferment any carbohydrate that was hydrolyzed by the other acholeplasmas, and their hexokinase activities were low, like the activities of the nonfermentative organisms *M. arthritidis* and *M. bovis genitalium*.

The International Commission on Systematic Bacteriology Taxonomic Subcommittee has indicated that the ability of an organism to synthesize carotenoid pigments should be determined if the organism is being considered for membership in the family *Acholeplasmataceae* (21). In our study, the absence of carotenoids in the horse isolates was indicated by the lack of appreciable adsorption of light waves at 438 nm by extracts of 500-ml portions of broth cultures. Recently, Smith et al. have shown that *A. axanthum* (20) and *A. modicum* (13) can produce carotenoids when large volumes (yield from more than 50 liters) of cells and absorption at a shorter wavelength are used. If the same methods were used with the horse isolates, carotenoids might be detected also in these organisms.

Phytone and Soytone have significant effects on the growth of the isolates which we studied. The responses of the isolates to peptone indicated that these strains require an additional source of essential amino acids. However, it is difficult to determine the nutritional requirements of acholeplasmas. On initial isolation, *A. axanthum* strains S743^T and S410 were reported to require fatty acid supplements, in the form of Tween 80, for adequate growth (23). Cells from later passages of these organisms did not appear to need additional fatty acids (6). A more specific test will be required to determine the growth responses of our isolates to peptone.

From the results described above, we conclude that strains H23M^T, H7M, H15M, and H23V constitute a new species in the genus *Acholeplasma*. For this species we propose the name *Acholeplasma parvum* (par'vum. L. adj. *parvus* small, intended to refer to the poor

TABLE 2. Serological relationships of *Acholeplasma* strains, as determined by the indirect immunofluorescence test

Strain	Reciprocal indirect fluorescent antibody titer of rabbit antiserum against strain:								
	PG8 ^T	BTS39 ^T	S743 ^T	PG49 ^T	19L ^T	C112 ^T	C1 ^T	72-043 ^T	H23M ^T
<i>A. laidlawii</i> PG8 ^T	3,200	— ^a	—	—	200	—	200	—	—
<i>A. granularum</i> BTS39 ^T	—	3,200	—	400	100	—	200	100	—
<i>A. axanthum</i> S743 ^T	—	—	3,200	—	—	—	—	—	—
<i>A. modicum</i> PG49 ^T	—	—	—	3,200	—	—	—	—	—
<i>A. oculi</i> 19L ^T	400	—	100	—	3,200	200	200	—	—
<i>A. equifetale</i> C112 ^T	—	—	100	—	400	3,200	—	—	—
<i>A. hippikon</i> C1 ^T	200	—	—	—	200	—	6,400	200	—
<i>A. morum</i> 72-043 ^T	100	—	—	100	200	100	400	6,400	—
<i>A. parvum</i> H23M ^T	—	—	—	—	100	—	—	—	3,200

^a —, No fluorescence at a dilution of 1:100.

biochemical activities and tiny colonies of this organism). Strain H23M is the type strain of *A. parvum*, and a culture of this strain has been deposited in the American Type Culture Collection as strain ATCC 29892.

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