Acholeplasma bactoclasticum sp. n., an Anaerobic Mycoplasma from the Bovine Rumen

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A strain of a strictly anaerobic, filterable, bacteriolytic microorganism has been isolated from the bovine rumen. It has the microscopic and colonial morphology characteristic of mycoplasmas and is resistant to penicillin G. Sterols are not required for growth, and physiological properties show it to be distinct from *Acholeplasma laidlawii*, *A. granularum*, and *A. axanthum*. It is proposed that the organism be named *Acholeplasma bactoclasticum* sp. n. The type strain is ATCC 27112.

Microorganisms causing a partial digestion of rumen bacterial cells included in an agar culture medium were initially thought to meet their energy needs by a fermentation of protein (6, p. 79). A rapid digestion of the casein in skim milk was observed, and addition of glucose to the medium did not greatly increase the size of the colonies.

The work reported here describes the results of experiments and observations identifying a similar microorganism as a new species of *Acholeplasma* establishing some of its characteristics.

MATERIALS AND METHODS

Bacterial strain. The strain reported here was isolated from rumen fluid siphoned from a fistulated Jersey heifer. It was deposited in the American Type Culture Collection, Rockville, Md. as ATCC 27112.

Preparation of media and supplements. The organism was cultured anaerobically under 100% CO₂ by the roll-tube technique (7). Cysteine (0.03%) and NaHCO₃ (0.05%) (both wt/vol in final concentration) were added anaerobically with a syringe before autoclaving. The 3% Na₂S·9H₂O solution routinely added to provide a low redox potential was prepared by dissolving the crystals in boiled deionized water cooled under O₂-free N₂, tubed anaerobically under N₂, and autoclaved. Final concentration in the medium was 0.03% Na₂S·9H₂O. Carbohydrate solutions were autoclaved under CO₂ to minimize alkaline destruction during sterilization. Sulfide and carbohydrate solutions were injected aseptically and anaerobically into the melted medium held at 48 C. The osmotic pressure of the media used was about 7 atmospheres, and the pH was 6.8.

Skim milk was autoclaved in 10-ml volumes under N₂ in butyl-stoppered tubes.

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A mineral salts basal medium was prepared by adding four volumes of deionized water to one volume each of mineral solutions A (0.3% KH₂PO₄, 0.6% NaCl, 0.3% (NH₄)₂SO₄, 0.06% MgSO₄·7H₂O, 0.06% CaCl₂·2H₂O) and B (0.3% K₂HPO₄), all w/v.

Minfav medium. Minerals salts-volatile fatty acid-vitamin (Minfav) medium was prepared by adding vitamins and volatile fatty acids to the mineral salts medium. The vitamin solution contained the following, dissolved in 500 ml of water: biotin, 1 mg; calcium pantothenate, 200 mg; folic acid, 1 mg; inositol, 1,000 mg; niacin, 200 mg; riboflavine, 100 mg; and thiamine hydrochloride, 200 mg. Each 10 ml of medium contained 10 μ liters of this solution. The volatile acid mixture contained 17 ml of acetic acid, 6 ml of propionic acid, 4 ml of butyric acid, 1 ml of *n*-valeric acid, 1 ml of isovaleric acid, and 1 ml of DL-methyl butyric acid; 0.03 ml of the mixture was neutralized with NaOH and added to each 10 ml of culture.

CRF medium. Clarified rumen fluid (CRF) medium was similar to the mineral salts medium except that two volumes of rumen fluid freed of cells by centrifugation were substituted for two volumes of deionized water. Rumen fluid was centrifuged at 25,000 $\times$ g for 15 min, and the supernatant fluid was added to the cooled mineral salts solution. Rumen fluid medium (RF medium) was prepared by using uncentrifuged rumen fluid freed of protozoa and plant material.

Skim milk medium. This medium, used for viable counts, contained 0.2 ml of sterile skim milk added to 4.25 ml of rumen fluid-agar medium.

CC medium. Concentrated cells medium (CC medium), relatively free of protozoa and plant material, was prepared by allowing the rumen liquor, as collected, to incubate at 38 C. Fermentation gases carried plant matter to the surface, the protozoa sank to the bottom, and the rumen fluid containing bacteria was drawn off from the middle part of the container with a 50-ml pipette.

This rumen fluid was centrifuged at 25,000 $\times$ g for 15 min. The pellet was suspended with CRF medium to one-tenth the original volume, and one part of this

10X concentrated bacterial suspension was added to one part of mineral salt solution and one part of deionized water.

Tryptone (1%, Difco), 3% tryptone-soy broth (Oxoid), 1.3% nutrient broth (Oxoid), and 3.7% brain heart infusion (Oxoid) were prepared under 100% CO₂ and buffered with 0.5% NaHCO₃.

Isolation procedures. Samples of rumen fluid were siphoned from a fistulated Jersey heifer and poured into a flask until it was almost full. The flask was stoppered, insulated, and quickly transferred to the laboratory. Oxygen-free CO₂ was bubbled through the sample to displace the air above it, and 0.5 ml of the stirred fluid was diluted with 1-ml syringes through tubes of anaerobic medium. Lower dilutions were in sterile 33% RF medium and higher ones were in the CC medium containing 1.5% agar, which was melted and held at 48 C. The viable count was not detectably changed by holding as long as 2 h at this temperature before rolling the tubes in ice water. Tubes were always rolled within 30 min after inoculation. The cultures were incubated at 39 C or 45 C and examined periodically for clear zones due to partial digestion of the rumen bacteria in the medium.

Pure cultures were obtained by picking individual colonies with a Pasteur pipette drawn out to a thin capillary and subculturing in agar medium until, in two successive dilution series from a colony in high dilution, only the clearing colonies appeared and their numbers in successive tubes decreased in approximate agreement with the dilution. There were no problems with contamination.

Hydrogen measurement. Hydrogen was measured on a Perkin-Elmer thermal conductivity vapor fractometer 154B with a silica gel column and N₂ carrier gas. The instrument detected 10³ μmol of H₂ in a 0.5-ml sample.

The volume of excess gas in the culture and control tubes was measured at atmospheric pressure with a 5- or 10-ml syringe and injected back into the tube after each measurement. A sample in excess of 0.5 ml was then drawn into a water-lubricated, sterile 1-ml glass syringe through a 21-gauge needle. The tip of the needle was drawn into the rubber of the stopper to allow the gas to come to atmospheric pressure. The volume was noted, the needle was withdrawn, the volume was reduced to 0.5 ml, and the gas was injected into the fractometer.

Ammonia determination. When ammonia was to be measured, the (NH₄)₂SO₄ (3 g/liter) in the mineral salts solution was omitted, and Na₂SO₄ (2.2 g/liter) was added. Ammonia was assayed by the method of Ternberg and Hershey (11).

Gas chromatography of fatty acids. Samples of culture medium were centrifuged for 15 min at 25,000 × g, and then a 0.7-ml sample was cooled to 4 C and acidified with 0.3 ml of 85% phosphoric acid. After acidification, the sample was rapidly filtered through a 0.22-μm membrane filter by using a glass syringe with a "Swinney" adapter, and kept at 4 C before injection directly into a Hewlett-Packard 700 flame ionization, dual-column gas chromatograph fitted with a stainless steel column (60 by 1/8 inches) packed with FFAP (14% polyethylene glycol dinitrophthalic acid ester on chromosorb W). The carrier

gas was helium at a flow rate of 13 ml/min. The column temperature was 160 C.

Determination of fermentation products with uniformly labeled ¹⁴C-galactose. The ¹⁴C-galactose was supplied in 20% ethanol solution. A 12-μliter amount was transferred with a micro-syringe to each of two culture tubes. The ethanol was evaporated in a stream of air, and 0.6 ml of 9% galactose was added to each tube under CO₂. Sterile, anaerobic RF medium was prepared, and 9.4 ml of the medium was added to the galactose which had been sterilized under CO₂. Control tubes lacked the ¹⁴C-galactose. Initial 1-ml samples were removed after inoculation with 0.1 ml of a CRF/milk culture, and the cultures were incubated at 43 C for 1 week.

A 1-ml amount of 10 N sulfuric acid was injected through the stopper of the culture tube, and the tube was shaken. The released CO₂ was collected in a water-lubricated 10-ml syringe while the tube and syringe were shaken to insure equilibrium of CO₂ between gas and liquid. The volume of gas in the syringe as well as the pressure were noted at room temperature, and the gas was injected into a stoppered flask containing 10 ml of 2,2,2-nitrotetraethanolamine, a sample of which was assayed with the scintillation counter.

Samples of the acidified culture (0.5 or 1.0 ml) were chromatographed on a Celite column by the method of Wiseman and Irvin (13). Samples of the eluant were assayed for ¹⁴C in a liquid scintillation counter (Nuclear-Chicago Mk II).

The ¹⁴C in trichloroacetic acid-insoluble material was assayed by collecting the 5% cold trichloroacetic acid precipitate from a sample of culture on a 0.45-μm membrane filter (Millipore Corp.). The filter was washed with 5% trichloroacetic acid, dried, and dissolved in the counting fluid, 0.4 g of 1,4 bis[2-(5 phenyloxazoly)]-benzene (POPOP, Packard Corp.) and 4 g of 2,5 diphenyloxazole in 1 liter of toluene.

Preparation of samples for electron microscopy. Colonies in CRF-agar were prefixed for 1 h in a Kellenberger buffer containing 0.2% glutaraldehyde, 0.1 g of OsO₄/10 ml, and sufficient sucrose to make the buffer and the medium isotonic. The buffer contained 5 ml of Veronal acetate (2.94 g of sodium barbiturate, 1.94 g of hydrated sodium acetate, and 3.4 g of NaCl in 300 ml), 13 ml of water, 0.25 ml of CaCl₂ (0.01 M), and 7.0 ml of 0.1 M HCl. The pH of the buffer was adjusted to 6 with HCl.

After prefixation, the colonies were rinsed with buffer and fixed overnight in buffer containing 1% osmium tetroxide. The agar blocks containing the fixed colonies were dehydrated in alcohol, washed in propylene oxide, and embedded in "Bojax #1A" (52.1% dodecenyl succinic anhydride, 34.7% Araldite 60005, 1.7% dibutyl phthalate, and 11.5% Epon 812). Benzyltrimethylamine was added as a catalyst, and the embedded blocks were sectioned with a diamond knife.

Serology. The slide agglutination test was used to detect cross-reaction of the antigen with various antisera (Table 1). The antisera were diluted in 0.85% NaCl, and one drop of each dilution was mixed with one drop of antigen suspension. The mixture was gently shaken on a rotary shaker for 3 min, and then

the various dilutions were examined for flocculated cells.

The antigen was grown in 4-liter cultures of 33% CRF medium with vitamins and volatile fatty acids added. After harvesting, the cells were washed once and suspended in diluent to give an optical density at 600 nm of 3.0. The diluent was prepared by mixing equal parts of 0.067 M KH_2PO_4 (9.073 g/liter) and 0.067 M NaHPO_4 (9.465 g/liter), giving a pH of 6.8. This was diluted 10 times with 0.85% NaCl, and aqueous merthiolate was added to a final concentration of 1:10,000.

Lytic activity. CRF-milk culture supernatant fluid containing 0.1% mercaptoethanol was precipitated with 65% ammonium sulfate under 100% CO_2 . After centrifugation in air, the precipitate was taken up in 0.1 M phosphate buffer (pH 7) containing 0.1% mercaptoethanol, dialyzed, and concentrated by pressure dialysis with a UM-2 membrane (Diaflow, Amicon Corp.). For measurement of lytic activity, small samples of this concentrated solution were added to substrate in stoppered tubes containing 100% CO_2 -

bicarbonate buffer (pH 7), 0.1% mercaptoethanol, and 1 ml of H_2S . Incubation was at 50 C.

Escherichia coli murein sacculi were prepared by the method of Martin and Frank (9).

RESULTS

Numbers in the rumen. Table 2 shows the colony counts of samples of rumen contents collected at different times from fistulated cows, a sheep, hay, and water. Penicillin G (5,000 units/ml) could be added to decrease the growth of most bacterial colonies, allowing surer detection of the microorganism with no decrease in count.

The hay tested (5 g) was collected from the cow's stall at the time a rumen fluid sample was taken. It was mixed with 180 ml of autoclaved rumen fluid and broken up in a Waring blender under CO_2 . A 5-g sample of the rumen fluid

TABLE 1. *Strains of Mycoplasma and Acholeplasma from bovine and avian sources for slide agglutination*

Organism	Strain	Origin	Source
<i>M. bovis genitalium</i>	B6P	Fabricant	Bovine vagina
<i>M. bovis genitalium</i>	1367	UCD ^a	Bovine vagina
<i>M. bovis mastitidis</i>	B38P	Fabricant	Bovine mastitic tissue
<i>M. bovis mastitidis</i>	01	UCD	Bovine mastitic tissue
<i>M. bovis rhinis</i>	FXT	Fabricant	Bovine joints
<i>A. laidlawii</i>	B6P	Fabricant	Bovine endometrium
<i>A. laidlawii</i>	1063	UCD	Tank milk
<i>A. laidlawii</i> subsp. <i>inocuum</i>	1527	H. E. Adler	Chicken sinus
3507 I	B142P	Fabricant	Bovine vagina
3509 K	B74P	Fabricant	Bovine nose
3511 M	B144P	Fabricant	Bovine joints

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TABLE 2. *Culture counts of cytotlastic organisms*

Expt. no.	Date	Source	Diet	Colony-forming units/ml
1	11/4/65	Jersey rumen contents	Alfalfa hay	4×10^6
2	12/11/65	Jersey rumen contents	Alfalfa hay	6×10^5
3	2/1/66	Jersey rumen contents	Alfalfa hay	4×10^6
4 ^a	3/11/68	Jersey rumen contents	Alfalfa hay + grain	9×10^6
5 ^a	3/11/68	Holstein 1 rumen contents	Alfalfa hay + grain	3×10^4
6 ^a	3/11/68	Holstein 2 rumen contents	Alfalfa hay	1×10^6
7 ^a	11/4/68	Jersey rumen contents	Alfalfa hay + grain	4×10^6
8 ^a	3/28/69	Sheep rumen contents	Alfalfa pellets	3×10^5
9 ^a	3/30/69	Sheep rumen contents	Alfalfa pellets	6×10^5
10	8/30/69	Jersey rumen contents	Alfalfa hay	4×10^6
		Jersey rumen contents ^b	Alfalfa hay	4×10^6
		5 g of alfalfa hay		0
11	10/27/69	Jersey rumen contents	Alfalfa hay	1.4×10^5
		Jersey rumen contents	Alfalfa hay	2.0×10^5
		Drinking water		8×10^0
		Drinking water		1.4×10^1

^a Penicillin G (5,000 $\mu\text{liters/ml}$) in medium.

^b Sample mixed in Waring blender to simulate treatment of hay sample.

FIG. 1. Photomicrographs of fresh mounts of cultures in clarified rumen fluid medium.

was similarly treated. Counts were made by decimal dilution of the samples through 30% CRF-agar containing *E. coli* cells plus penicillin and compared with similar counts made without blending. Samples of drinking water from the stall contained a few colony-forming units probably derived from cells entering the water as the animal drank. Clearing zones were not detected in agar series inoculated from two samples of human feces and one sample of deer rumen contents (*Odocoileus hemionus hemionus*).

Morphology. The organism is gram negative, coccoid, and varies in size from less than 0.5 to 2.0 μ m in diameter. Figure 1a shows the size variability typical in CRF-agar medium. In liquid culture, clusters of cells and short chains are common (Fig. 1b and c). Large cells frequently appear to have surface projections (Fig. 1d and e) which are difficult to resolve with phase microscopy. These large cells are fragile and lyse easily in wet mounts. In old liquid cultures and in the centers of colonies, ghosts of these large cells, sometimes containing inclusions, are common.

Electron micrographs of sections through agar-grown colonies (Fig. 2) show cells of irregular outline bounded by a unit membrane, with no distinguishable cell wall. Some of the sections appear to be through clusters of cells similar to those seen with phase microscopy. The small membrane-bound vesicles may represent sections through projections from larger cells or through the smallest colony-forming units.

Surface colonies on CRF-agar reach a diameter of 1 to 1.5 mm after 5 days of incubation at 43 C. They appear white when viewed with reflected light and have the morphology typical of the *Mycoplasmatales* (Fig. 3 and 4). The colonies stain blue with Dienes stain (2).

Comparison with some known strains. The

fistulated animals in which the microorganism has been found have all appeared healthy over a 5-year period. A milk (0.2 ml) CRF culture in which the casein had cleared was injected into the yolk sac of chicken embryos, with autoclaved culture and sterile saline injected into controls. Table 3 shows the number of surviving embryos after 7 days of incubation. No deaths occurred in a second passage when yolk from two of these eggs was inoculated into 7-day yolk sacs. No viable cells developed in 30% CRF-milk broth inoculated from yolk sacs of

FIG. 2. Electron micrographs of a section through a colony grown on the surface of clarified rumen fluid agar.

FIG. 3. Photomicrographs of surface colonies. Bar represents 1 mm.

FIG. 4. Photomicrograph of surface colony. Magnification $\times 75$.

TABLE 3. Effect of inoculation of 7-day chicken embryos with the new microorganism

Inoculum	First passage		Second passage	
	No. of embryos inoculated	No. of embryos dead (7 days)	No. of embryos inoculated	No. of embryos dead (7 days)
Viable culture	18	1	18	0
Autoclaved culture	15	1		
Sterile saline	12	0	12	0

either group. No symptoms were detectable after accidental self-inoculation of one author with about 0.2 ml of viable culture.

Antisera against two strains of *M. bovis genitalium*, two of *M. mastitidis*, one strain of *M. bovirhinis*, and three strains of *A. laidlawii* did not agglutinate the microorganism when its homologous titer was 180. *M. gallisepticum* was not agglutinated by antiserum against the new isolate.

Lytic activity. Colonies growing in CRF-skim milk medium were surrounded by a somewhat oval zone of clearing (Fig. 5) and smaller zones were seen around colonies in CC-agar medium. Suspensions of autoclaved rumen bacteria were lysed by ammonium sulfate-precipitated culture supernatant fluid (Fig. 6), whereas live cells

were less readily attacked. A similar result was obtained with autoclaved and live suspensions of *E. coli*, *Salmonella typhimurium*, and *Spirillum serpens* (Table 4). Murein sacculi of *E. coli* were lysed by a similar preparation (Fig. 7).

Freeze-dried *M. lysodeikticus* cells (Bactolyszyme Substrate, Difco) were not lysed by the culture supernatant fluid. Autoclaving and chloroform-methanol (2:1) extraction, which facilitated lysis of *E. coli*, *S. typhimurium*, and *S. serpens*, did not render the *M. lysodeikticus* cells susceptible to lysis (Table 5).

Other properties. The microorganism failed to grow on agar plates of CRF medium containing cysteine and Na_2S , either aerobically or under CO_2 in Brewer jars. Growth occurred anaerobically in roll tubes of the same

FIG. 5. Colony of *Acholeplasma bactoclasticum* surrounded by clearing due to casein digestion.

addition of either penicillin or thallium acetate, and has not developed penicillin sensitivity.

Guinea pig, cow, or sheep blood at a concentration of 5% (vol/vol) in CRF-agar medium containing 10 μ mol of galactose per ml

FIG. 6. Lysis of live and autoclaved rumen bacteria by ammonium sulfate-precipitated lytic factor. Autoclaved cells ($\square$), autoclaved cells control ($\blacksquare$), live cells ($\circ$), live cells control ($\bullet$).

TABLE 4. Lysis of heat-killed bacterial cells by ammonium sulfate precipitate of culture supernatant

Substrate ^a	Initial OD	Final OD	OD change in 100 min
Autoclaved cells			
<i>Spirillum serpens</i>	.46	.12	-.32
<i>S. serpens</i> control	.44	.36	-.10
<i>Salmonella typhimurium</i>	.41	.14	-.27
<i>S. typhimurium</i> control	.29	.27	-.02
<i>Escherichia coli</i>	.80	.29	-.51
<i>E. coli</i> control	.64	.56	-.08
Live cells			
<i>Spirillum serpens</i>	.56	.19	-.37
<i>S. serpens</i> control	.55	.25	-.30
<i>Salmonella typhimurium</i>	.48	.39	-.09
<i>S. typhimurium</i> control	.36	.31	-.05
<i>E. coli</i>	.95	.89	-.06
<i>E. coli</i> control	.72	.64	-.08

^a In the control tubes the lytic factor was boiled in buffer before substrate addition.

medium at 36, 42, and 45 C but could not be detected after 2 weeks of incubation at 30 or 47 C. Growth was inhibited by 20 μ g of terramycin/ml but not by 5,000 units of penicillin G per ml. Thallium acetate (0.025%) was not inhibitory in our experiments, but Allison and Robinson (*personal communication*) indicated that the thallium may have been precipitated by the sulfide used as a reducing agent. The microorganism has been subcultured weekly for 4 years in liquid medium without

was not hemolyzed. Suspensions of tristearin, olive oil, maize oil, or tributyrin added to a similar medium at 1% (v/v) concentration were not cleared. Arginine was not fermented.

The organism was filtered through a 0.45- μ m filter (Millipore Corp.). At 10 lb/in² positive pressure, about 2% of a suspension of 5.1×10^5 cells/ml was cultured from the filtrate. On other occasions, a few cells were recovered after passing a broth culture through a 0.22- μ m membrane filter.

FIG. 7. Decrease in turbidity of R-layer suspension during incubation with ammonium sulfate-precipitated lytic factor in phosphate buffer (pH 6.8) at 50 C. Factor (▲), boiled factor (●).

TABLE 5. Incubation of *M. lysodeikticus* cells in culture supernatant fluid

Substrate ^a	OD change in 60 min
Autoclaved <i>E. coli</i>	-.33, -.29
Autoclaved <i>E. coli</i> control	-.11
<i>M. lysodeikticus</i>	-.02, -.02
<i>M. lysodeikticus</i> control	-.01, -.01
Autoclaved <i>M. lysodeikticus</i>	+.01, 0
Autoclaved <i>M. lysodeikticus</i> control	0
Extracted <i>M. lysodeikticus</i>	+.02, 0
Extracted <i>M. lysodeikticus</i> control	+.01, 0

^a In the control tubes the lytic factor was boiled in buffer before substrate addition.

TABLE 6. Change in OD at 4 days in subcultures in mineral salts plus *E. coli* medium supplemented with various nutrients

Supplement	Subculture					
	1	2	2a	3 ^a	4 ^a	5 ^a
I None	-.08	-0.05	-0.40 ^b	-0.36	-0.05	0
II Volatile fatty acid mixture	-0.09	-0.02	-0.29 ^b	-0.31	0	0
III 10 μg of cholesterol/ml	-0.08	-0.03	-0.25 ^b	-0.22	-0.06	0
IV Vitamin mixture	-0.11	0	-0.37 ^b	-0.36	-0.06	0
V Hemin 0.001% (wt/vol)	-0.11	-0.01	-0.34 ^b	-0.39	-0.06	0
VI Volatile fatty acid, hemin vitamins	-0.41	-0.41		NM ^c	NM	NM

^a Subcultures 3, 4, and 5 of all except supplement VI are from subculture 2a of the respective supplements.

^b Tube 2 incubated further after inoculation with 0.1 ml of 33% CRF-milk culture.

^c NM, Not measured.

Nutrition. Mixed rumen bacteria added to 2% CRF medium increased cell counts (Fig. 8); a similar result was obtained with *E. coli* cells. Subculture experiments show that growth, indicated by clearing of the *E. coli* suspension,

FIG. 8. Effect, on colony-forming units per milliliter, of addition of a suspension of rumen bacteria to 2% CRF medium; 2% CRF (▲), 10 ml of 2% CRF plus 0.05 ml of cell suspension (●), 10 ml of 2% CRF plus 2.0 ml of cell suspension (○).

is not sustained in *E. coli*-mineral salts medium unless volatile fatty acids, hemin, and vitamins or CRF are present (Table 6). The Minfav medium containing galactose and autoclaved *E. coli* cells (0.5 mg/ml) alone has supported

growth in more than 30 consecutive subcultures without the addition of sterols. No growth was detectable in 1% tryptone (Difco), 1.3% nutrient broth, 3.7% brain heart infusion, or 3% tryptone-soy broth, or in the same media supplemented with a volatile fatty acid mixture, galactose, or autoclaved *E. coli* cells.

Fermentation products. Gas-liquid chromatographic determination of fermentation products was attempted after growth in a medium containing 33% CRF and 20 μ mol of galactose per ml. The results of the assay are depicted in Fig. 9. Comparisons of peak areas indicated that 4 and 20 μ mol of acetic acid per ml was produced in duplicate cultures 3 and 4, respectively. This method was not sufficiently sensitive to measure accurately the small increases when the initial medium contained so much volatile fatty acid added with the rumen fluid. Butyric acid and the compounds giving the slight shoulders on the propionic and butyric acid peaks seem to be used during growth. Lactic acid formation, assayed by the method of Barker and Summerson (1), was 7 and 5 μ mol/ml, and galactose utilization was 19.3 and 19.9 μ mol/ml in cultures 3 and 4, respectively.

To increase the analytical precision, uniformly labeled ^{14}C -galactose was fermented. The products are shown in Table 7. Formic, acetic, and lactic acids, hydrogen, and CO_2 were main products, with traces of propionic acid. The counts in the trichloroacetic acid precipitate represent minimum values, as some cell lysis is to be expected after 10 days of incubation. Calculation of the oxidation-reduction balance of the fermentation products suggests that an unidentified reduced product is formed. The cultures were not tested for ethanol.

The microorganism is classified in the order *Mycoplasmatales* on the basis of colonial morphology, microscopic morphology, ability to pass a 0.45- μ m membrane filter at 10 lb/in², and resistance to penicillin G.

Repeated subculturing in liquid medium without addition of penicillin has not resulted in loss of these properties.

The microorganism is able to grow in a sterol-free medium containing *E. coli*, vitamins, volatile fatty acids, hemin, and galactose, and has therefore been assigned to the family *Acholeplasmataceae*, genus *Acholeplasma* (3).

The new isolate differs from the previously described species of *Acholeplasma*, *A. axanthum*, *A. laidlawii*, and *A. granularum*, in several physiological characters. All mycoplasmas which grow anaerobically can also grow

aerobically (12). This organism has remained obligately anaerobic during 4 years of in vitro cultivation. The formation of an enzyme capable of hydrolyzing peptidoglycan has not been described before among the *Mycoplasmatales*. The microorganism ferments ribose, xylose, arabinose, and lactose, not previously reported as fermented by mycoplasmas (4), and also galactose and sucrose, reportedly fermented only by avian strains (14). By use of the slide-agglutination technique for preliminary serological examination, no relationship was detected between the new strain and a limited number of available strains isolated from cattle. On the basis of these properties, the microorganism is classified as a new species, *Acholeplasma bactoclasticum* (*bact-*) part of the stem of the Gr. dim. noun *bacterium*, a small rod; Gr. adj. *clasticus* breaking; M. L. adj. *bactoclasticus* bacteria-breaking.

Penicillin-resistant bacteriolytic colonies similar in appearance to *A. bactoclasticum* have been found in all studied samples of rumen contents from both sheep and cows, though the viable count has not exceeded 10^7 /ml. The feed and water of the animals when tested did not

FIG. 9. Gas-liquid chromatographs from samples of acidified, filtered cultures; 1 and 2, before incubation; 3 and 4, after incubation. A, acetic acid; B, propionic acid; C, butyric acid.

TABLE 7. Recovery of labeled C from the fermentation of uniformly labeled ^{14}C -galactose by the new microorganism

Compound	Total counts/ml of sample (counts/min)		μMol	μmol of product/ μmol of galactose used
	Used	Produced		
Expt 1				
Galactose	2,988,770		44.017	
Butyric acid		3,512	0	
Propionic acid		109,197	3.125	0.073
Acetic acid		705,902	31.179	0.708
Formic acid		523,478	46.243	1.051
Lactic acid		840,090	23.081	0.524
CO_2		242,028	21.381	0.486
Trichloroacetic acid precipitate		207,214		
Total	2,988,770	2,627,909		
Total corrected		2,837,932		
Recovery (%)		95		
Hydrogen				Not measured
Label in cell material (%)		9.8		
Expt 2				
Galactose	671,928		9.89	1
Butyric acid		2,555	0.057	0.0057
Propionic acid		10,927	0.323	0.003
Acetic acid		257,887	11.368	1.15
Formic acid		76,433	6.752	0.683
Lactic acid		141,204	4.161	0.421
CO_2		41,394	3.66	0.370
Trichloroacetic acid precipitate		3,802		
Total	671,928	531,692		
Total corrected		566,992		
Recovery (%)		84		
Hydrogen				0.68
Label in cell material (%)		4.4		

contain significant numbers of these lytic organisms. In view of the volume of the rumen contents, it seems unlikely that the organism could enter the rumen in sufficient numbers from other sources within the animal, such as the nares or the buccal cavity, to maintain a count of about $10^6/\text{ml}$. The rumen is thus considered to be the habitat of the organism. The osmotic pressure of rumen fluid, measured as 7.1 and 7.8 atmospheres (6), is within the range of 6.8 to 14.0 atmospheres optimal for many mycoplasmas (8) and close to the estimated internal osmotic pressure of *A. laidlawii* (10).

The microorganism seems unlikely to be the L-form of a rumen bacterium, since it was isolated and subcultivated in the absence of cell wall inhibitors without any "reversion" to walled morphology being detected, and it is apparently capable of maintaining itself in the rumen environment.

Characteristics of *Acholeplasma bactoclasticum*. *A. bactoclasticum* is a free-living rumen organism. It is gram negative, usually coccoid, and has a cell envelope with a "unit-membrane" structure. Cells pass through a $0.45\text{-}\mu\text{m}$ membrane filter at 10 lb/in^2 . Surface colonies are small and have a central papilla. Growth depends on strictly anaerobic conditions in the medium.

Glucose, galactose, maltose, lactose, sucrose, arabinose, ribose, and xylose are fermented. Hydrogen, carbon dioxide, formic acid, acetic acid, and lactic acid are formed when galactose is fermented. Arginine is not fermented to yield ammonia. A rich medium with factors present in rumen fluid and *E. coli* cells is required. Cholesterol is not required for growth; neither are serum factors.

Skim milk is cleared by a proteolytic, extracellular enzyme, but ammonia is not formed. A variety of heat-killed cells, including

those of *E. coli* but not *M. lysodeikticus*, are lysed by an extracellular enzyme which attacks the peptidoglycan layer of the cell wall. Both of these lytic activities are sensitive to oxygen.

Chicken embryos are not killed when inoculated with *A. bactoclasticum*, and no hemolysis of bovine, ovine, or guinea-pig blood in an anaerobic agar medium could be detected.

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