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Growth Characteristics of Large- and Small-Colony Types of *Mycoplasma mycoides* subsp. *mycoides* on 5% Sheep Blood Agar

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Mycoplasma mycoides subsp. *mycoides* of the large-colony (LC) type was isolated in pure culture on 5% sheep blood agar plates inoculated with lung specimens from a 4-month-old Toggenburg goat. The growth characteristics of this isolate, of four known LC types, and of five known small-colony (SC) types of *M. mycoides* subsp. *mycoides* were compared on 5% sheep blood agar at 2, 5, and 7 days. The SC types were not visible at 2 days and did not grow larger than 0.1 mm, whereas the LC types were visible in 2 days and increased in diameter over 7 days to between 0.4 and 0.7 mm. These results indicate that growth on 5% sheep blood agar can be used as an additional marker in differentiating LC and SC types of *M. mycoides* subsp. *mycoides*.

Mycoplasma mycoides subsp. *mycoides* strains from goats and cattle are serologically indistinguishable, at least by the growth inhibition test (1, 4–7, 12, 16). These mycoplasmas have been differentiated into two colony types (large and small) on the basis of their growth and biochemical characteristics (5). The large-colony (LC) types grow to a greater turbidity in broth and form larger colonies on mycoplasma agar than do the small-colony (SC) types. Also, these LC types digest casein, liquify inspissated serum actively, and survive longer at 45°C. The LC types of *M. mycoides* subsp. *mycoides* have not been shown to be pathogenic for cattle except when trypanosomiasis is also present (12) or when large inocula (10^7 CFU/ml) are given intravenously (14). The strains of *M. mycoides* subsp. *mycoides* that cause contagious bovine pleuropneumonia are of the SC type. Such strains may occasionally be isolated from goats and are potentially capable of causing contagious bovine pleuropneumonia in cattle (4). However, these SC types of *M. mycoides* subsp. *mycoides* have not been recovered from either cattle or goats in the United States (11), although a number of reports document the occurrence of LC variants of *M. mycoides* subsp. *mycoides* in goats in the United States (2, 8, 15, 17). The occurrence of colonial variants within *M. mycoides* subsp. *mycoides* and the fact that these variants can differ in host specificity and pathogenicity has raised some confusing but important questions about this particular organism (11). The advantage of having other markers that may assist in the differentiation of these colony types is obvious.

Although most mycoplasmas usually require complex media for growth and primary isolation, some goat isolates will grow on blood agar (9, 13, 15, 17). One such strain of *M. mycoides* subsp. *mycoides* (LC type), isolated on blood agar, is described in this report, and the growth characteristics of four other LC and five SC type strains of *M. mycoides* subsp. *mycoides* are compared on 5% sheep blood agar. The differences observed in growth characterisitcs on blood agar between LC And SC type strains of *M. mycoides* subsp. *mycoides* are described as an additional marker for differentiating the two colony forms.

MATERIALS AND METHODS

Primary isolation. Tracheal-lung washes and lung tissue were collected aseptically at necropsy from a 4-month-old Toggenburg goat that died with pneumonia (17). Specimens were plated directly on both Trypticase soy agar containing 5% defibrinated sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and on mycoplasma agar plates (BBL) containing 20% horse serum.

Identification. The mycoplasma isolate was submitted to the Mycoplasma Section, Laboratory of Molecular Microbiology, National Institute of Allergy and

Infectious Diseases, Frederick, Md., where the disk growth inhibition test (3) and a direct fluorescent antibody test (6) were used to identify the organism. The isolate was tested against specific antisera or fluorescein-tagged conjugates to the following mycoplasma species: *M. alvi* (Ilsley), *M. bovigenitalium* (PG11), *M. bovirhinis* (PG43), *M. bovoculi* (M165/69), *M. dispar* (462/2), *M. mycoides* subsp. *mycoides* (PG1), *Mycoplasma* sp. bovine group 7 (B5P), *Mycoplasma* sp. bovine group L (California Calf 188), *M. verecundum* (107), *M. capricolum* (California Kid), *M. conjunctivae* (HRC581), *M. mycoides* subsp. *capri* (PG3), *M. ovipneumoniae* (St. 931), and *M. putrefaciens* (KS1).

The biochemical and growth tests used to characterize the isolate as the LC type of *M. mycoides* subsp. *mycoides* have been previously described (5). Briefly, casein liquefaction was determined by using a solid medium to which nonfat skim milk was added when the plates were poured. Serum liquefaction tests were carried out on a medium consisting of PPLO broth (Difco Laboratories, Detroit, Mich.) (20%) pig serum (75%), and fresh yeast extract (5%) followed by heating to 90°C until the medium had solidified. Survival of mycoplasmas at 45°C was determined by performing viability counts on cultures held at this temperature for 24 h. After exposure at 45°C, each sample was diluted in 10-fold steps in liquid medium, and drops of each dilution were placed on solid medium with calibrated pipettes. Plates and broths were incubated at 37°C for 5 days, after which time the colonies were counted and the number of CFU was calculated. Growth in liquid medium as indicated by turbidity was determined by measuring the optical densities of cultures in the exponential and stationary growth phases with a Spectronic 20 (Bausch & Lomb Inc., Rochester, N.Y.) photoelectric colorimeter at a wavelength of 650 nm.

Comparative growth studies. Three comparative growth studies were performed. The strains of *M. mycoides* subsp. *mycoides* used in comparisons with the isolate were the LC types Y goat, 74/2488, GM12, and HH, whereas the SC types were P goat, O goat, VOM, PG1, and Gladysdale. The origins of the P goat, O goat, Y goat, and VOM strains have been described (4, 5). The Gladysdale strain (bovine) and the HH strain (goat) originated from the Commonwealth Scientific and Industrial Research Organization, Division of Animal Health, Parkville, Victoria, Australia. The 74/2488 strain was obtained from Littlejohns and Cottew (10), and the GM12 strain was obtained from H. E. Adler, University of California at Davis.

In the first study, the three test media were: (i) ox blood agar (pH 7.5) consisting of 1.0% Difco Bacto-Peptone, 0.4% Lab Lemco (powdered beef extract; Oxoid Ltd., London, England), 0.5% sodium chloride, 1.5% Difco Bacto-Agar, and 10% defibrinated ox blood; (ii) mycoplasma agar consisting of Difco PPLO agar base (as per bottle) with 0.6% Albimi yeast autolysate, 20% inactivated swine serum (56°C for 30 min), 0.002% DNA, 1.0% Herdeschee yeast extract, 100 μg of ampicillin per ml, and 0.025% thallium acetate; and (iii) mycoplasma agar as described above with 10% defibrinated ox blood substituted for the swine serum. In the second study, the test media consisted of the blood agar base medium as described above (i) with 0, 2, 5, 10, or 20% ox blood. In the third study, Trypticase soy agar base containing 15 g of pancreatic digest of casein, 5 g of papaic digest of soybean meal, 5.0 g of sodium chloride, 15 g of agar, and 50 ml (5%) of defibrinated sheep blood per liter was used.

Overnight cultures of the organisms in Difco PPLO liquid medium (5) were plated to the test media, and the plates were incubated in a candle jar at 37°C for 7 days. The plates were examined daily for growth with a plate microscope containing an ocular reticule. Three to six of the largest isolated colonies were measured, and their mean diameter was recorded in millimeters.

RESULTS

Primary isolation. Pinpoint, alpha-hemolytic colonies were visible in pure culture after 2 days at 37°C on 5% sheep blood agar inoculated with lung specimens. After 7 days, these colonies were larger (0.6 mm), opaque, and resembled colonies of beta-hemolytic streptococci (Fig. 1). On mycoplasma agar at 2 days, colonies were larger than on blood agar and within 7 days were 2.0 to 2.5 mm in diameter.

Identification. The mycoplasma isolate was identified as *M. mycoides* subsp. *mycoides*. A 2-mm zone of growth inhibition was produced by the National Institutes of Health reference reagent antiserum to *M. mycoides* subsp. *mycoides* PG1. The test was repeated, using incubation for 3 days at room temperature (23°C) followed by 2 days at 37°C. This produced an inhibition zone of 9 mm. In the direct fluorescent antibody test,

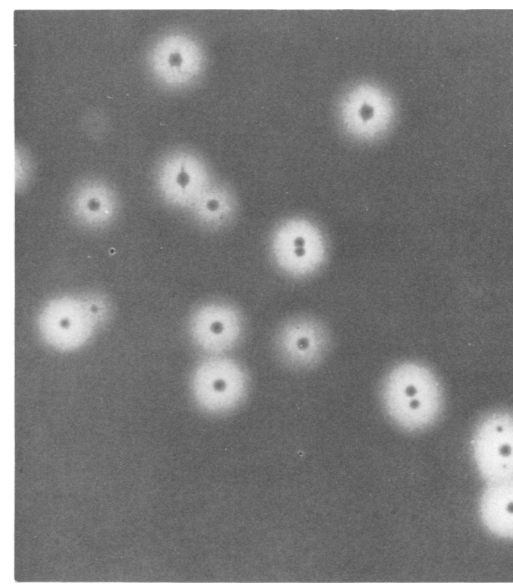

FIG. 1. Beta-hemolytic colonies of *M. mycoides* subsp. *mycoides* (LC type) at 7 days on Trypticase soy agar containing 5% defibrinated sheep blood (magnification, ×3).

TABLE 1. Survival of the isolate at 45°C compared with that of SC and LC types of *M. mycoides* subsp. *mycoides*

Time (h)	CFU (log_{10}/ml)		
	Gladysdale strain (SC type)	HH strain (LC type)	Isolate
0	9.13	9.11	9.47
2	8.93	8.97	9.47
8	7.39	9.06	8.77
24	3.60	8.90	6.93

the isolate reacted only with the National Institutes of Health Reference Reagent conjugate to PG1. This was verified by running an extinction titer (1:2,048) with the PG1 conjugate. It was further characterized as an LC type on the basis of its ability to digest casein, liquefy inspissated serum actively, survive longer at 45°C, grow to greater turbidity in broth, and form larger colonies on both mycoplasma and blood agar media than SC types of *M. mycoides* subsp. *mycoides*. The ability of the isolate to survive at 45°C for 24 h was compared with that of the HH strain, an LC type, and that of the Gladysdale strain, an SC type (Table 1).

After 48 h of incubation in liquid medium, the optical density of the HH strain, an LC type, was 0.236, that of the isolate described was 0.165, and that of the Gladysdale strain, an SC type, was 0.101. The optical density of the SC strain was only 0.121 after 5 days, at which time the optical density of the isolate was 0.185.

Comparative growth studies. Colony growth characteristics of the isolate were compared with those of the HH strain (LC type) and the Gladysdale strain (SC type) on blood and mycoplasma agars after 2 and 5 days of incubation (Table 2). No growth was observed on 10% ox blood agar plates inoculated with the SC type (Gladysdale strain) during the 5-day incubation period. In contrast, the isolate and the known LC type (HH strain) formed visible colonies in 2 days on each test medium (Table 2).

TABLE 3. Effects of ox blood concentration and length of incubation on colony size of *M. mycoides* subsp. *mycoides* strains

Concn of ox blood in medium (%)	Time (days)	Colony diam (mm)[a]	
		HH strain (LC type)	Isolate
0	2	<0.1	NG[b]
	5	≤0.3	NG
2	2	<0.1	<0.1
	5	≤0.4	≤0.1
5	2	<0.1	<0.1
	5	≤0.5	≤0.2
10	2	<0.1	<0.1
	5	≤0.5	≤0.35
20	2	<0.1	<0.1
	5	≤0.5	≤0.35

[a] The Gladysdale strain (SC type) did not grow with any concentration of ox blood.
[b] NG, No growth.

The effects of the concentration of ox blood in the medium on the colony growth of the isolate (LC type), the HH strain (LC type), and the Gladysdale strain (SC type) of *M. mycoides* subsp. *mycoides* are reported in Table 3. Increasing the concentration of ox blood incorporated in a blood agar medium above 10% failed to increase the diameter of colonies of the isolate (LC type) or the HH strain (LC type) of *M. mycoides* subsp. *mycoides*, but even at a 20% concentration of ox blood, no growth of the Gladysdale strain (SC type) was observed.

The growth characteristics of the isolate were compared with those of other known LC and SC types (Table 4). On 5% sheep blood agar, the SC types were not visible at 2 days and did not grow larger than 0.1 mm, whereas the LC types were

TABLE 2. Comparison of colony size of the isolate with known LC and SC type strains of *M. mycoides* subsp. *mycoides* on blood- and serum-enriched media at 2 and 5 days

Substrate	Time (days)	Colony diam (mm)		
		Gladysdale strain (SC type)	HH strain (LC type)	Isolate
Blood agar (10% ox blood)	2	NG[a]	0.2	<0.1
	5	NG	0.6	0.35
Mycoplasma agar (10% ox blood)	2	NG	1.0	0.7
	5	<0.1	1.5	1.1
Mycoplasma agar (20% swine serum)	2	0.5	2.5	2.3
	5	1.8	2.8	2.7

[a] NG, No growth.

TABLE 4. Comparative growth characteristics of known LC and SC types of *M. mycoides* subsp. *mycoides* on 5% sheep blood agar

Strain	Colony diam (mm) after incubation of:		
	2 days	5 days	7 days
M. mycoides subsp. *mycoides* (LC)			
Y goat	≤0.3	≤0.6	≤0.7
74/2488	≤0.2	≤0.45	≤0.5
Isolate (ATCC 33557)	≤0.1	≤0.33	≤0.4
GM12	≤0.25	≤0.45	≤0.5
HH	≤0.2	≤0.5	≤0.6
M. mycoides subsp. *mycoides* (SC)			
P goat	NG[a]	≤0.1	≤0.1
O goat	NG	≤0.1	≤0.1
VOM	NG	≤0.1	≤0.1
Pg1	NG	≤0.1	≤0.1
Gladysdale	NG	≤0.1	≤0.1

[a] NG, No growth.

0.1 to 0.3 mm in diameter after 2 days and increased over 7 days to 0.4 to 0.7 mm in diameter.

DISCUSSION

M. mycoides subsp. *mycoides* strains have been separated into two types by using simple biochemical and growth tests (5). This separation is significant since the two types differ in their pathogenicity for cattle.

The isolation (17) of *M. mycoides* subsp. *mycoides* on conventional blood agar provided the impetus to study the growth of LC and SC types of this species on this and other media. The obvious differences in colony size between five LC and five SC types of *M. mycoides* subsp. *mycoides* after 7 days of incubation on conventional sheep blood agar medium indicated the potential value of the use of growth on this medium as an additional characteristic for differentiating these types. It is suggested that mycoplasma isolates identified serologically as *M. mycoides* subsp. *mycoides* be subcultured from the mycoplasma agar plate or from the mycoplasma broth onto 5% sheep blood agar to determine whether the isolate is an LC or an SC type. Isolates which form colonies 0.4 to 0.7 mm in diameter within 7 days on 5% sheep blood agar can be considered to be the LC type. Clear-cut zones of beta-hemolysis around discrete colonies should also be seen with LC types. Aged liquid cultures of the SC types plated on sheep blood agar may cause hemolysis in the primary inoculum zone, even though growth may not occur in that zone, even after prolonged incubation. The zone of beta-hemolysis produced by the isolate and the other LC types increased when the plates were stored at 4 or 20°C for 8 h; however, the increase in the zone of hemolysis was not measured. Further investigations are necessary to define clearly the conditions under which beta-hemolysis is produced by *M. mycoides* subsp. *mycoides* strains.

Finally, it is useful for microbiologists processing specimens from goats and cattle to be aware that some mycoplasmas may grow on conventional blood agar and that the size of the colony formed on 5% sheep blood agar at 7 days can be useful in differentiating LC and SC types of *M. mycoides* subsp. *mycoides*. This isolate has been deposited with the American Type Culture Collection where it is listed as ATCC 33557.

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