Acholeplasma equifetale and *Acholeplasma hippikon*, Two New Species from Aborted Horse Fetuses

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Six *Acholeplasma* strains, C1, C112, C117, C581, C589, and PC536, isolated from the lungs, and in one case also from the liver, of aborted horse fetuses exhibited serological properties distinguishing them from the known *Acholeplasma* species. From the results obtained in growth inhibition, metabolic-inhibition, indirect immunofluorescence, and double-immunodiffusion tests, it can be concluded that these strains belong to two new species, for which the names *Acholeplasma equifetale* and *Acholeplasma hippikon* are proposed. *A. equifetale* contains strains C112, C117, C581, and C589, of which C112 (= ATCC 29724) is the type strain. *A. hippikon* contains strain C1 and PC536, of which C1 (= ATCC 29725) is the type strain.

Recently a number of publications have reported on the isolation of *acholeplasmas* from a great variety of vertebrate hosts. In horses, *acholeplasmas* were found in the respiratory tract by Allam et al. (2) and by Ogata et al. (12), and we isolated *acholeplasmas* from the lungs and livers of aborted horse fetuses (11). Ten strains were cultivated (11). Of these, four were identified as *Acholeplasma laidlawii*. The remaining six isolates did not belong to any of the known *Acholeplasma* species: *A. laidlawii*, *A. granularum*, *A. axanthum*, *A. modicum*, and *A. oculi*. These unidentified organisms were studied to determine whether they belong to new, heretofore undescribed, species.

MATERIALS AND METHODS

Acholeplasma strains. All of the *Acholeplasma* strains used in this study were isolated from the lungs of aborted horse fetuses; one of them was also isolated from liver. The designations and sources of the strains are given in Table 1. The *Acholeplasma* strains used for comparative serological examinations are listed in Table 2. The six isolates were cloned three times by picking single colonies. The fluid culture was filtered through a 450-nm filter, and dilutions of the filtrate were plated on solid medium to produce well-separated colonies.

Culture medium. The *Acholeplasma* strains were cultivated in a medium of the following composition: heart infusion broth (Oxoid), 720 ml; unheated horse serum, 200 ml; 25% (wt/vol) fresh yeast extract, 10 ml; deoxyribonucleic acid (Sigma Chemical Co.), 0.02 g; 1.25% (wt/vol) thallium acetate, 10 ml; and penicillin, 2×10^6 IU. Agar plates were prepared by adding 10 g of agar (Oxoid) to the fluid medium.

Morphological studies. *Acholeplasma* colonies were studied by using a stereo microscope (Leitz). Organisms from fluid cultures were studied by dark-field and phase-contrast microscopy (Leitz).

Filtration characteristics. Filterability of the

Acholeplasma strains was determined by using Swinex-25 adapters and membrane filters (Millipore Corp.) with a pore diameter of 450 or 200 nm.

Reversion experiments. The isolated strains were passaged seven consecutive times on a substrate without penicillin and thallium acetate. Passages one to five, inclusive, were performed on solid media by sliding inverted agar blocks across the agar surfaces. In the sixth passage, the agar blocks were inoculated into noninhibiting fluid medium and, after incubation, the organisms were examined by dark-field microscopy. The seventh passage was made on solid medium.

Differentiation of *acholeplasmas* from mycoplasmas. The ability of the organisms to grow at 22°C and in medium without serum was tested. The test for cholesterol requirement was carried out as described by Edward (7). The following substrates were used: (i) serum-free medium (meat extract, 10 g; peptone [Oxoid], 10 g; NaCl, 5 g; agar [Oxoid], 10 g; distilled water, 1 liter); (ii) as (i) but with 19 µg of palmitic acid per ml and 0.5% bovine serum albumin added; (iii) as (ii) but with 5 µg of cholesterol per ml added. Inhibition of growth by sodium polyanethanol-sulfonate (Liquoid, Roche Diagnostics) and by digitonin was tested by the disk method (9).

Biochemical tests. Breakdown of the following carbohydrates (1%) was determined by the method of Aluotto et al. (3): glucose, lactose, maltose, saccharose, galactose, fructose, arabinose, sorbitol, rhamnose, xylose, mannose, trehalose, dulcitol, inositol, and salicin. The *acholeplasmas* were also examined for their ability to hydrolyze arginine (1%) and urea (1%), to reduce 2,3,5-triphenyltetrazolium chloride (0.2%) and potassium tellurite (0.00125, 0.0025, and 0.005%), to hydrolyze gelatin, to digest casein and coagulated serum, and to produce phosphatase (3). The production of "film and spots" (8) was determined on an agar medium supplemented with 10% egg yolk emulsion (Oxoid) and 20% horse serum and on a medium containing 10% egg yolk emulsion and 20% swine serum.

Production of carotenoids. The ability to synthesize carotenoid pigments was investigated in a medium containing sodium acetate according to the procedure

TABLE 1. Designations and sources of the strains studied

Strain designation	Source
C1	Lung of aborted horse fetus
C112	Lung of aborted horse fetus
C117	Lung of aborted horse fetus
C581	Lung and liver of aborted horse fetus
C589	Lung of aborted horse fetus
PC536	Lung of aborted horse fetus

TABLE 2. Sources of the *Acholeplasma* strains used for serological comparison

<i>Acholeplasma</i> strain	Source ^a
<i>A. laidlawii</i> A PG8	NIH, catalog no. M-728-001-584
<i>A. granularum</i> BTS-39	FAO/WHO International Reference Center for Animal Mycoplasmas, Aarhus, Denmark
<i>A. oculi</i> 19L	FAO/WHO International Reference Center for Animal Mycoplasmas, Aarhus, Denmark
<i>A. modicum</i> PG49	FAO/WHO International Reference Center for Animal Mycoplasmas, Aarhus, Denmark
<i>A. axanthum</i> S-743	NCTC, catalog no. 10138

^a Abbreviations: FAO/WHO, Food and Agricultural Organization, World Health Organization; NIH, National Institutes of Health, Bethesda, Md.; NCTC, National Collection of Type Cultures, London, England.

of Razin and Cleverdon (14), as subsequently modified by Tully and Razin (18).

Hemolysis and hemadsorption. Hemolysis was tested by the overlay technique (3). Hemadsorption was determined as described by Sobeslavsky et al. (16). Both tests were performed with equine, bovine, ovine, canine, rabbit, guinea pig, and chicken erythrocytes.

Electrophoresis of cell proteins. Electrophoretic patterns of the cell proteins of strains C112 and C1, as well as of the *Acholeplasma* strains listed in Table 2, were obtained using the techniques described by Razin and Rottem (15) and as modified for horizontal flat-gel polyacrylamide electrophoresis by Boden and Kirchhoff (4).

Preparation of antisera. For the preparation of antisera, organisms were cultivated in the following medium: phosphate-buffered saline, 765 ml; heated swine serum, 200 ml; 25% (wt/vol) fresh yeast extract, 10 ml; glucose, 2.5 g; hydrolyzed lactalbumin, 5 g; 1.25% (wt/vol) thallium acetate, 10 ml; and penicillin, 2×10^6 IU. The *acholeplasmas* were passaged six to

eight times in this medium to eliminate traces of horse serum. They were then washed two times in buffered saline, resuspended in 0.01 of the initial volume, emulsified in the same volume of complete Freund adjuvant (Difco), and administered to rabbits by the method of Morton and Roberts (10). The sera produced by this method were used for the serological tests.

Serological tests. The *acholeplasmas* were compared by using the disk growth inhibition test (GIT) of Clyde (5), the metabolic-inhibition test (MIT) as described by Taylor-Robinson et al. (17), the indirect immunofluorescence test (IFT) of colonies (6), and the double-immunodiffusion technique (13). For the GIT, agar plates without yeast extract and with serum fraction (1%) were used. Each antigen was diluted 1:100, 1:500, and 1:1,000 and was then tested against undiluted antiserum. The plates were incubated at 37°C and read shortly after development of colonies. The MIT was performed in microtiter plates. Besides undiluted antigen, dilutions of 1:10, 1:100, 1:1,000, and 1:10,000 were tested against the serum dilutions in a medium containing glucose or maltose (1%), horse serum (20%), and yeast extract (1%) in Difco PPLO broth. The microtiter plates were incubated at 37°C. In the GIT and MIT, tests with the optimal antigen concentration were selected. In the IFT, only young colonies (1 and 2 days old) were used because autofluorescence was encountered with older colonies. For gel diffusion, organisms were washed twice in saline (0.25 M), suspended in 1% sodium deoxycholate in phosphate-buffered saline (pH 8.5), representing a 100-fold concentration, and kept overnight at room temperature. The extract was centrifuged (20,000 $\times g$ for 30 min), and the supernatant, after dialysis against distilled water, was used as antigen.

RESULTS

Morphological and cultural characteristics. Colonies of all isolates studied exhibited the typical "fried-egg" morphology, with a marked central spot, on solid media. Growth in fluid media reached a maximum between 2 and 3 days of incubation at 37°C. All isolates grew both aerobically and under 5% CO₂. Phase-contrast and dark-field microscopy of the fluid cultures showed pleomorphic organisms, with coccoid elements dominating. The colonial and cellular morphologies of the isolates were indistinguishable from those of established species of the genus *Acholeplasma*.

Filterability and reversion. All of the strains passed 450- and 200-nm filters with a decrease of about 10² colony-forming units per ml. There was no reversion to cell wall forms during seven passages on a medium without penicillin and thallium acetate.

Differentiation of *acholeplasmas* from mycoplasmas. All of the isolates multiplied at 22°C. Growth occurred on substrate containing cholesterol (iii) as well as on substrate with palmitic acid and bovine serum albumin (ii)

added and on substrate without supplements (i). All of the isolates were resistant to 20% sodium polyanetholsulfonate and 1.5% digitonin.

Biochemical characteristics. The results of the biochemical investigations of the isolates are given in Table 3. All of the strains showed the same biochemical features. They were found to ferment various carbohydrates aerobically as well as anaerobically. All of the investigated carbohydrates were utilized; however, acid production varied, depending on the carbohydrates used, and was not always reproducible. Strong acid production (pH 5 and lower) occurred in media containing saccharose, glucose, fructose, maltose, or galactose. The degree of acid production with a given carbohydrate appeared to correlate closely with the number of colony-forming units that developed. All strains were found to be unable to hydrolyze arginine and urea. Reduction of 2,3,5-triphenyltetrazolium chloride was observed in fluid medium only, and even then it was not always reproducible; reduction did not occur on solid medium. Potassium tellurite was reduced by all strains, and the reduction was influenced by various factors, especially the size of the colonies, the duration of incubation, and the concentration of the potassium tellurite. Potassium tellurite reactions were negative on plates with dense growth; large, single colonies usually developed a black coloration, indicative of a positive reaction. The intensity of the coloration increased with the length of incubation. Generally, the most intense coloration was attained after 7 days of incubation. All of the isolates were inhibited by a 0.005% concentration of potassium tellurite; therefore, lower concentrations (0.0025 and 0.00125%) were used. The intensity of the col-

oration was not influenced by a diminution of the concentration of the potassium tellurite.

Digestion of coagulated serum, hydrolysis of gelatin, digestion of casein, and phosphatase activity were not observed. Production of "film and spots" was found on a medium with 10% egg yolk emulsion and 20% horse serum after incubation for 7 days but not on a medium with 10% egg yolk emulsion and 20% swine serum.

Production of carotenoids was not observed.

Hemolysis occurred with equine, bovine, ovine, canine, rabbit, guinea pig, and chicken erythrocytes. Hemadsorption did not occur with these erythrocytes.

Electrophoresis of cell proteins. The electrophoretic pattern of the cell proteins of C1 differed from that of strain C112, and both patterns differed from those of type strains of established *Acholeplasma* species (listed in Table 2) (Fig. 1).

Serology. In serological investigations, the six *Acholeplasma* strains were tested with antisera against strains C1, C112, and C589 by IFT, GIT, and MIT (Table 4). The investigations indicated that the *Acholeplasma* isolates belong to two serologically different groups. Strains C1 and PC536 belong to one group, and strains C112, C117, C581, and C589 belong to another group.

For comparison with the type strains of the established *Acholeplasma* species listed in Table 2, strains C1 and C112 were used in the IFT, GIT, MIT, and double-immunodiffusion test. In the IFT, some cross-reactions were observed (Table 5). Compared with the titers obtained for the homologous antisera, these were on a very low level.

Cross-reactions between the type strains of

TABLE 3. *Characteristics of six Acholeplasma strains isolated from horses*

Characteristic	Reactions ^a of <i>Acholeplasma</i> strain:					
	C1	C112	C117	C581	C589	PC536
Fermentation of carbohydrates	+	+	+	+	+	+
Hydrolysis of arginine	—	—	—	—	—	—
Hydrolysis of urea	—	—	—	—	—	—
Reduction of tetrazolium chloride	(+)	(+)	(+)	(+)	(+)	(+)
Reduction of potassium tellurite	+	+	+	+	+	+
Digestion of coagulated serum	—	—	—	—	—	—
Hydrolysis of gelatin	—	—	—	—	—	—
Digestion of casein	—	—	—	—	—	—
Production of phosphatase	—	—	—	—	—	—
Production of "film and spots"	+	+	+	+	+	+
Production of carotenoids	—	—	—	—	—	—
Hemolysis of equine, bovine, ovine, canine, rabbit, guinea pig, and chicken erythrocytes	+	+	+	+	+	+
Adsorption of equine, bovine, ovine, canine, rabbit, guinea pig, and chicken erythrocytes	—	—	—	—	—	—

^a +, Positive reaction; —, negative reaction; (+), variable reaction.

FIG. 1. Electrophoretic patterns of the cell proteins of the type strains of established *Acholeplasma* species and of strains C1 and C112. (A) *A. laidlawii* PG8, (B) *A. granularum* BTS39, (C) *A. modicum* PG49, (D) *A. axanthum* S-743, (E) *A. oculi* 19L, (F) strain C112, and (G) strain C1.

TABLE 4. Serological comparison of the isolated *Acholeplasma* strains with antisera against C1, C112, and C589 in IFT, GIT, and MIT

Strain	Antiserum against C1			Antiserum against C112			Antiserum against C589		
	IFT ^a	GIT ^b	MIT ^c	IFT ^a	GIT ^b	MIT ^c	IFT ^a	GIT ^b	MIT ^c
C1	+	5-7	>1,024	-	1-2	-	-	-	-
C112	-	-	-	+	6-8	>1,024	+	5-6	256
C117	-	-	-	+	4-5	512	+	4-5	256
C581	-	-	-	+	5	512	+	4-5	512
C589	-	-	-	+	4-6	>1,024	+	4-6	>1,024
PC536	+	3-5	>1,024	-	-	-	-	-	-

^a +, Positive reaction; -, negative reaction.

^b Inhibition zone in millimeters.

^c Reciprocals of serum titer.

Acholeplasma species listed in Table 2 were also observed in the GIT (Table 6). The cross-reactions appeared as zones of inhibition 1.5 to 3.0 mm in diameter. Zones of inhibition in homologous reactions ranged between 4 and 8 mm. Strain C112 showed a weak cross-reaction (2 mm) with antiserum against *A. granularum* BTS-39. Strain C112 was slightly inhibited (1- to 2-mm zone of inhibition) by antisera against *A. laidlawii* PG8, *A. granularum* BTS-39, and strain C1. Cross-reactions relating to strains C112 and C1 were generally weaker than those that occurred between the type strains of the *Acholeplasma* species listed in Table 2.

The greatest serological specificity, and therefore the greatest difference between the established acholeplasmas and the horse strains, was observed with the MIT (Table 7). Cross-reactions with antisera against the strains of the other *Acholeplasma* species were observed with *A. modicum* PG49. Strains C112 and C1 did not cross-react with other acholeplasmas.

The results of the gel diffusion tests are shown

in Table 8. The reactions of the antigens with the homologous and heterologous antisera gave six to eight and one to three precipitation lines, respectively.

DISCUSSION

The morphology of the colonies, the pleomorphic cell form in fluid culture, the passage through 450 and 200-nm filters, and the stability on noninhibiting substrates indicate that the isolates studied belong to the order *Mycoplasmatales*.

Growth on serum-free and cholesterol-free medium, growth at 22°C, and resistance to sodium polyanethol sulfonate and digitonin indicate that the isolates belong to the family *Acholeplasmataceae*. In biochemical investigations, all of the isolates showed the same features. The serological differences observed between isolates C1 and PC536, on the one hand, and isolates C112, C117, C581, and C589, on the other, indicate that the isolates belong to two different taxa. Representative strains of each

TABLE 5. *Serological relationships of Achleplasma strains determined by indirect immunofluorescence tests*

Antigen	Antiserum against:						C112	C1
	<i>A. laidlawii</i> PG8	<i>A. granu- larum</i> BTS- 39	<i>A. axan- thum</i> S-743	<i>A. modicum</i> PG49	<i>A. oculi</i> 19L			
<i>A. laidlawii</i> PG8	2,000 ^a							
<i>A. granularum</i> BTS-39		3,500						
<i>A. axanthum</i> S-743			6,000					200
<i>A. modicum</i> PG49		100	500	8,000				
<i>A. oculi</i> 19L	100				8,000			
C112						2,500		
C1								6,000

^a Figures are reciprocals of serum titers.TABLE 6. *Serological relationships of Achleplasma strains determined by growth inhibition tests*

Antigen	Antiserum against:						C112	C1
	<i>A. laidlawii</i> PG8	<i>A. granu- larum</i> BTS- 39	<i>A. axan- thum</i> S-743	<i>A. modicum</i> PG49	<i>A. oculi</i> 19L			
<i>A. laidlawii</i> PG8	5-8 ^a	2-3	2				1-2	1-2
<i>A. granularum</i> BTS-39	1.5-2	5-8						
<i>A. axanthum</i> S743			5-7					
<i>A. modicum</i> PG49	1.5	2-2.5	2-2.5	4-6	1.5-2.5			2-2.5
<i>A. oculi</i> 19L	2-3	2-3	2-2.5		5-7 ^b			2-2.5
C112		2				6-8		
C1	2	1-2				1-2		5-7

^a Figures are zones of inhibition in millimeters.^b Occasionally, some colonies grew within the zone of inhibition.TABLE 7. *Serological relationships of Achleplasma strains determined by the metabolic-inhibition test*

Antigen	Antiserum against:						C112	C1
	<i>A. laidlawii</i> PG8	<i>A. granu- larum</i> BTS- 39	<i>A. axan- thum</i> S-743	<i>A. modicum</i> PG49	<i>A. oculi</i> 19L			
<i>A. laidlawii</i> PG8	512 ^a							
<i>A. granularum</i> BTS-39		>1,024						
<i>A. axanthum</i> S-743			128					
<i>A. modicum</i> PG49	128	32	16	>1,024		8		8
<i>A. oculi</i> 19L					>1,024			
C112						>1,024		
C1								>1,024

^a Figures are reciprocals of serum titers.TABLE 8. *Serological relationships of Achleplasma strains determined by the double-immunodiffusion technique*

Antigen	Antiserum against:						C112	C1
	<i>A. laidlawii</i> PG8	<i>A. granu- larum</i> BTS- 39	<i>A. axan- thum</i> S-743	<i>A. modicum</i> PG49	<i>A. oculi</i> 19L			
<i>A. laidlawii</i> PG8	6 ^a	2	1	2	2	1	1	1
<i>A. granularum</i> BTS39	3	8	2	2	2	3	1	1
<i>A. axanthum</i> S-743	2	1	6	1	1	2	1	1
<i>A. modicum</i> PG49	2	2	3	7	2	1	1	1
<i>A. oculi</i> 19L	1	3	1	2	7	3	1	1
C112	2	2	1	1	2	6	2	2
C1	2	3	1	2	3	2	7	7

^a Figures are numbers of precipitation lines.

group (C1 and C112) differed from the established *Acholeplasma* species when tested by the IFT, GIT, MIT, and double-immunodiffusion test. Finally, the electrophoretic patterns of the cell proteins of strains C1 and C112 differed from each other and from those of the type strains of *Acholeplasma* species listed in Table 2.

From these results, it can be concluded that the six isolates belong to two new species of the genus *Acholeplasma*. For the species containing strains C112, C117, C581, and C589, the name *Acholeplasma equifetale* (L. noun *equus* horse; L. adj. *fetalis* fetal; M.L. adj. *equifetale* pertaining to the horse fetus) is proposed. For the other species, which contains strains C1 and PC536, the name *Acholeplasma hippikon* (Gr. adj. *hippikon* pertaining to the horse) is proposed. The type strain of *A. equifetale* is C112 and the type strain of *A. hippikon* is C1. These strains have been deposited in the American Type Culture Collection (ATCC), Rockville, Md., under the numbers 29724 and 29725, respectively, and in the National Collection of Type Cultures (NCTC), London, England.

Recently, it was brought to my attention that an acholeplasma which may belong to *A. equifetale* was found by Allam and Lemke (1) in the respiratory tract of an apparently healthy horse.

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REPRINT REQUESTS

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