Mycoplasma subdolum, a New Species Isolated from Horses

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The growth characteristics, biochemical activities, and serological relationships of a group of mycoplasmas that were originally described as capable of splitting urea were reexamined. Most of the strains examined were isolated from the genital tract of mares and stallions or from the tissues of aborted equine fetuses, but one was obtained from the respiratory tract and another was obtained from the brain of a horse. The colonial morphology and growth characteristics of these isolates were those of the classical "large-colony" mycoplasmas rather than those of species of *Ureaplasma*. The mycoplasmas hydrolyzed arginine, were inhibited by sodium polyanethol sulfonate and digitonin, and failed to grow without cholesterol. Thus, they had the properties of a typical arginine-degrading species of *Mycoplasma*. The strains produced a slow but restricted increase in the pH of some types of urea-containing broth, but these pH changes were smaller and less rapid than those given by strains of *Ureaplasma*. Apart from these properties, the strains were unremarkable in their biochemical activities, although they could be distinguished from strains of *M. equirhinis*, an arginine-degrading species found in horses, by their failure to produce "film and spots" on serum-containing media. The serological results and electrophoresis of cell proteins suggest that the strains examined constitute a fairly homogeneous group. A representative strain, TB, was shown to be serologically distinct from strains of 48 species of *Mycoplasma* and of 3 species of *Acholeplasma*. These species included 8 found in horses and 24 which metabolized arginine or arginine and glucose. On the basis of these results, it is suggested that strain TB and related strains constitute a new species, for which the name *Mycoplasma subdolum* is proposed. Strain TB (= NCTC 10175 = ATCC 29870) is the type strain.

In an earlier communication (10), a group of mycoplasmas isolated in Germany from the cervix of infertile mares (15) and from aborted equine fetuses (13) was described as being able to degrade both urea and arginine. These strains produced a rise in the pH of urea-containing broth prepared by the method of Aluotto et al. (2), and it was thought that they were able to hydrolyze urea. According to the present classification of the *Mollicutes* (5), this property would have placed these strains in the urea-hydrolyzing genus *Ureaplasma*. Subsequently, C. J. Howard (personal communication) reported that the growth characteristics of a representative strain (strain TB) from infertile mares were not those of a species of *Ureaplasma*. The same strain was also examined by M. C. Shepard and C. D. Lunceford (personal communication) and found to be distinct in several respects from *Ureaplasma* species: the equine mycoplasma gave a negative reaction in two tests for urease production (25; M. C. Shep-

ard and C. D. Lunceford, Bacteriol. Proc., p. 83, 1970); its growth was not inhibited by acetohydroxamic acid (1,000 µg/ml), 5 iodo-2'-deoxyuridine (250 µg/ml), thallium acetate (1:500), or erythromycin (25 µg/ml); and its optimal reaction for growth was pH 7.4, not pH 6.0. Moreover, it grew in a medium supplemented with arginine (0.1%, wt/vol) but not with the same concentration of urea.

At about this time, two arginine-degrading strains of *Mycoplasma*, one from the genital tract and one from the respiratory tract of horses, were isolated at the Lister Institute and found to be serologically related to a representative of the German strains.

In the light of these observations, the earlier results were reappraised, and fuller investigations of nine strains isolated in Germany and of two isolated in Britain were carried out. This paper summarizes the characteristics of this group of mycoplasmas and presents evidence that it represents a new species of *Mycoplasma*.

MATERIALS AND METHODS

Mycoplasma strains. Strains TB, T11, T201, T205, T206, T414, and TE were isolated from the

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cervix of infertile mares as described by Krabisch et al. (15). Strains PC1618 and C98 were obtained from the lungs of aborted equine fetuses (13). All investigations of these nine strains were carried out at the Mikrobiologisches Institut der Tierärztlichen Hochschule, Hannover. Strain 910 was isolated from a nasopharyngeal swab from a young thoroughbred (21); four clones (A, B, C, and D) were examined. Strain H8360 was obtained from a cervical swab from an apparently healthy mare. It was isolated in two urea-containing media: U2 broth at pH 6.2 (8) and a urea-manganese agar (pH 6.5) prepared according to a schedule from the Mycoplasma Reference Laboratory (Norwich, England). H8360 clone A was derived from the isolate in U2 broth, and H8360 clone B was derived from that on urea-manganese agar. Strains 910 and H8360 were compared with strain TB at the Lister Institute, London. All 11 strains were cloned as recommended by the Subcommittee on the Taxonomy of the *Mycoplasmatales* (27) and were tested for reversion to a bacterial form by five to seven passages soon after isolation on media without penicillin or thallium acetate (10, 17).

Six other strains from horses were not cloned. Strain C56 from an aborted fetus and M234 from semen were isolated in Hannover; strain st2823 was isolated by J. Halen (Institut National de Recherches Veterinaires, Brussels) from semen. Three strains were sent by A. R. S. Moorthy (University of Queensland, Australia): PS12 from the penis of a stallion with breeding difficulty (18), AFSC4 from the stomach of an aborted fetus (19), and 233H/B from a brain.

The equine mycoplasmas *A. equifetale* C112, *A. hippikon* C1, *M. equigenitalum* T37, *M. equirhinis* M432/72, *M. felis* strains "*M. equipharyngis*" and equine N29B, *M. pulmonis* equine strain N14, and the unnamed strain *Mycoplasma* sp. equine N3 were those described by Kirchhoff (10-12) and Allam and Lemcke (1). The ureaplasma M3720 from a human genital tract was kindly supplied by B. E. Andrews (Mycoplasma Reference Laboratory). Mycoplasmas from other hosts were from the sources previously indicated (1, 10).

Media. The medium used in investigations carried out in Hannover was that described by Krabisch et al. (15); all cultures were incubated in air with 5% CO₂. At the Lister Institute, the medium was deoxyribonucleic acid (DNA) agar containing 10% (vol/vol) inactivated human serum (1) or broth containing 20% inactivated human serum (21). Fluid cultures were incubated aerobically, and cultures on agar were incubated in 5% CO₂ in N₂ (anaerobically) except for certain biochemical tests, in which duplicate plates were incubated aerobically in a moist atmosphere.

Morphology. Centrifuged deposits of broth cultures in the logarithmic phase (3,500 × *g* for 30 min) were examined by phase-contrast microscopy. Films of the deposits were also fixed in methanol, stained with Giemsa, and examined by light microscopy. For examination with an electron microscope (Phillips EM 300; accelerating voltage of 80 kV), centrifuged deposits were fixed, sectioned, and stained with ruthenium red as described by Howard and Gourlay (9).

Biochemical tests. Tests for carbohydrate fermentation, arginine hydrolysis, tetrazolium and tellurite

reduction, liquefaction of serum, hydrolysis of gelatin, digestion of casein, phosphatase production, and formation of "film and spots" were carried out as described by Aluotto et al. (2). All tests included known positive and negative cultures as controls. The German isolates were also tested for tetrazolium reduction in fluid medium and for tellurite reduction in media containing concentrations of the substrate lower than the recommended concentrations (2), which were somewhat inhibitory. In both laboratories film-and-spot formation was tested on a medium containing horse serum (20%, vol/vol) (2) and on media containing egg yolk (1, 6). Egg yolk emulsion (10%, vol/vol) was added to DNA medium containing inactivated human serum (10%, vol/vol), to a medium containing swine serum (20%, vol/vol), and to a medium containing horse serum (20%, vol/vol).

The growth of various strains in several types of urea-containing broth was examined. Strains TB, T11, T201, T205, T206, T414, TE, PC1618, and C98 were grown in urea broth prepared according to the formula of Aluotto et al. (2) and in the U9 medium described by Shepard and Lunceford (26). Four clones of strain 910, two clones of H8360, and strain TB were grown in three types of urea-containing broth. One, supplemented with inactivated horse serum, was prepared by the method of Aluotto et al. (2) and adjusted to pH 7.2. The second was a freeze-dried broth (FDTL) at pH 6.5, kindly supplied by G. D. Windsor (Wellcome Research Laboratories, Beckenham, Kent). The third was a modified DNA medium (1) containing inactivated pig serum (10%, vol/vol) and urea (0.05%, vol/vol) adjusted to pH 6.5. A recognized arginine-hydrolyzing strain, *M. arthritidis* PG6, and a ureaplasma, M3720, were included as controls. The inoculum, 0.02 ml of an actively growing culture, was added to 1 ml of medium in 2-ml screw-cap vials.

The ability to grow in a medium without serum or at 22°C was tested by the method of Leach (17). Inhibition by sodium polyanethol sulfonate and digitonin was tested by the disk method (7). The sterol requirements of strains TB and H8360 clone A were determined by the method of Edward (4), except that the basal medium consisted of DNA agar (1) without serum. The sterol-nonrequiring species *A. laidlawii* (PG8) was included in the tests as a control.

Hemolysis was tested by the overlay technique (2). Hemadsorption of erythrocytes to colonies was determined by the method of Del Giudice and Pavia (Bacteriol. Proc., p. 71, 1964). Both tests were performed with equine, bovine, ovine, canine, rabbit, guinea pig, and chicken erythrocytes.

Electrophoresis of cell proteins. Polyacrylamide disk electrophoresis was carried out in urea-acetic acid gels (23).

Antisera. For the preparation of antisera to strains TB, T11, T201, T205, T414, and PC 1618, organisms were cultivated in a medium containing: phosphate-buffered saline, 765 ml; inactivated swine serum, 200 ml; fresh yeast extract (25%, wt/vol), 10 ml; glucose, 2.5 g; lactalbumin hydrolysate, 5 g; thallium acetate (1.25%, wt/vol) 10 ml, and penicillin, 2 × 10⁶ IU. The mycoplasmas were passaged six to eight times in this medium to eliminate traces of horse serum. They were then washed twice in buffered saline, resuspended to

0.01 of the initial volume, emulsified in the same volume of complete Freund adjuvant (Difco Laboratories), and administered to rabbits by the method of Morton and Roberts (20). Cultures grown in rabbit serum broth were used to prepare antiserum to strain H8360 clone A by a method previously described (1). Sources of antisera against recognized species of *Mycoplasma* are listed in Table 1. Antisera from several sources were available against some species.

Serological tests. Disk growth inhibition (GI) tests were performed by the method of Clyde (3), and metabolism inhibition (MI) tests were performed by the method of Purcell et al. (22) with arginine as the substrate. The indirect fluorescent-antibody (IFA) test was carried out on colonies grown on agar (24).

RESULTS

A description of the 11 isolates follows.

Growth characteristics. Although different types of agar medium were used in the two laboratories, all of the isolates produced "fried-egg" colonies typical of species of *Mycoplasma* or *Acholeplasma*. Examination by phase-contrast or of Giemsa-stained preparations showed that broth cultures consisted mainly of small coccal and coccobacillary forms. Some ring forms of various sizes were also visible in phase-contrast preparations. Electron microscopy of thin sections revealed predominantly round or oval forms. The limiting membrane had a typical trilaminar structure. Outside of the membrane a small amount of amorphous material was shown by the ruthenium red stain, but there was no true capsule. The internal structure comprised electron-dense ribosomes, together with a fine reticulum of DNA (Fig. 1). The nine German strains were filterable through membranes (Millipore Corp.) with average pore diameters of 450 and 220 nm, with a decrease of about 10^2 colony-forming units per ml. The two British isolates easily passed a 450-nm filter during cloning but were not otherwise tested for filterability. None of the strains reverted to a bacterial form after five to seven passages on inhibitor-free media.

All of the strains were inhibited by sodium polyanethol sulfonate and digitonin. In addition, the German strains failed to grow at 22°C and could not be passaged in serum-free medium; the British strains were not thus tested. In tests for sterol requirement, washed, filtered suspensions of strains TB and H8360 clone A produced no colonies on the basal sterol-free agar or on basal medium supplemented with bovine serum albumin (fraction V; 0.5%, wt/vol) and palmitic acid (10 µg/ml) but without cholesterol. The same inocula produced heavy growth on basal medium supplemented with human serum (20%, vol/vol) or with bovine serum albumin (fraction V), palmitic acid, and various concentrations (5

to 100 µg/ml) of cholesterol. Growth was less heavy at a cholesterol concentration of 1 µg/ml, and no growth occurred at 0.2 µg/ml. In contrast, the sterol-independent strain *A. laidlawii*, which was used as a control, grew on basal medium alone or on basal medium supplemented only with bovine serum albumin (fraction V) and palmitic acid.

Biochemical properties. The biochemical reactions of the strains are summarized in Table 2. Results obtained in the two laboratories were consistent, apart from phosphatase production and the formation of film and spots. Phosphatase production was observed under aerobic conditions in Hannover, but only anaerobically at the Lister Institute. Film-and-spot formation was observed in only one laboratory and then only on the medium containing 20% (vol/vol) horse serum and 10% (vol/vol) egg yolk emulsion. Six of the nine strains tested were positive on this medium.

During exhaustive testing of the nine German strains against erythrocytes from seven species, the type of hemolysis that developed was found to be influenced by various factors, especially the age, size, and density of the mycoplasma colonies. Beta-hemolysis was frequently observed around well-isolated colonies, but it could not be consistently produced. As with other *Mycoplasma* species, the type of hemolysis was variable, even when the sensitive overlay technique was used.

None of the strains tested in Shepard U9 urea broth produced an increase in pH after incubation for 14 days. As previously noted (10), strains grown in urea broth prepared by the method of Aluotto et al. (2) produced an increase in pH. Increases of 0.4 to 1.0 pH unit occurred over a period of 5 to 15 days. Similarly, increases of 0.7 to 1.0 and 0.9 to 1.3 pH units were noted in FDTL and DNA broth, respectively. The increases in pH often occurred gradually over a period of several days. *M. arthritidis* produced similar changes. In contrast, ureaplasma M3720 caused the pH of all three media to rise to at least 8.0 within 18 to 42 h.

Comparison of the new arginine-degrading strains. The 11 strains that were cloned appear to constitute a homogeneous serological group. The nine German strains were tested against antisera to six of them, including strain TB, in both GI and IFA tests. All of the strains were inhibited in GI tests to the same extent with any one antiserum. Inhibition zones were 3 to 8 mm in diameter, depending on the serum and culture conditions. In IFA tests, all strains fluoresced strongly with each antiserum at 1:500, the highest dilution tested. No inhibition or fluorescence was observed with any of the corre-

TABLE 1. *Mycoplasma strains and antisera used in serological tests*^a

Species	Tests carried out at:			
	MIT		LI	
	Strain	Source of antiserum	Strain	Source of antiserum
<i>Acholeplasma</i>				
1. <i>A. laidlawii</i> ^b	PG8	MA	NT	
2. <i>A. equifetale</i> ^b	C112	MIT	NT	
3. <i>A. hippikon</i> ^b	C1	MIT	NT	
<i>Mycoplasma</i> : arginine degrading				
4. <i>M. hominis</i>	PG21	MA and NIH	SC4	LI
5. <i>M. salivarium</i>	PG20	MA and NIH	B3	LI
6. <i>M. orale</i>	CH19299	MIT and MA	837	LI
7. <i>M. buccale</i>	CH20247	MA and NIH	CH20247	NIH
8. <i>M. faucium</i>	DC333	IRC	DC333	NIH
9. <i>M. primum</i>	HRC292	MIT and NIH	Navel	LI
10. <i>M. arginini</i>	G230	MIT and NIH	G230	NIH
11. <i>M. arthritidis</i>	PG6, PG27	NIH and MA	PG6, PG27	NIH and LI
12. <i>M. gateae</i>	CS	MIT	CS	M. Gois
13. <i>M. gallinarum</i>	PG16	MIT and NIH	Fowl, PG16	LI and NIH
14. <i>M. spumans</i>	PG13	MIT and IRC	PG13	NIH
15. <i>M. maculosum</i>	PG15	MIT and IRC	PG15	NIH
16. <i>M. alkalescens</i>	BBS.803	NIH	L52/D12	MRL
17. <i>M. iners</i>	PG30	NIH	M (avian)	LI
18. <i>M. hyosynoviae</i>	S16	CVM	S16	IRC
19. <i>M. meleagridis</i>	17529	IRC	17529	NIH
20. <i>M. lipophilum</i>	MaBy	IRC	MaBy	M. Barile
21. <i>M. canadense</i>	NT		NCTC 10152	MRL
22. <i>M. equirhinis</i> ^b	M432/72	MIT	M432/72	LI
23. <i>M. opalescens</i>	MH5408	MIT	NT	
<i>Mycoplasma</i> : arginine degrading and glycolytic				
24. <i>M. fermentans</i>	PG18	MIT and MA	PG18	LI and NIH
25. <i>M. capricolum</i>	California kid	IRC	pp. goat	J. Tully
26. <i>M. moatsii</i>	MK405	IRC	NT	
27. <i>M. alvi</i>	NT		Ilseley	IRAD
<i>Mycoplasma</i> : Arginine nondegrading and nonglycolytic				
28. <i>M. agalactiae</i>	PG2	IRC	Agalactia	LI
29. <i>M. bovis genitalium</i>	PG11	MIT and NIH	PG11	LI and NIH
30. <i>M. verecundum</i>	107	MIT	107	IRAD
31. <i>M. bovis</i>	NT		Ab/1	IRAD
<i>Mycoplasma</i> : glycolytic				
32. <i>M. felis</i> ^b	" <i>M. equipharyngis</i> "	MIT	Equine N29B	LI
			CO	LI
33. <i>M. equigenitalium</i> ^b	T37	MIT	NT	
34. <i>M. pulmonis</i> ^b	PG34	MIT	Equine N14	LI
35. <i>Mycoplasma</i> sp. equine N3 ^b	NT		N3	LI
36. <i>M. pneumoniae</i>	FH, Eaton	MIT and MA	FH	LI and NIH
37. <i>M. hyorhinis</i>	S7, GDL	MIT and MA	S7	NIH
38. <i>M. bovoculi</i>	M165/69	IRC	M165/69	MRL
39. <i>M. canis</i>	PG14	MIT	PG14	NIH
40. <i>M. edwardii</i>	PG24	MIT	PG24	IRC
41. <i>M. neurolyticum</i>	ATCC 15649	MIT	KSA, type A	LI and NIH
42. <i>M. gallisepticum</i>	PG31	MIT	X95, PG31	LI and NIH
43. <i>M. bovirhinis</i>	PG43	MIT	PG43	NIH
44. <i>Mycoplasma</i> sp. bovine group 7	NT		PG50	MRL
45. <i>M. dispar</i>	NT		462/2	IRC
46. <i>M. mycoides</i> subsp. <i>mycoides</i>	NT		G1/61	LI
47. <i>M. mycoides</i> subsp. <i>capri</i>	NT		PG3	LI and NIH
48. <i>M. conjunctivae</i>	NT		HRC 581	M. Barile
49. <i>M. feliminutum</i>	NT		Ben	IRC
50. <i>M. anatis</i>	NT		1340	NIH
51. <i>Mycoplasma</i> sp. avian group C	NT		AM	LI

^a Abbreviations: NT, not tested; MIT, Mikrobiologisches Institut der Tierärztlichen Hochschule, Hannover, Germany (H. Kirchhoff and J. Heilmann); MA, Microbiological Associates, Bethesda, Md., U.S.A.; NIH, National Institutes of Health, Bethesda, Md., U.S.A. (reference reagents); LI, Lister Institute, London, England (R. Lemcke); IRC, FAO/WHO International Reference Center for Animal Mycoplasma, Aarhus, Denmark (E. A. Freundt); MRL, Mycoplasma Reference Laboratory, Public Health Laboratory Service, Norwich, England (R. H. Leach); CVM, College of Veterinary Medicine, Iowa State University, Ames, Iowa, U.S.A. (R. F. Ross); IRAD, Institute for Research on Animal Diseases, Compton, Berkshire, England (C. J. Howard and R. N. Gourlay).

^b Species found in horses.

FIG. 1. Electron micrograph of strain TB; 24-h-old culture in 20% human serum broth, thin-section preparation stained with ruthenium red. Bar represents 200 nm. Inset: Higher magnification showing triple-layered unit membrane (M). Bar represents 100 nm.

sponding preimmunization sera. In MI tests with antisera to strains TB and T11, all nine strains gave titers of $>1/1,024$. Clones of the two British isolates and strain TB were inhibited in GI tests by antiserum to the cervical strain H8360 clone A but not by the corresponding preimmune serum.

The cell proteins of strains TB, 910 clone A, and H8360 clone A were also compared. The electrophoretic patterns of these are shown in Fig. 2 in comparison with those given by two strains of another arginine-degrading mycoplasma, *M. arthritidis*. It can be seen that those of TB, 910, and H8360 are almost identical but are different from those of the two strains of *M. arthritidis*.

Of the uncloned isolates, strains C56, M234, and st2823 were identified as belonging to the same group by GI, IFA, and MI tests in which antiserum to strain TB was used. The Australian isolates PS12, AFSC4, and 233H/B were inhibited by antiserum against H8360 clone A in GI tests but not by the corresponding preimmunization serum or by antisera to 18 other arginine-degrading species of *Mycoplasma*.

Comparison with other equine orga-

nisms. The nine German strains were tested in GI, IFA, and MI tests against six other organisms found in horses: *Acholeplasma laidlawii*, *A. equifetale*, *A. hippikon*, *Mycoplasma equigentialium*, *M. equirhinis*, and *M. felis* ("*M. equipharyngis*"). Reciprocal tests of these strains against antisera to TB, T11, T201, T205, T414, and PC1618 were also carried out. No cross-reactions were observed between the nine strains and strains of any of the other species.

Strain TB, four clones of strain 910, and two clones of strain H8360 were compared in GI and IFA tests with strains of four species of *Mycoplasma* found in horses: *M. equirhinis*, *M. felis* (equine strain N29B and feline strain CO), *M. pulmonis* (equine strain N14 and rodent strains Ash and M1), and *Mycoplasma* sp. equine N3. These organisms were also tested against antiserum to strain H8360 clone A. No cross-reactions were observed.

The new arginine-degrading strains were thus serologically distinct from three species of *Acholeplasma* and five species of *Mycoplasma* found in horses. Of these, only *M. equirhinis* hydrolyzed arginine; the rest were glycolytic.

Comparison with other arginine-degrad-

TABLE 2. *Biochemical reactions of a new arginine-degrading mycoplasma*

Reaction	Strains tested at:	
	MIT ^a (aerobic/ anaerobic)	LI ^b (aerobic/ anaerobic)
Metabolism of glucose	-/-	-/-
Metabolism of other carbohydrates ^c	-/-	NT ^d
Hydrolysis of arginine	+/+	+/+
Phosphatase production	+/-NT	-/+
Serum liquefaction	-	-/NT
Hydrolysis of gelatin	-	NT
Hydrolysis of casein	-	NT
Tetrazolium reduction	-/- ^e	-/-
Tellurite reduction: 0.005%	v/-NT	v/-
0.0025%	v/NT	NT
0.00125%	v/NT	NT
Film-and-spot formation	v ^f /NT	-/-
Hemolysis of erythrocytes:		
Sheep, horse, cattle, dog, rabbit, guinea pig, chicken	α - β ^h	α - α'
Hemadsorption:		
Sheep, horse, cattle, dog, rabbit, guinea pig, and chicken erythrocytes	-	NT

^a MIT, Mikrobiologisches Institut der Tierärztlichen Hochschule, Hannover, Germany. Strains TB, T11, T201, T205, T206, T414, TE, FC168, and C98.

^b LI, Lister Institute, London, England. Strains TB, 910 (four clones), and H8360 (two clones).

^c Lactose, maltose, saccharose, galactose, fructose, arabinose, sorbitol, rhamnose, xylose, mannose, trehalose, dulcitol, inositol, and salicin.

^d NT, Not tested.

^e Tested in fluid and solid media.

^f v, Variable.

^g Six of nine strains positive on medium containing 20% (vol/vol) horse serum and 10% (vol/vol) egg yolk emulsion; negative on other media.

^h Beta-hemolysis around well-isolated colonies.

ing mycoplasmas. In Hannover, strain TB was tested in GI, IFA, and MI tests against antisera to all of the arginine-degrading and all of the arginine-degrading and glycolytic strains listed in Table 1 except those of *M. canadense* and *M. alvi*. The eight other German isolates and strains T11, T201, T205, T206, T414, and TE were similarly tested against 12 of these species (Table 1, no. 4 to 7, 9 to 15, and 24). No inhibition of TB or related strains was observed in GI tests with any of the antisera. Nor was there any inhibition in MI tests at 1:4, the lowest antiserum dilution tested. No fluorescence was obtained in IFA tests with any of the antisera at dilutions of 1:10 and 1:50. Reciprocal tests of strains of the named species against antisera to strain TB and related strains were not carried out.

At the Lister Institute, strains TB, 910 (four

clones), and H8360 (two clones) were tested in GI tests against antisera to all of the arginine-degrading and all of the arginine-degrading and glycolytic strains listed in Table 1 except those of *M. opalescens* and *M. moatsii*. None of the antisera inhibited these strains, whereas all except the antiserum to *M. maculosum* gave satisfactory zones of inhibition (>2 mm of complete inhibition) with the homologous species. Reciprocal GI tests of all the species except *M. opalescens* and *M. moatsii* against antiserum to H8360 clone A were also carried out. None of the species was inhibited by this antiserum, which produced inhibition zones at least 5 mm in diameter with strains TB, 910, and H8360.

In addition to GI tests, IFA tests with antiserum to strain H8360 clone A were performed on *M. alkalescens*, *M. hyosynoviae*, *M. meleagridis*, *M. lipophilum*, *M. canadense*, *M. equirhinis*, *M. capricolum*, and *M. alvi*. Reciprocal IFA tests on strain TB were also carried out with antisera to six of these species; antisera suitable for IFA were not available against *M. meleagridis*, *M. lipophilum*, or *M. canadense*. No cross-reactions were observed between strain TB and any of these species in IFA tests.

If the results from the two laboratories are considered together, strain TB appears to be distinct by at least two serological tests from 24 species of *Mycoplasma*, including the equine mycoplasma *M. equirhinis*, that share its ability to hydrolyze arginine.

FIG. 2. Polyacrylamide disk electrophoresis of cell proteins of strain TB (A), strain H8360 clone A (B), strain 910 clone A (C), and two strains of *M. arthritidis* (PG6 [D] and PG27 [E]).

Comparison with arginine-nondegrading mycoplasmas. Strain TB was tested by GI, IFA, and MI against antisera to three of the four arginine-nondegrading, nonglycolytic *Mycoplasma* species listed in Table 1 (no. 28 to 30) and to eight species of glycolytic mycoplasma (Table 1, no. 36 to 43) from hosts other than the horse. The eight other German strains were also tested against most of these antisera. No reactions were observed between the new arginine-degrading strains and any of these antisera.

Strains TB, 910 (four clones), and H8360 (two clones) were tested by GI or IFA against the same range of antisera and also against antisera to *M. bovis* and eight other glycolytic *Mycoplasma* species (Table 1, no. 31 and 44 to 51). None of these antisera reacted with the new arginine-degrading strains. All of the antisera except that against *M. agalactiae* gave a satisfactory homologous reaction in at least one type of test. When the homologous reaction was unsatisfactory in either type of test, antiserum to H8360 clone A was tested against that species.

Thus, the results of the two laboratories indicate that strain TB and the strains related to it are serologically distinct from 4 arginine-nondegrading, nonglycolytic *Mycoplasma* species and 16 glycolytic *Mycoplasma* species from hosts other than horses.

DISCUSSION

The arginine-degrading mycoplasmas represented by strain TB were first isolated from the cervix of mares (15) and from the tissues of aborted equine fetuses (13). Of the 17 strains examined in the course of the studies reported here, 8 were obtained from the cervix, 3 were obtained from the semen or penis of stallions, and 4 were obtained from aborted fetuses. Of the two remaining strains, one was isolated from a brain at postmortem, but the significance of its presence in this location is uncertain. The other, strain 910, was isolated from a nasopharyngeal swab during a survey that involved repeated swabbing of selected horses in seven stables (21). Although a total of 227 nasopharyngeal swabs was examined during this survey and 151 were positive for mycoplasmas, strain 910 was the only isolate belonging to this group. Moreover, this type of mycoplasma was never isolated during earlier investigations of mycoplasmas in equine respiratory tracts (1, 14). Present evidence thus suggests that this equine mycoplasma is primarily a parasite of the genital tract. In humans, *M. hominis*, a mycoplasma found in the genital tract, is also sometimes isolated from the oropharynx.

The morphology and growth characteristics of these organisms, their filterability, and their fail-

ure to revert to bacterial forms suggest that they are typical mycoplasmas. Since sterol dependence was demonstrated in two representative strains and all of the strains included in the study were inhibited by sodium polyanethol sulfonate and digitonin, it is apparent that they should be included in the sterol-requiring genus, *Mycoplasma*. Moreover, all of the strains metabolized arginine, a property not detected in any recognized sterol-nonrequiring *Acholeplasma* species. Although the ability of these strains to grow in certain types of urea broth and produce an increase in pH was at first misleading, their colonial morphology and growth characteristics were different from those of ureaplasmas. In addition, a pH increase did not occur in all types of urea broth, and the direct test for urease gave no evidence of urease activity in a representative strain (Shepard and Lunceford, personal communication). It is possible that the increase in pH observed in some types of urea broth was due to traces of arginine in some of the medium constituents. Nevertheless, the ability of these strains to grow in urea broth and the isolation of one strain (H8360) on media designed for ureaplasmas shows that they are able to grow and replicate at pH values below those generally recommended for species of *Mycoplasma*.

Apart from their ability to degrade arginine, the biochemical reactions of strain TB and the strains related to it were not distinctive. The only property that was of practical use for distinguishing these strains from those of *M. equirhinis*, the other arginine-degrading mycoplasma found in horses, was their failure to produce film and spots on serum-containing media. Whereas *M. equirhinis* consistently produced this phenomenon on all types of serum-containing media if incubation was extended beyond 2 to 3 days, strain TB and related strains never did so. In fact film-and-spot formation was observed only on a medium containing egg yolk as well as 20% (vol/vol) serum and then not with all strains.

Serologically the strains appear to form a homogeneous group. Comparison of the electrophoretic patterns of cell proteins, although restricted to three strains, two from the genital and one from the respiratory tract, also indicates this. In accordance with the principles laid down by the Subcommittee on the Taxonomy of the *Mycoplasmatales* (27), the most intensive serological comparisons were made with those mycoplasmas found in the same host and those having the same metabolic characteristics, in this case the ability to hydrolyze arginine. However, comparisons with arginine-nondegrading species were also made by at least one serological method. Overall, no cross-reactions were ob-

served with any of the 3 species of *Acholeplasma* or the 48 species of *Mycoplasma* listed in Table 1. It therefore seems justifiable to regard this group (nine German strains and two British strains) of arginine-degrading mycoplasmas from horses as a new species of *Mycoplasma*, for which the name *Mycoplasma subdolum* sp. nov. (sub'do.lum. L. adj. *subdolum* somewhat deceptive) is proposed. The specific epithet alludes to the somewhat deceptive color change produced by the organism in some types of urea-containing broth that resulted in the original description of the strains as urea hydrolyzing. Strain TB, which has been examined in both laboratories, is the type strain of *M. subdolum*. Cultures of this strain have been deposited in the National Collection of Type Cultures (NCTC), London, and in the American Type Culture Collection (ATCC), Rockville, Md., where they have been assigned the numbers 10175 and 29870, respectively.

At present there is little evidence that *M. subdolum* is pathogenic to its host. Although the original cervical isolates were obtained from a group of infertile mares, this mycoplasma was isolated from only a small percentage of the mares (15). Moreover, strain H8360 was obtained from the cervix of an apparently normal mare. Four strains were obtained from the tissues of aborted fetuses, but several species of *Acholeplasma*, the glycolytic species *M. equigenitalium*, and *M. bovis genitalium* have also been isolated from aborted fetuses (10, 11, 13, 16, 19). Thus, no consistent pattern of infection has emerged to implicate *M. subdolum* as a cause of fetal wastage in the horse. Of the three strains obtained from stallions, that from the penis came from an animal with breeding difficulties (18), but there was no information about the two animals that yielded positive semen samples. It is clear that no conclusions can be drawn from such fragmentary evidence and that the pathogenicity of *M. subdolum* requires further investigation.

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