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Morphology of *Ureaplasma urealyticum* (T-Mycoplasma) Organisms and Colonies

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The morphology of *Ureaplasm urealyticum* in broth cultures was studied by phase-contrast microscopy. Most organisms appeared singly or in pairs. Long filaments and long chains of cocci, common in classical mycoplasma cultures, were not observed. On solid medium, *U. urealyticum* produced "fried-egg" colonies which developed according to the scheme suggested by Razin and Oliver (J. Gen. Microbiol., 1961) for the morphogenesis of the classical mycoplasma colonies. The formation of the peripheral zone of the colonies followed that of the central zone only when growth conditions were adequate. Hence, the appearance of peripheral zones, and consequently the larger colony size, can be taken as an indicator of improved growth conditions. Incubation in an atmosphere of 100% CO_2 resulted in significantly larger colonies than in an atmosphere of N_2, O_2, or air. CO_2 acts as a buffer, keeping the pH at the optimal range for *Ureaplasma* growth (pH 6.0 to 6.5) in the presence of the ammonia produced from the urea hydrolyzed by the organisms. The addition to the medium of 0.01 M urea together with 0.01 M putrescine enabled better growth than with urea alone. Small amounts of phosphate improved growth in an atmosphere of CO_2, apparently fulfilling a nutritional role. Under nitrogen, higher phosphate concentrations were required for good growth, apparently serving as a buffer as well as a nutrient. Sodium chloride and sucrose which had been added to increase the tonicity of the medium inhibited growth above 0.1 M. An increase in the agar concentration above 2% resulted in decreased colony size. Likewise, prolonged drying of the agar plates caused a marked decrease in colony size, mostly affecting the peripheral zone. The addition of both urea and putrescine to the growth medium and incubation in a humidified CO_2 atmosphere are recommended for improved growth and formation of fried-egg colonies of *U. urealyticum* on agar. It must be emphasized that these experiments were carried out with a laboratory-adapted strain.

The T-mycoplasmas differ from the classical mycoplasma in at least two basic properties: their ability to hydrolyze urea (5, 20, 29, 33) and their failure to grow in the conventional mycoplasma media to titers higher than 10^7 colony-forming units (CFU) per ml (35). Associated with their poor growth in liquid media is their tiny colony size on solid media, giving rise to the trivial name T-mycoplasmas ("T" from tiny colonies; 28). The T-mycoplasmas were recently provided with the status of a separate genus, *Ureaplasma*, within the family *Mycoplasmataceae* (35). Yet, our knowledge of their biology is still meager, and their phylogenetic relationship to the other organisms included in the *Mycoplasmataceae*, the so-called classical mycoplasmas, is not clearly understood. One aim of the present investigation was to study the morphology of ureaplasmas by phase-contrast microscopy, under conditions minimizing artifact formation, and to compare it with the morphology of the classical mycoplasmas; another aim was to elucidate the factors influencing *Ureaplasma* colony shape and size and to determine whether the morphogenesis of *Ureaplasma* colonies follows the pattern proposed for classical mycoplasma colonies (24). The results presented in this communication show that the ureaplasmas resemble the classical mycoplasmas in gross morphology of the organisms and in morphogenesis of their colonies.

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MATERIALS AND METHODS

Organisms and growth medium. *Ureaplasma urealyticum* (strain 960) was originally supplied by M. C. Shepard (Camp Lejeune, N.C.). Sterile basal

broth prepared from 50 g of beef heart infusion and 10 g of peptone (Difco Laboratories, Detroit, Mich.) per liter was supplemented with 5% (vol/vol) unheated horse serum (Microbiological Associates, Inc., Bethesda, Md.), 10% (vol/vol) fresh yeast extract (10), 1% (vol/vol) phosphate-buffered saline (10× concentrate; Grand Island Biological Co., Grand Island, N.Y.), 1,000 U of penicillin G per ml, 0.002% phenol red, 0.01 M urea, and 0.01 M putrescine hydrochloride (Sigma Chemical Co., St. Louis, Mo.). The pH was adjusted with 1 N HCl to a value between 6.0 and 6.5, and the medium was filtered through a membrane filter (0.22-μm average pore diameter; Millipore Corp., Bedford, Mass.). Agar medium was prepared by the addition of 1.4% (wt/vol) agar (Difco) to the basal broth. Ten-milliliter volumes of the agar medium were dispensed into polystyrene petri plates (60-mm diameter; Falcon Plastics, Oxnard, Calif.). For some experiments polystyrene petri plates (100-mm diameter) divided into four compartments were used; in this case, 8-ml volumes of agar were added to each quadrant of the plate.

Growth conditions. The agar plates were dried for 10 min with their lids ajar in a laminar-flow hood. A 24-h culture of *U. urealyticum* (containing about 10^6 CFU/ml) was centrifuged at 18,000 × *g* for 15 min at room temperature, and the sedimented organisms were resuspended in an equal volume of basal broth. The cell suspension was then diluted 1:100, 1:200, 1:400, and 1:800 in basal broth. By use of a calibrated pipette, 0.01-ml drops from the various dilutions were carefully placed on the surface of the dried agar plates. Immediately after the drops had dried, the lids were replaced and the plates were transferred to anaerobic jars (Anaerobic Systems, BBL, Cockeysville, Md.) containing a moist gauze pad. The atmosphere in the jar was then changed to CO_2, N_2, or O_2 by flushing the jars for several minutes with the desired gas taken directly from gas tanks. The jars were then sealed with silicone grease and incubated at 37°C for 5 days.

Determination of colony number and size. The number of colonies was determined after 5 days of incubation at 37°C by using a Leitz inverted microscope (×3.5 objective and ×15 eyepiece). For best results, drops producing between 30 and 100 colonies were selected, and the diameters of all of the colonies in the drop area were measured with a calibrated Filer micrometer eyepiece. Colonies were photographed with type PB Polaroid camera (binocular model Mic-1625) using Polaroid black and white film type 107.

Phase-contrast microscopy. Small drops of broth cultures were put on glass slides, covered with cover slips, and examined with a Zeiss Photomicroscope II, using a ×100 neofluor-phase objective, a ×1.6 Optovar, and a ×12.5 eyepiece, giving a total magnification of ×2,300. The organisms were photographed as soon as they settled on the slide, using Kodak Tri-X pan (ASA400) film.

RESULTS

Morphology of organism. Figure 1 shows photomicrographs of *U. urealyticum* growing in filtered broth. To minimize artifact formation, the culture was not centrifuged, fixed, or treated in any other way before its examination in the phase-contrast microscope. Filtration of the medium before its inoculation with *U. urealyticum* was essential in order to remove particulate matter which could resemble *Ureaplasma* cells. Figure 1 shows the morphology of the organisms in broth and the various forms of cell associations which closely resemble those seen in *Mycoplasma* and *Acholeplasma* cultures (2, 23). The only difference is that far fewer organisms could be seen in the microscopic field, a finding which agrees with the much lower number of viable organisms in *U. urealyticum* broth cultures (10^6 to 10^7 CFU/ml as compared to more than 10^9 CFU/ml in the classical mycoplasma cultures). In addition, we did not observe long filamentous forms or long chains of cocci, which are characteristic of logarithmically- growing cultures of many classical mycoplasmas (23).

Factors influencing colony morphology and size. (i) Urea, putrescine, and the gas phase. Previous results from this laboratory (19) showed that putrescine can replace urea in liquid *Ureaplasma* media, following the adaptation of the organisms to this amine. The effects of putrescine and urea were, therefore, assessed in our experimental system, in which *Ureaplasma* growth was estimated according to colony number and size. Previous reports (5, 30) indicated a beneficial effect of CO_2 on *Ureaplasma* growth; consequently, we combined the examination of the effects of the gaseous environment on colony size with those of urea and putrescine. Figure 2 shows that incubation in an atmosphere of 100% CO_2 resulted in significantly larger colonies compared to incubation in an atmosphere of N_2, O_2, or air. The figure also shows that supplementation of the medium with a mixture of urea and putrescine improved growth under CO_2, though a reverse effect was seen under O_2 (Fig. 2). The pH of the agar plates incubated under CO_2 remained acid, whereas that of the plates incubated under N_2, O_2, or air became alkaline after the development of colonies. The removal of the plates from the CO_2 jar resulted in an alkaline shift of the pH of the medium within 10 to 30 min, depending on the number and size of the colonies on the plate. Since horse serum is expected to contain both urea and putrescine, the effects of urea and putrescine were also tested in a medium containing dialyzed calf serum, which is essentially free of these compounds (17). Table 1 shows that the medium containing dialyzed calf serum without urea or putrescine failed to support the growth of *U. urealyticum*.

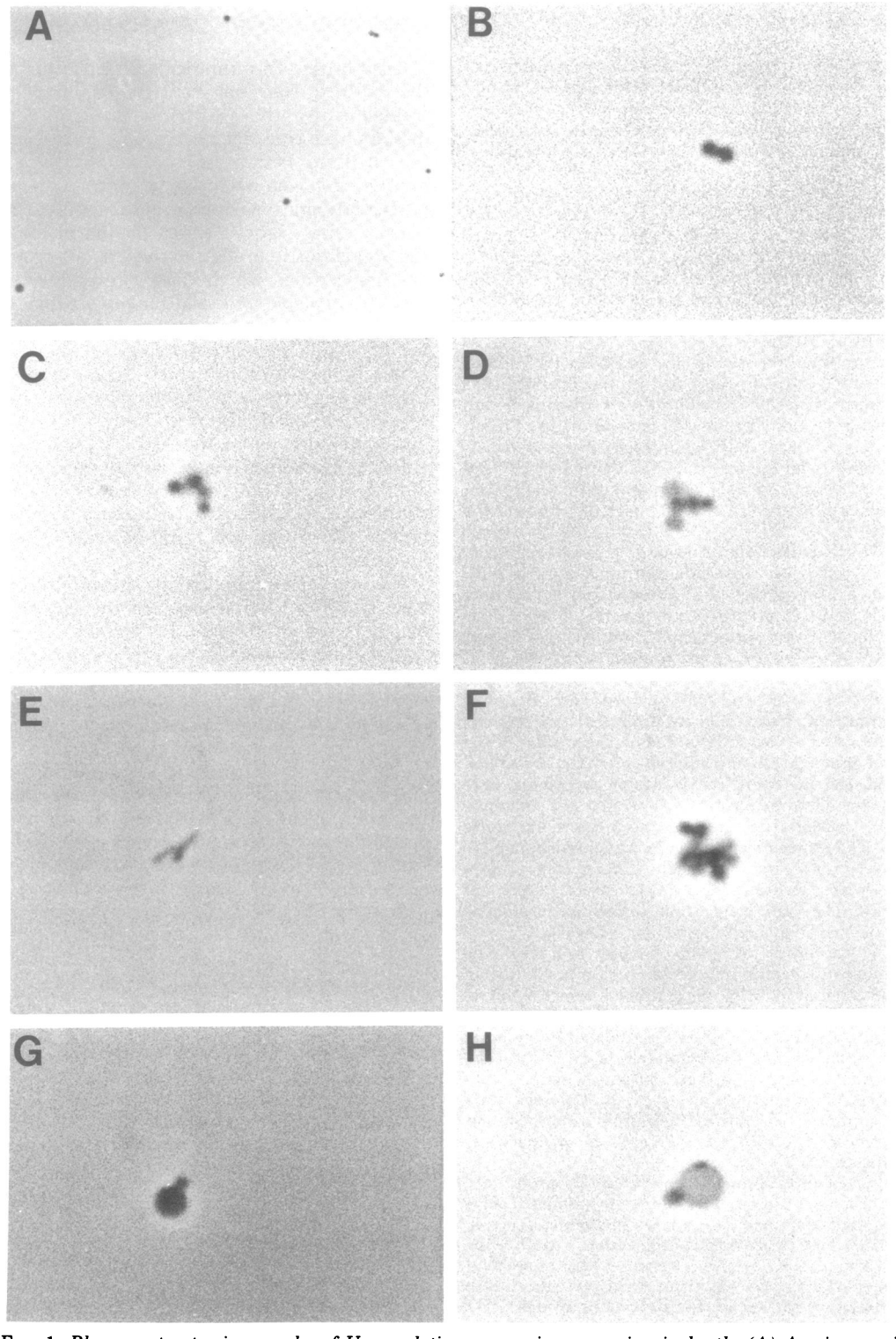

FIG. 1. *Phase-contrast micrographs of U. urealyticum organisms growing in broth. (A) A microscopic field of a 20-h culture. The scarcity of organisms in the field is noticeable. ×1,075. (B through H) Various cell associations including: a diplococcus form (B); a short chain of cocci (C); a branching chain of cocci (D); a short and branching filamentous form (E); a group of organisms (F); and "budding" forms consisting of a "normal-looking" cell associated with a swollen cell or ghost (G and H). ×2,560.*

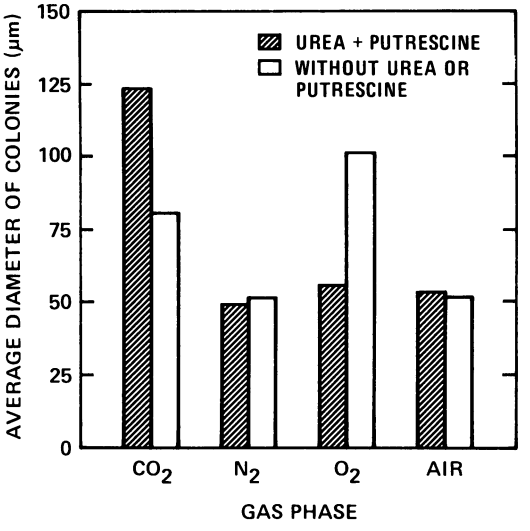

FIG. 2. *Effect of the gas phase and added urea plus putrescine on colony size. Organisms were grown on the solid medium containing 5% horse serum as described in Materials and Methods.*

TABLE 1. *Effects of urea and putrescine on the growth of U. urealyticum on agar containing dialyzed calf serum or horse serum*[a]

Urea (0.01 M)	Putrescine (0.01 M)	No. of colonies (mean diameter [μm])	
		5% Dialyzed calf serum	5% Horse serum
0	0	0	72
	0		(105)
+	−	9	55
		(124 μm)	(140)
0	+	0	90
			(110)
+	+	73	87
		(125 μm)	(235)

[a] The atmosphere was 100% CO_2. The strain was not adapted to grow with putrescine alone.

The addition of 0.01 M urea alone supported very poor growth, as evidenced by the small number of colonies. Putrescine by itself did not support growth, but the combination of urea and putrescine supported good growth, as indicated by the larger number of colonies that developed on the agar. The beneficial effect of putrescine could also be seen in the medium containing the nondialyzed horse serum, where the addition of putrescine together with urea improved growth considerably, as evidenced by the much larger colony size (Table 1). Subsequently, a mixture of urea and putrescine was routinely added to all our growth media.

(ii) Serum concentration. In the experiments shown in Table 1 and Fig. 2, the concentration of serum in the medium was 5% (vol/vol). This concentration was found to be adequate, since an increase of the serum content above 5% did not increase the number or size of the colonies. In fact, when the growth medium was supplemented with urea + putrescine and incubated under 100% CO_2, the serum content could be reduced to 2.5% without affecting growth. However, no growth occurred when the serum was totally excluded from the medium. In view of these results, 5% (vol/vol) horse serum was included in all experiments. It has to be emphasized that all our experiments were carried out with a laboratory-adapted strain, so that 5% (vol/vol) of horse serum may not be optimal for the growth of fresh *U. urealyticum* isolates.

(iii) pH and phosphate concentration. Figure 3 shows that the maximum colony size was obtained at pH 6.15, though the size of colonies at pH 5.75 or 6.70 was not much smaller. Phosphate was added to the growth medium to serve as a pH buffer. While our work was in progress, we became aware of a report by Romano et al. (25) showing that the incorporation of phosphate buffer into the growth medium of *U. urealyticum* considerably increased colony size. Therefore, we were interested in attempting to differentiate the effect of phosphate as a buffer from its effect as a nutrient. Figure 4 shows that

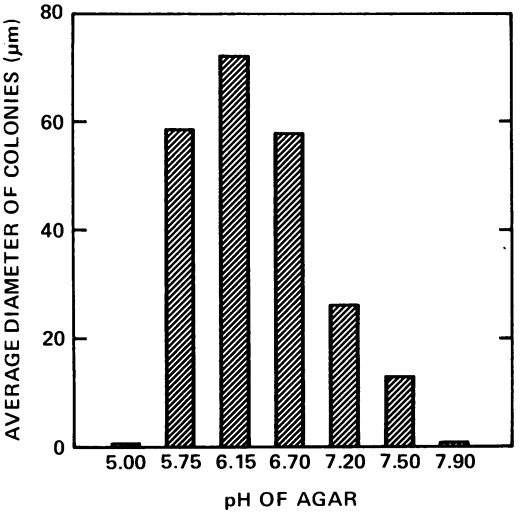

FIG. 3. *Effect of pH on colony size. The medium, as described in Materials and Methods, was buffered with 0.05 M K_2HPO_4 and adjusted with NaOH or HCl to the different pH values. Incubation was under N_2 instead of CO_2 to prevent the acidification of the medium by the CO_2. The colonies were measured after only 3 days of incubation.*

the effect of phosphate depended on the gas phase: under nitrogen, its addition, at least up to 100 mM, improved growth, only at low concentrations, whereas higher concentrations inhibited growth.

(iv) Tonicity. The tonicity of the growth medium may considerably influence the growth of mycoplasmas and spiroplasmas (12, 27). Table 2 shows that the addition of NaCl or sucrose to the growth medium did not enhance the growth of *U. urealyticum* and, at concentrations of 0.10 M and higher, the added solutes inhibited growth. Also, from this table it can be seen that NaCl or sucrose did not act as substitutes for urea and putrescine, as evidenced by the poorer growth in the medium not supplemented with urea and putrescine.

Agar concentration and moisture. As had been found for other mycoplasmas (24), the agar concentration enabling the best growth of *U. urealyticum* was about 1.4% (wt/vol). An increase in the agar concentration above 2% resulted in decreased colony size, and with 3% agar the inhibition of growth was almost complete. Prolonged drying of the agar plates in the laminar-flow hood also affected their ability to support growth. Thus, lengthening the drying time for more than an hour caused a marked decrease in colony size, mostly affecting the peripheral zone, and plates dried for over 2 h completely lost their ability to support growth of *U. urealyticum*.

(v) Other factors influencing colony shape and size. *U. urealyticum* colonies grown under N_2, O_2, or air, but not under CO_2, on agar containing urea produced small, dark granules found mostly at the periphery of the colony. The granules appeared only after the growth medium turned alkaline, so that colonies on plates kept under 100% CO_2 were devoid of granules. However, the granules were produced when the plates were taken out of the CO_2 jar and kept in air so that their pH became alkaline (Fig. 5). Another interesting observation, for which we have no explanation as yet, was a marked increase in the size of the peripheral zone of colonies developing in areas where an unidentified precipitated material covered the agar surface (Fig. 6).

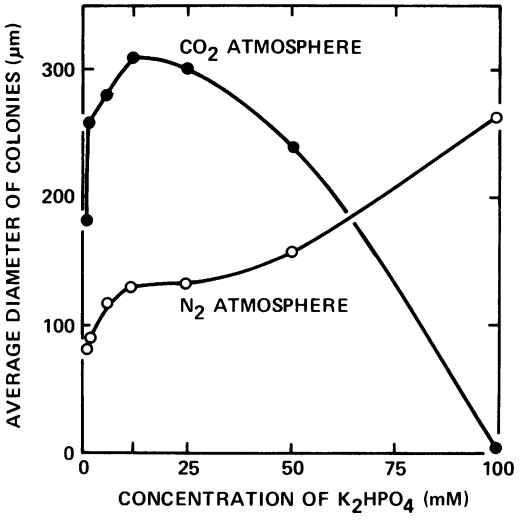

FIG. 4. *Effect of the phosphate buffer concentration of the growth medium on colony size. The solid medium used was that described in Materials and Methods.*

DISCUSSION

Phase-contrast microscopy of unfixed ureaplasmas in liquid culture confirms their morphological similarity to classical mycoplasmas, which were observed by electron microscopy (1, 26). A rough estimate of the diameter of the unswollen coccoid *Ureaplasma* cells, based on measurements of their image on the phase-contrast micrographs, gives values ranging between 0.3 to 0.8 μm, resembling those found for the classical mycoplasmas (23). However, long filaments and long chains of cocci, common in fast-growing classical mycoplasma cultures, were not found in the *Ureaplasma* cultures. This may be taken in support of the thesis (21,

TABLE 2. *Effects of changes in tonicity of the medium on U. urealyticum growth*

Concn (M) of solute added to the medium	No. of colonies[a]			
	NaCl		Sucrose	
	Urea + putrescine added	Without addition of urea + putrescine	Urea + putrescine added	Without addition of urea + putrescine
0	32 (165 μm)	14 (85 μm)	43 (151 μm)	13 (70 μm)
0.05	35 (126 μm)	41 (70 μm)	41 (122 μm)	3 (77 μm)
0.10	32 (75 μm)	38 (78 μm)	17 (101 μm)	NG
0.17	NG	25 (72 μm)	NG	NG
0.25	NG	NG	NG	NG

[a] The number in parentheses is the average diameter. NG, No growth.

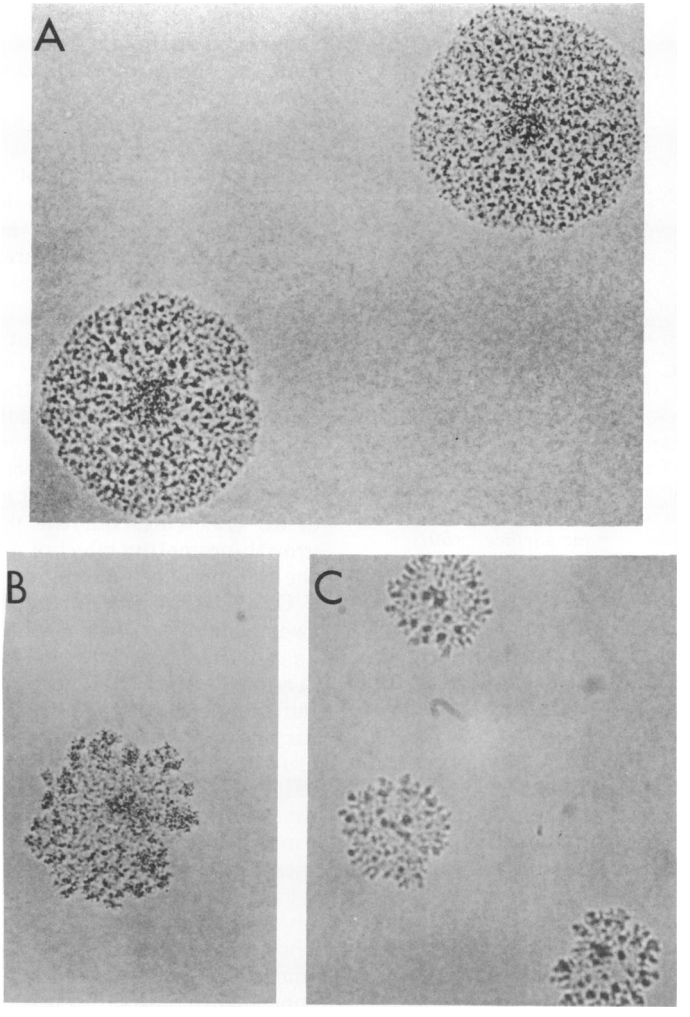

FIG. 5. *U. urealyticum colonies. (A) Five-day colonies from a medium supplemented with urea plus putrescine and incubated under 100% CO_2. The central and peripheral zones of the colonies are discernible. ×240. (B) A colony from the same medium showing convoluted borders and dark granules appearing after alkalinization of the growth medium. ×240. (C) Similar colonies but with heavier granules. ×100.*

23) that filaments appear only under conditions enabling rapid growth, when cytoplasmic division lags behind genome replication. A similar phenomenon has also been recorded for wall-covered bacteria. *Arthrobacter* sp. (14) and other bacteria (11) growing in continuous culture produced long rods when their growth rate was at a maximum and coccoid bodies when their growth rate was lower. Our finding that most of the cells in *Ureaplasma* cultures appear singly or in pairs supports the suggestion of Furness (9), based on sonic oscillation and ultraviolet inactivation curves, that the CFU of *Ureaplasma* cultures mostly consists of single organisms.

Our study indicates that the morphogenesis of *Ureaplasma* colonies follows the same pattern as that suggested for the classical mycoplasma colonies (24). This pattern consists of an initial spherical growth inside the agar (the central zone), followed by spreading of the organisms into the thin free-water film on the agar, forming the peripheral zone. Factors that retard growth were shown to inhibit the formation of the peripheral zone. Razin and Oliver suggested in 1961 (24) that the tiny *Ureaplasma* colonies are only composed of the central zone embedded in the agar, as a result of the inadequacy of the then available media to support good growth of ureaplasma. Ford's failure in 1962 (3) to transfer ureaplasmas on solid media by the conventional inverted agar block

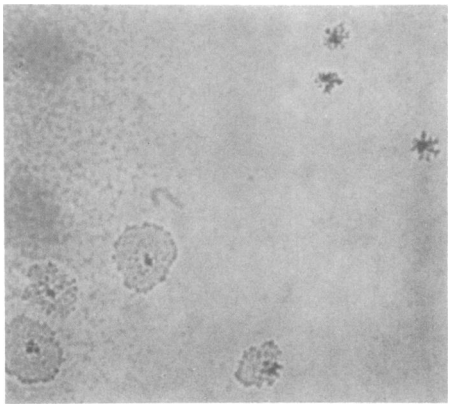

FIG. 6. *U. urealyticum colonies developing in areas where unidentified material has precipitated on the agar surface. The large dimensions and fried-egg shape of these colonies can be contrasted with the minute and irregular shape of the colonies growing on the adjacent uncovered agar surface.* ×50.

technique can also be taken to indicate the absence of peripheral zones in the colonies. Subsequent improvements in the growth media for ureaplasmas, including the addition of urea (6, 33), strengthening the buffering capacity of the medium by incubation under 20% CO_2 [4, 29; by inclusion of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (16) or by phosphate buffer], and simply increasing the volume of the agar in the plates (8, 13), resulted in larger colonies and the appearance of peripheral zones.

Our finding that an atmosphere of CO_2 most markedly improves the growth of *U. urealyticum* can be explained by the excellent buffering capacity of CO_2 in the acidic pH range, between 6.0 and 6.5, which had been found by Shepard and Lunceford (31) to be optimal for *Ureaplasma* growth. This optimal pH can be kept with CO_2, even in the presence of excessive amounts of ammonia derived from urea hydrolysis (19).

Although isolated *U. urealyticum* colonies developing under CO_2 very frequently exceeded 200 μm in diameter, the mass of organisms included in them was apparently much smaller than that in classical mycoplasma colonies of the same diameter. This was evidenced by the thinness of the *Ureaplasma* colonies, a factor which made their observation by the naked eye very difficult. Our data (19) showing that, under CO_2, the maximum titer of viable ureaplasmas (CFU per milliliter) in liquid culture is not much higher than that under N_2, though the stationary phase under CO_2 is longer, appear to be in accord with the smaller mass of cells in *Ureaplasma* colonies.

Our results confirm those of Shepard and Lunceford (33) and Ford and MacDonald (5) by showing that the dialysis of the serum component of the growth medium abolished its growth-promoting activity. Working with dialyzed horse serum, the above-mentioned authors were able to restore its growth-promoting activity by the addition of urea. Our results with dialyzed calf serum indicate that the addition of urea alone is not as effective as the addition of a mixture of urea and putrescine. Although in the experiment shown in Table 1 putrescine by itself could not support growth in the dialyzed calf serum medium, we were able to adapt the *U. urealyticum* strain to grow with putrescine alone, without the addition of urea, confirming the previous results of Masover and Hayflick (18). The fact that our experiment was carried out in an atmosphere of CO_2, combined with observations of Ford et al. (4) and Masover et al. (19), rules out the possibility that the growth-promoting effect of urea is derived from the CO_2 produced during urea hydrolysis.

Our experiments appear to differentiate between the effects of phosphate as a buffer and as a nutrient in *Ureaplasma* growth. In the presence of a CO_2 atmosphere, where the buffering effect of the phosphate was not necessary, low concentrations of phosphate improved growth, possibly indicating a nutritional effect (Fig. 4). Under a nitrogen atmosphere, the marked improvement in growth by increasing concentrations of phosphate may be due to the increased buffering capacity of the medium in addition to the nutritive value of the phosphate. It is not clear why high concentrations of phosphate inhibited growth under CO_2 but not under N_2. Growth inhibition by high phosphate concentrations has been recorded for classical mycoplasmas (22) as well as for ureaplasmas (33).

An increase in the tonicity of the growth medium by adding NaCl or sucrose not only failed to improve the growth the *U. urealyticum* but inhibited growth above certain concentrations of these solutes. This may be correlated with the findings of Makki (15) that the addition of 5% (wt/vol) of glucose, fructose, fucose, or maltose to the growth medium completely inhibited *Ureaplasma* growth.

The nature of the dark granules appearing on the periphery of the colonies, when the medium becomes alkaline due to the ammonia released from urea, is uncertain. The granules may represent amorphous ammonium magnesium phosphate, a compound shown to be produced during *Ureaplasma* growth (7, 26, 36), or insoluble metallic oxides of Mg^{2+} or Mn^{2+} produced by the ammonium hydroxide accumulated in the growth medium (31, 34).

As a result of the present study, we would

recommend the addition of both urea and putrescine to the growth medium and the incubation of the cultures in an atmosphere of CO_2. An atmosphere rich in CO_2 can be produced either by flushing the jar containing the plates with CO_2 from a tank or by placing a small piece of dry ice in the jar. Incubation under CO_2 will considerably increase the size of most colonies and could, thus, facilitate the isolation and identification of *U. urealyticum* from clinical material.

ACKNOWLEDGMENT

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