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Mycoplasma hominis: Growth, Reproduction, and Isolation of Small Viable Cells[1]

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Improved methods for studying the growth of *Mycoplasma hominis* (ATCC 14027) have been developed, involving modified growth conditions and preparation of the organisms under minimally distorting conditions. Cells so prepared from batch cultures show relatively uniform exponential growth and appear to be dividing by binary fission; but pleomorphic forms appear upon further incubation. Similar behavior was demonstrated by another laboratory-adapted strain and by three clinical isolates, and therefore seems characteristic of the species. The pleomorphic populations contain small forms having diameters within the 100- to 250-nm size range reported for "elementary bodies." Such forms were isolated from this strain of *M. hominis* by sequential filtration using gravity alone, after cell aggregates were dispersed by Pronase treatment. Of the small bodies which traversed membranes of 220-nm pore size, a negligible number grew in liquid or on solid media, suggesting that these were not essential reproductive units in a life cycle, but involution forms due to growth in an altered environment.

The bewildering diversity of evidence and opinion on mycoplasma reproduction (1, 9, 21) partly reflects fundamental and clearly demonstrable differences between species. However, much of the confusion could have arisen from imperfect understanding of their growth requirements and from overlooking their liability to manipulative distortion. We attempted to determine the true morphological changes during growth of a readily cultivable species, *Mycoplasma hominis*, when cells were produced and prepared for microscopy under optimal conditions. Studies undertaken to determine a minimally distorting technique for microscopy are outlined elsewhere (23).

Experiments are recorded in which the reproductive pattern of *M. hominis* was established. Filtration has been applied to the isolation of cells under pressure or negative pressure (5, 15, 20). For this study we adopted filtration through membranes by gravity alone. Special attention was given to the smallest cells, spheres of 100 to 250 nm in diameter, commonly known as "elementary bodies," which are regarded generally as the minimum reproductive units.

(This work is based on a thesis submitted by J. Robertson to the faculty of graduate studies, McGill University, in partial fulfilment of the Ph.D. degree requirements.)

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MATERIALS AND METHODS

Organisms and cultural conditions. *M. hominis* strain 14027 came from the American Type Culture Collection, Washington, D.C. Strains 721, 1024, and 1035 were obtained from the (Clinical Bacteriology Laboratory) Department of Microbiology and Immunology, McGill University, Montreal, and strain 463 was obtained from W. Bredt, Institut für Medizinische Mikrobiologie, Johannes Gutenberg-Universität, Mainz, Germany. Ten percent (vol/vol) of glycerol was added to logarithmic cultures of each strain, and these were stored in 2-ml portions at −70 C.

Media. "Improved" broth medium (pH 7.3; 384 mosmol/kg consisted of PPLO (pleuropneumonia-like organism) broth without crystal violet (Difco Laboratories, Detroit, Mich.), 2.1 g; *N*-2 hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (Calbiochem, Los Angeles, Calif.), 1.19 g; and 80 ml of deionized, glass-distilled water. After being autoclaved at 121 C for 15 min, this basal medium was supplemented with 10 ml of fresh yeast extract and 10 ml of pooled normal horse serum (Institut de Microbiologie et d'Hygiène, Université de Montréal, Laval des Rapides, Quebec), both supplements having been sterilized by filtration. The serum was found mycoplasma free and had good growth-promoting properties.

Broth medium for strain 463 (pH 7.8 267 mosmol/kg) consisted of PPLO broth without crystal violet, 1.47 g; distilled water, 70 ml, agamma horse serum (Microbiological Associates, Bethesda, Md.); 20 ml; and yeast extract, 10 ml. Because of the claim that strain 463 gave filamentous growth, this strain was investigated in the medium described (17) except that gelatin was omitted.

Agar medium consisted of PPLO agar, 3.5 g; L-arginine monohydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio), 0.5 g; and 80 ml of distilled water. The sterile agar received the same supplements as the "improved" broth medium. The pH was about 7.4.

Diluting fluid (pH 7.3; 252 mosmol/kg) consisted of 2.1 g of PPLO broth without crystal violet in 99 ml of water to which 1 ml of horse serum was added.

All media were free of antimicrobial agents. Glassware was silicone-coated. The pH was adjusted with HCl or NaOH. Osmolal concentrations were measured by the freezing-point depression technique, with an Osmette precision osmometer (Precision Systems, Framington, Mass.). The values given in milliosmoles per kilogram are the mean of two readings.

Inoculum preparation and incubation conditions. Preparation of inocula has been described (23). Fifty-milliliter cultures in 250-ml Erlenmeyer flasks were incubated in a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 125 rpm, and always at 37 C.

Growth measurement. Population changes were monitored by nephelometry measurements made against an incubated medium blank in a Turner Model 110 fluorometer (G. K. Turner Associates, Palo Alto, Calif.) with a no. 22 orange filter and illumination expanded three times. Nephelometry readings had been previously correlated with viable cell counts (VCC). For these a 0.5-ml sample of culture was diluted in 4.5 ml of diluting fluid and the suspension was subjected to gentle sonic treatment for 20 s (Sontegrator System 40, Ultrasonic Industries, Inc., Hicksville, N.Y.), the period which gave maximal dispersal of cells in both logarithmic and late stationary-phase cultures. Aliquots of 0.025 ml of appropriate dilutions were plated in quadruplicate on agar. After 3 days of incubation colonies were enumerated and the numbers expressed as colony-forming units (CFU) per ml.

Electron microscopy. Cells were pre-fixed in situ by a method reported elsewhere (23). Negative-contrast preparations were made with 1% (wt/vol) phosphotungstic acid (PTA) (pH 7.4; 34 mosmol/kg), isoosmolal ammonium molybdate (pH 7.4; 384 mosmol/kg) or 1% (wt/vol) sodium zirconium glycolate (SZG) (pH 7.4; 115 mosmol/kg). SZG generally gave the least intense positive staining. Whole cell preparations were made at frequent intervals during logarithmic and stationary phase growth, and throughout incubation of the cultures, for periods up to 3 weeks. The other strains were studied only during logarithmic growth and the early stationary phase, and again 9 h later.

For ultrastructural studies, pre-fixed cells were placed in 5% (vol/vol) glutaraldehyde (pH 7.4; 920 mosmol/kg) for 1 h at 4 C, followed by 2% (wt/vol) osmium tetroxide (pH 7.4), for 1 h at 23 C and then 1% (wt/vol) uranylacetate for 1 h at 23 C. Sorenson phosphate buffer (pH 7.4) was used for glutaraldehyde and osmium fixative solutions and for all washes. After fixation, cells were dehydrated in a graded series of alcohols and propylene oxide and then embedded in Araldite. Sections were stained for 5 min in 3% (wt/vol) uranyl acetate in 50% (wt/vol) ethanol (pH 4.8), 45 s in Reynolds lead citrate, and then examined in Philips EM 300 electron microscope at 60 kV.

Sequential filtration of M. hominis cultures. A 1% (vol/vol) inoculum of *M. hominis* 14027 was introduced into broth medium. After incubation periods of 12 h and 3, 7, and 14 days respectively, 20-ml samples were removed and sonicated. One-half milliliter was used for VCC and the rest was applied, in 2-ml volumes, to a special filtration apparatus. This consisted of a barrel of a 10-ml syringe attached to a Swinnex-25 filter holder with a sterile MF membrane filter (Millipore Corporation, Bedford, Mass.) of 3.0-μm average pore diameter (a.p.d.). The apparatus, used in an upright position, had been prerinsed with 10 ml of hot distilled water to removed detergent. Samples (at 23 C) passively traversed the membrane. The procedure was considered complete when 1 min elapsed without a drop of filtrate appearing in the collection tube. Ten milliliters was applied sequentially to membranes of decreasing pore size (1.2 μm, 800, 650, 450, and 220 nm), a procedure which altogether required about 10 min. VCC were made after each step.

Use of enzymes for recovery of small viable cells. Cells of *M. hominis* 14027 from late stationary (24 h) and decline phase cultures (3 and 7 days) were collected by centrifugation at 3,090 $\times$ *g* for 15 min at 4 C (23), and suspended in Dulbecco phosphate-buffered saline (PBS) (pH 7.4, 270 mosmol/kg), to one-half the original volume. At a constant 37 C, each of three enzyme solutions deoxyribonuclease [DNase] trypsin, and pronase) or, as control solutions, enzyme solvent or PBS, was added to an equal volume of cell suspension. After mixing, samples were removed for VCC and the flasks were incubated on the shaker at 125 rpm. One-milliliter samples were taken at suitable intervals, diluted in 9 ml of broth at 23 C, and thoroughly mixed. VCC were made on the first sample, both before and after sonication treatment. Subsequent samples were not sonic treatment. Subsequent samples were not sonicated. The remaining enzyme-treated suspensions were filtered sequentially through membranes of 3.0 μm (to remove clumps), 450 and 220 nm a.p.d. In addition to plating the usual 0.025-ml samples, 1 ml of filtrate was put on agar and another 1 ml in 9 ml of broth medium. The enzyme solutions were: 0.5 mg of DNase per ml (DNase 2$\times$ crystallized, Nutritional Biochemicals Corporation, Cleveland, Ohio), in 0.02 M $MgSO_4$, pH 7.2; 0.25% (wt/vol) 0.1% (wt/vol) protease (Pronase, B grade, Calbiochem, La Jolla, Calif.) in PBS, pH 7.0. These solutions were sterilized by filtration and stored at -20 C until use.

Viability of the small particles. A 400-ml culture of *M. hominis* 14027 was incubated for 12 h after reaching stationary growth, the cells were collected and suspended in 100 ml of PBS. As described above, equal volumes of this suspension and Pronase solution were incubated for 5 min when the reaction was stopped by the addition of an equal volume of broth

medium at 23 C. The suspension was filtered sequentially through membranes of 3.0 μm, and 450 and 220 nm. After each step, samples were taken for VCC; 100- and 200-ml volumes of the 450- and 220-nm filtrates, respectively, were fixed with glutaraldehyde at 23 C. Ten milliliters of the 220-nm filtrate were added to 40 ml of broth medium and incubated. The fixed cells were collected at 12,350 × *g* for 15 min, and the supernatant was centrifuged at 24,150 × *g* for any residual particles. Cells from each filtrate were pooled, washed, collected at the higher speed, and suspended in water to one-fiftieth and one two-hundredth, respectively, of the original volumes. Total cell counts were made according to the loop-drop method (24).

RESULTS

Changes in population and morphology during growth. The pattern of growth for *M. hominis* 14027 in improved broth, agitated during incubation, is shown in Fig. 1. In this instance, with an inoculum volume of 0.1% (13.3 $\log_2$ CFU per ml), the lag phase was about 40 min. However, its duration varied inversely with inoculum size and could be less than 1 min when 20% was used. The mean generation time of the culture illustrated was 98 min. This rate was fairly consistent for a given batch of medium, but varied from 73 to 120 min with different lots of serum supplement. The maximum population reached was CFU per ml, and in this instance was about 4×10^8 (28.6 $\log_2$) CFU per ml. The half-life during cultural decline (between 48 and 105 h of incubation) was 9 h. The decline continued slowly; after 15 days the CFU still numbered more than one-half the initial total.

Cells of *M. hominis*, cultured and prepared for electron microscopy as described, showed no changes in gross morphology during the relatively short lag phase. Cells in actively growing cultures were typically round to rod shaped, as previously reported (23). Some of the rods were dumbbell shaped (Fig. 2a). Thin sections of such cells displayed conventional ultrastructural features (Fig. 2b). Electron-transparent regions at both poles of the long forms contained fibrils 2 to 3 nm wide, presumably deoxyribonucleic acid. In the surrounding cytoplasm were dense particles of a maximum diameter of 17.5 nm, which is within the size range of ribosomes of mycoplasmas and other prokaryotic cells. These particles increased in numbers during log-phase growth but diminished later in exponential growth. In the sections shown in Fig. 2b they are randomly distributed, with a tendency to linear arrangement, running parallel and about 6 nm from the inner leaflet of the membrane. The membranes, about 6 nm wide, are narrower than the 7- to 11-nm width characteristic of most biomembranes. Sometimes the outer leaflet of the membrane appeared thicker than the inner leaflet (Fig. 2b,d).

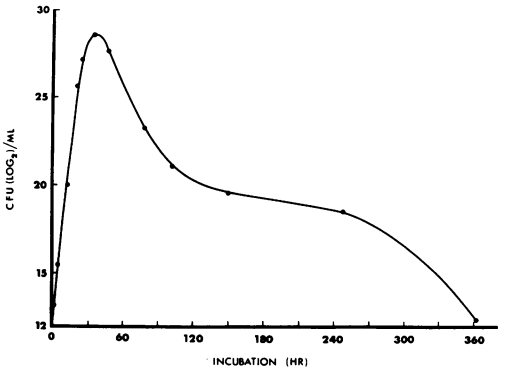

FIG. 1. *Growth of M. hominis 14027 under optimal cultural conditions.*

In logarithmic phase cultures, most cells would be expected to be replicating. Longitudinal sections through the bilobed, dumbbell-shaped forms found in both whole cell (Fig. 2a) and thin-sectioned preparations (Fig. 2c) might be in the final stages of binary fission through formation of protomembranes (Fig. 2d). By the end of exponential growth, round forms predominated over rods and there was an apparent increase in cell mass. Cells of both forms were still present 12 h later in the late stationary phase. Grossly abnormal forms were now also evident. Some of these showed great variations in size (Fig. 3a, b), whereas others suggested aberrant division (Fig. 3c, d). After about 3 days of incubation, the incidence of such bizarre forms diminished and the culture was comprised of mostly round forms, some of which were within the 100- to 250-nm diameter reported for "elementary bodies." Very small bodies were occasionally seen in clusters or chains (Fig. 3e). These round and oval bodies, 85 to 300 nm in diameter, resembled the very minute (10 to 100 nm) globular elements described during the growth of *Mycoplasma pneumoniae*, whose nature has not been established (6). Cultures which followed for 3 weeks remained viable but showed no further morphological changes.

Four other strains of *M. hominis*, one laboratory adapted (463), and three clinical isolates (721, 1024, 1035), grown under the conditions established for strain 14027, had a comparable growth pattern. Morphological changes were also basically similar, the relative uniformity of logarithmic phase cultures (Fig. 3f,h) yielding to pleomorphism by the late stationary phase (Fig. 3g,i).

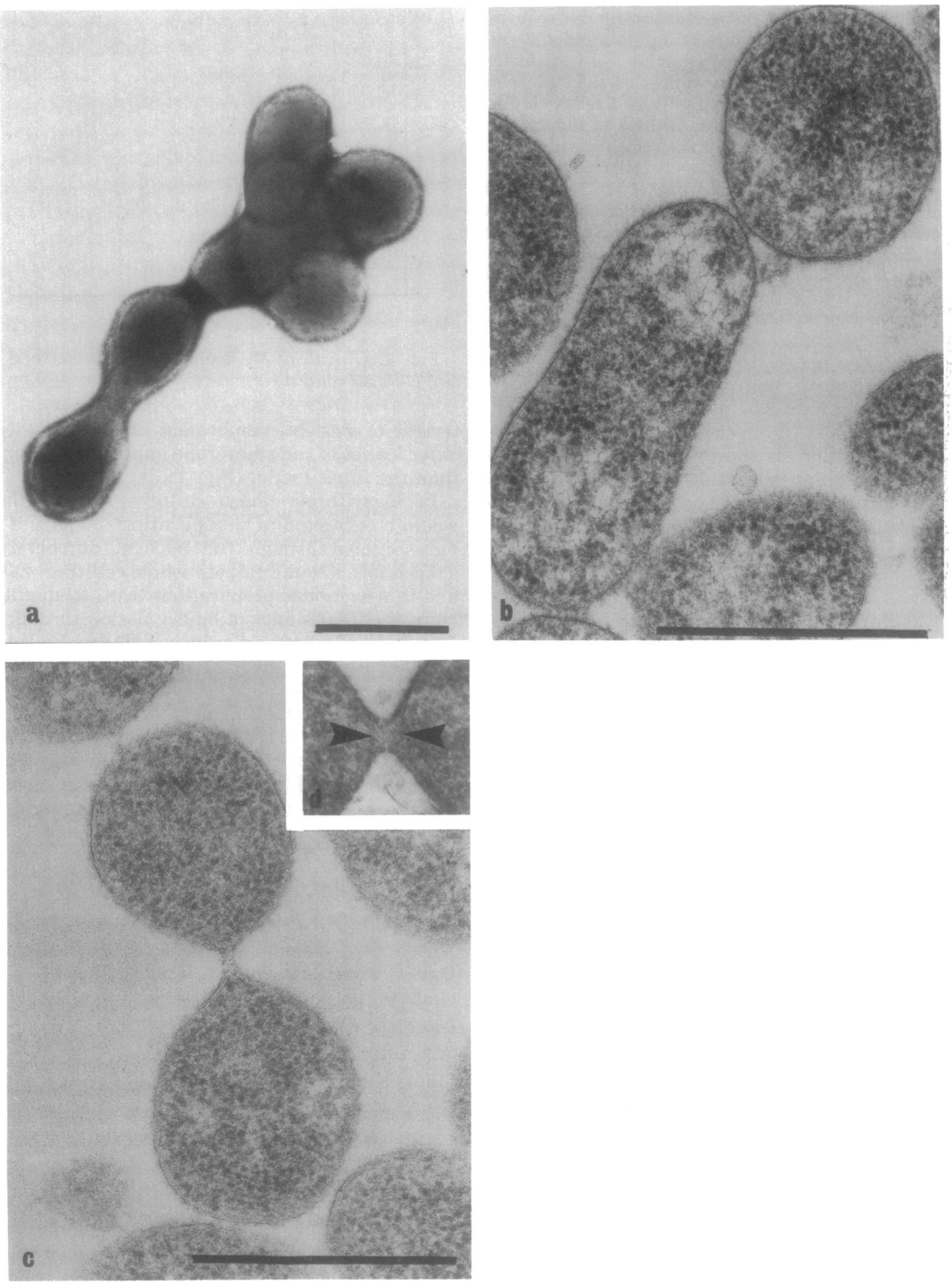

FIG. 2. *Preparations of M. hominis 14027 cells from cultures in exponential growth. (a) Whole cells of the dumbbell type. (PTA) × 40,000. Thin sections showing (b) usual ultrastructural features: membrane, ribosomes, and regions of deoxyribonucleic acid. (c) A dumbbell-shaped form. (d) Arrows indicate possible protomembranes arising at the point of constriction. × 80,000. The bars represent 0.5 μm.*

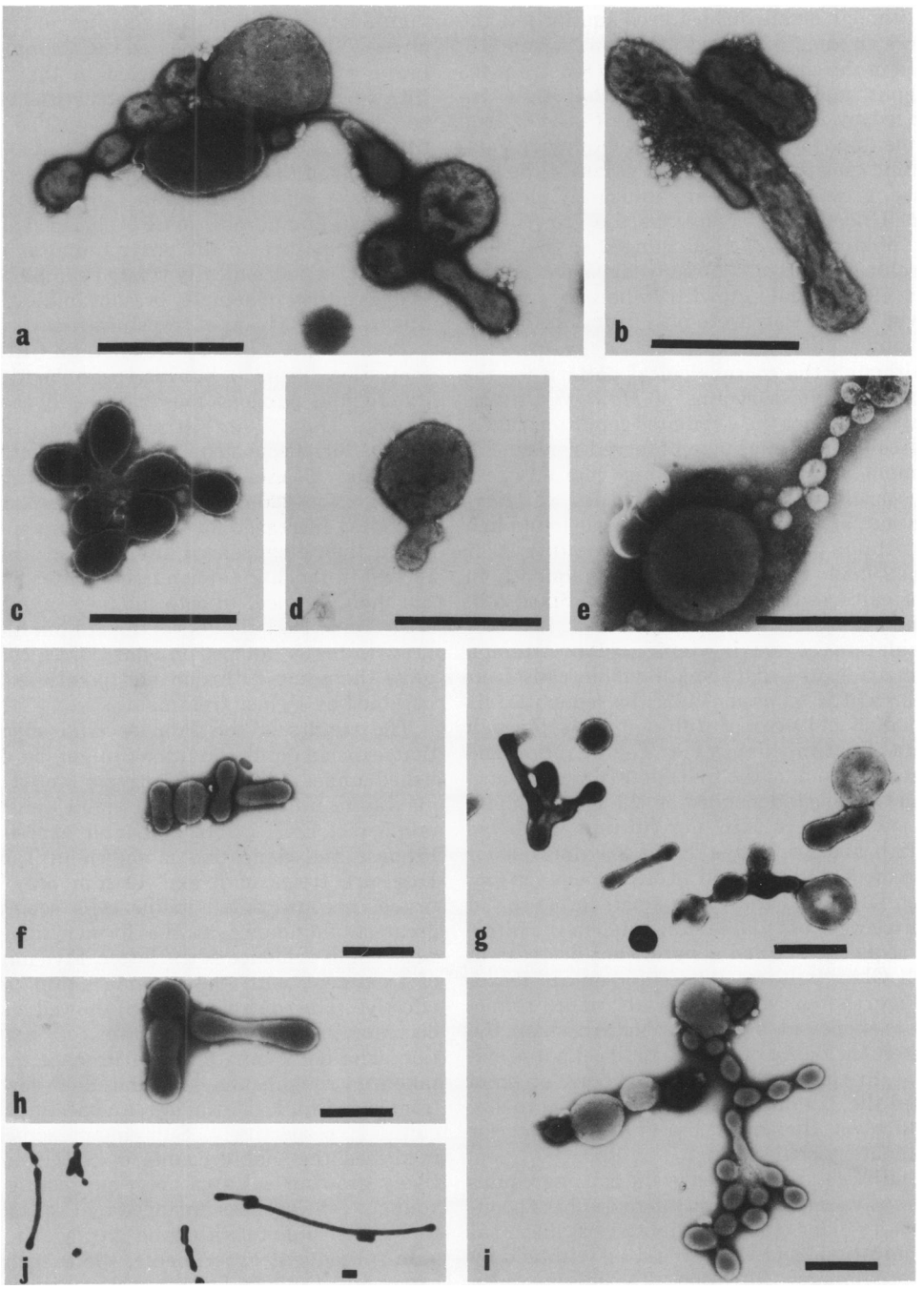

FIG. 3. *(a to e) Cells from cultures of M. hominis 14027 in late stationary and decline phases of growth. The wide range of size in round and long forms is shown in (a) and (b). Apparent growth with incomplete division and attempted division after minimum growth are depicted in (c) and (d). Many minute forms appear in (e). ×20,000. (f to j) Cells from cultures of other M. hominis strains. Cells on the left are from logarithmic phase cultures, while those on the right are from late stationary phase cultures. Strain 721 (f, g), and strain 463 (h, i, j). Cells (f) to (i) were grown under the optimal conditions described. ×10,000. (j) Cells were cultured in the special broth for strain 463. ×2,000. Preparations (a) to (d) were made with PTA, (e) to (j) with SZG. The bars represent 1 μm.*

Although strain 463 behaved similarly to the others in the "improved" medium, when cultured in the special broth medium for strain 463 without agitation, the generation time increased by about one-half, whereas very long filamentous forms became evident during logarithmic growth (Fig. 3j). By the late stationary phase, growth had become unusually pleomorphic. Under these conditions the growth pattern of strain 14027 also changed so that pleomorphic forms were already apparent during late exponential growth, whereas in "improved" broth they were only found in the late stationary phase.

Although the morphological pattern for the cultures of these strains of *M. hominis* was consistent under the stipulated conditions, unusual cell types might be observed rarely. For example, in the late stationary phase, strain 463 contained an occasional beaded structure (Fig. 4a); a single budding form was found in a logarithmic phase culture of *M. hominis* 14027 (Fig. 4b); and rosettes appeared infrequently in older cultures (Fig. 4c,d). A few nonfixed cells of the same strain were fringed (Fig. 4e).

Isolation of small viable cells. Attempts were made to isolate small viable cells from cultures of *M. hominis* 14027, by sequential filtration of cultures of different ages through filters of diminishing a.p.d. The filtration end point (the a.p.d. of the first filter through which no CFU passed) depended on the growth phase and age of the culture. For cultures incubated for 12 h and for 3 days it was 220 nm, but for those incubated for 7 and 14 days it was 450 nm, which suggested that very small forms in old cultures were not viable. Each step in the filtration sequence showed a significant decrease in CFU. Older cultures tended to contain larger cell aggregates, which were also more refractory to dispersal by sonication. Although the greatest incidence of viable small cells was evidently in the younger culture, none of them passed the 220-nm membrane, whose pore size would cover the upper limits of diameter for "elementary bodies."

Smaller cells entangled in the cell aggregates frequently seen in electron micrographs of populations in older cultures might resist dispersal by sonication, and then would be withheld by the preliminary filtration. To liberate such trapped cells the suspensions were exposed to enzymes before sequential filtration. The effect of DNase on cells from late stationary cultures of *M. hominis* is shown in Table 1. The maximum number of CFU found in preparations exposed to the enzyme for 15 min was five times that of the sonicated sample; but the number of viable small cells in the filtrates did not increase. Very few CFU passed the 450-nm membrane and none was detected in the 220-nm filtrate. Three-day cultures showed a similar pattern, whereas 7-day cultures yielded sterile filtrates.

Results of the trypsin treatment on logarithmic-phase cells are shown in Table 2. Although the maximum number of CFU obtained after 15 min of exposure to the enzyme approximated the VCC of the sonically treated sample, a few viable cells were actually present in the 220-nm filtrate after 30 min of trypsinization.

Pronase had a lesser effect on total CFU than sonication (Table 3), but increased the filtrability of viable particles more noticeably than did trypsin. The 450-nm filtrate yielded the most CFU after suspensions had reacted with Pronase for 10 min. The results of filtrations through the 220-nm membrane are complex. The CFU increased for the first 5 min of treatment, then disappeared after 10 min; they returned in much greater numbers after 20 min, but then declined. In both the trypsin and Pronase trials, 1-ml aliquots of the 220-nm filtrates grew in broth but not on agar. Older cultures gave the same filtration end points as those obtained by DNase treatment.

The results of the Pronase trial suggested that experimental conditions might be established under which this enzyme would yield maximum numbers of small viable cells. The number of CFU before and after exposure to Pronase and sonication is shown in Table 4. Exposure times of 5 and 10 min only were tested, because small viable cells seemed in greatest abundance at the former time and were not in evidence at the latter. Five minutes of treatment with the enzyme again proved effective. The controls (in PBS) showed a slight decrease in CFU, possibly from cell aggregation. The enhancing effect of Pronase may be taken as roughly 30, 110, and 100% for cells from logarithmic, stationary, and decline phase cultures, respectively. Subsequent sonication increased the viable counts of cells from actively growing cultures only, suggesting that younger cells are less damaged by Pronase and thus better able to withstand ultrasound.

In subsequent experiments, the suspensions exposed to Pronase for 5 min were filtered sequentially. In every instance, the number of CFU was greater in the enzyme-treated preparations than in the control suspensions. The greatest enhancement in the recovery of CFU (from 20 to 37,000—almost 2,000 times) was found in the 220-nm membrane filtrates of the late-stationary phase culture. No viable units

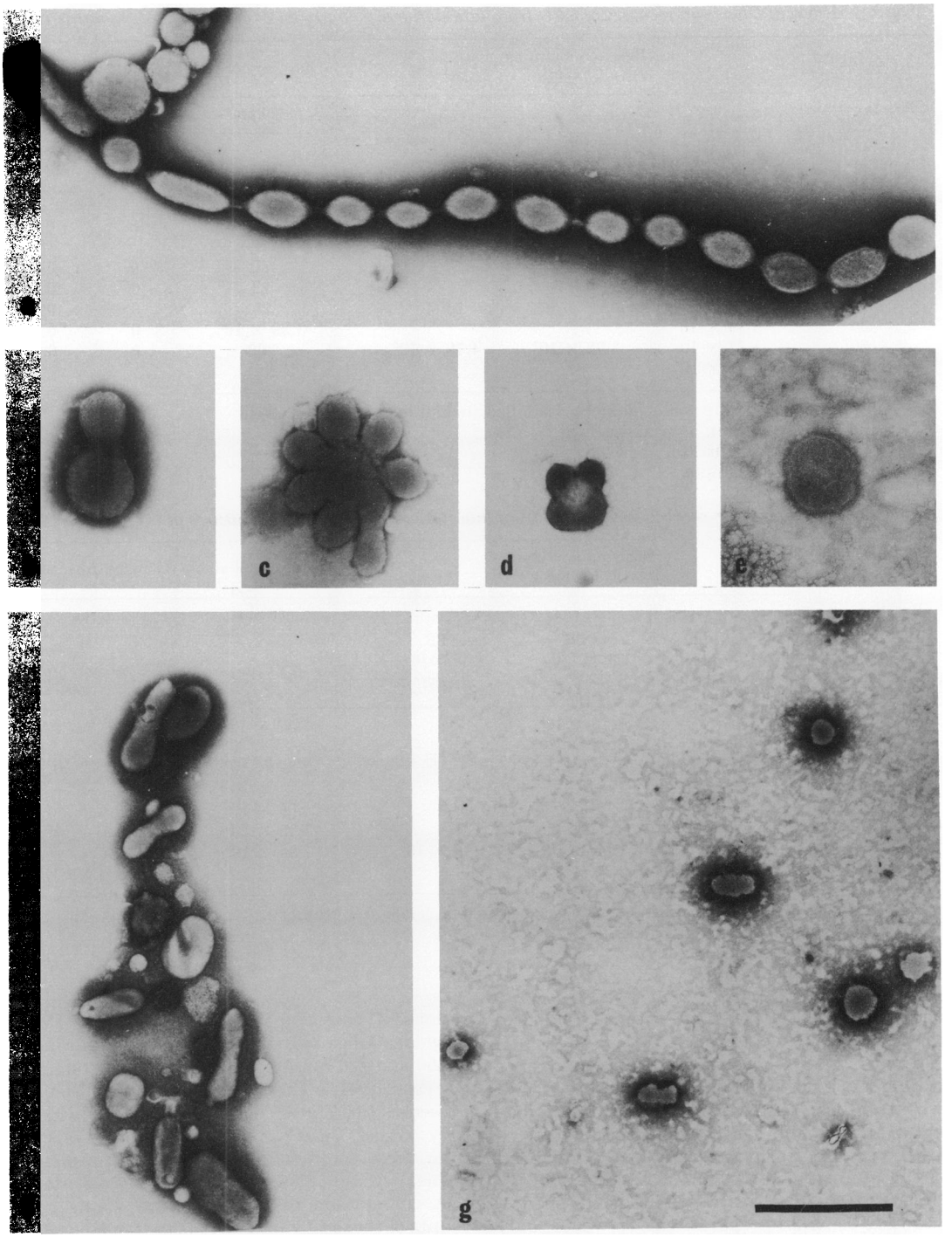

FIG. 4. *Other forms found in cultures of M. hominis. Rare (a) beaded, (b) budding, (c) and (d) fragmenting cells, and (e) a fringed sphere. Cells isolated by Pronase treatment and passage through membranes of (f) 450 nm and (g) 220 nm a.p.d. Micrograph (a) is of strain 463 grown in the special broth medium for that strain. The other micrographs are of strain 14027 from cultures in "improved" broth. Preparations were made with SZG (a, c to g), PTA (b), and ammonium molybdate (e). The bar represents 1 μm.*

TABLE 1. *Effect of DNase on recovery of CFU from late stationary phase (24 h) cultures of M. hominis 14027*

Reaction time (min)	Reaction mixtures[a] CFU/ml			Recovery of viable cells after filtration		
				450 nm	220 nm	
	DNase (pH 7.2)	PBS (pH 7.2)	$MgSO_4$ solution (pH 7.2)	CFU/ml	CFU/ml	Growth in broth medium[b]
0		3.21×10^7		0[c]	0	0
		1.21×10^{8d}				
	4.59×10^7			0	0	0
5	5.81×10^7			12	0	0
15	6.12×10^8			25	0	0
30	5.74×10^7			3.39×10^3	0	0
60	5.11×10^7	3.72×10^7	4.79×10^7	300	0	0
120	5.19×10^7					

[a] The enzyme and buffer solutions are described in Materials and Methods.
[b] Detected by growth on agar subculture.
[c] No CFU in 1 ml of filtrate.
[d] Sonically treated sample.

TABLE 2. *Effect of trypsin on recovery of CFU from late stationary phase (24 h) cultures of M. hominis 14027*

Reaction time (min)	Reaction mixtures[a] (CFU/ml)			Recovery of viable cells after filtration		
				450 nm	220 nm	
	Trypsin (pH 8.0)	PBS (pH 7.2)	PBS (pH 8.0)	CFU/ml	CFU/ml	Growth in broth medium[b]
0		1.76×10^8		1.32×10^5	0[c]	0
		2.14×10^{8d}				
	2.29×10^8			2.22×10^5	0	0
5	2.28×10^8			3.71×10^5	0	+
15	2.54×10^8			1.08×10^6	0	+
30	1.51×10^8			3.10×10^6	20	+
60	2.58×10^7	1.55×10^8	1.46×10^8	1.94×10^6	0	+
120	0					

[a] The enzyme and buffer solutions are described in Materials and Methods.
[b] Detected by growth on agar subculture.
[c] No CFU in 1 ml of filtrate.
[d] Sonically treated sample.

passed the 220- and 450-nm filters in cultures incubated for 3 and 7 days.

Small cells able to pass without assistance through membranes of 220 nm should fall within the size range of elementary bodies. The above experiments indicate that such cells were indeed present in cultures of *M. hominis* 14027, but only during late stationary phase growth, thus corresponding with the small cells seen in negative-contrast preparations. However, only some of the filtrates contained these small cells and then in extremely small numbers. As their incidence was increased by Pronase treatment, this enzyme was used for isolation of the particles on a larger scale. Negative-contrast preparations of particles isolated by filtering Pronase-treated suspensions through membranes of 450 and 220 nm are illustrated in Fig. 4f and g, respectively. Their least dimensions never exceeded the stated pore size through which they passed. Cells having any diameter larger than these could still pass through the membranes without distortion if properly oriented.

A comparison of the total and viable cell counts of these filtrates reveals that of 3.6×10^7 cells which passed the 450-nm membranes, 2.45×10^7 or about 67% were viable, whereas of 4.55×10^6 cells passing the 220-nm membranes, only 1.27×10^3, or abut 0.03%, were capable of growth. The latter filtrate was used as inocu-

lum for a culture. After an extended lag phase of about 10 h, growth proceeded at the same rate and to the same final population as with an inoculum of logarithmic phase cells under identical and optimal conditions.

DISCUSSION

Mycoplasmas are generally cultivated for microscope studies without special consideration for their responsiveness to the nutritional and physical conditions of their environment. A culture of indeterminate age or growth phase may be introduced into an arbitrarily selected medium and simply incubated until turbidity dictates harvesting. Only a single sample may be prepared for microscopy and then in thin section, and without regard to the plasticity of these cells. Attempts have been made to clarify *Mycoplasma* reproduction while avoiding these differences in approach (4, 10, 11, 12, 14, 19). Indeed, replication by binary fission has been postulated for a number of *Mycoplasma* species, including *M. hominis* (10), a system supported by analogy with other prokaryotic cells. However, this report apparently represents the first detailed study of the main changes in morphology at every phase of growth.

Minor modifications to a classic *Mycoplasma* medium (13) and cultural agitation during incubation improved the growth of *M. hominis* 14027. The improvements included shorter generation time during exponential growth, high

TABLE 3. *Effect of Pronase on recovery of CFU from late stationary phase (24 h) cultures of M. hominis 14027*

Reaction time (min)	Reaction mixtures[a] (CFU/ml)		Recovery of viable cells after filtration		
			450 nm	220 nm	
	Pronase (pH 7.0)	PBS (pH 7.0)	CFU/ml	CFU/ml	Growth in broth medium[b]
0		1.01×10^8 1.65×10^8[d]	1.97×10^2	0[c]	+
	1.31×10^8		8.61×10^4	55	+
5	1.44×10^8		2.22×10^5	88	+
10	1.37×10^8		2.46×10^5	0	+
15	7.82×10^7		7.50×10^4	IF[e]	+
20	5.09×10^7		6.19×10^4	860	+
30	3.60×10^7	8.18×10^7	7.46×10^4	260	+
60	8.75×10^6				

[a] The enzyme and buffer solutions are described in Materials and Methods.
[b] Detected by growth on agar subculture.
[c] No CFU in 1 ml of filtrate.
[d] Sonically treated sample.
[e] IF, Insufficient fluid.

TABLE 4. *Effect of Pronase and sonication on CFU of M. hominis 14027*

Growth phase	CFU/ml				
	Cell suspension	Reaction times			
		5 min			10 min
		PBS	Pronase	Pronase and sonication	Pronase
Late logarithmic (12 h)	1.15×10^7	1.00×10^7	1.29×10^7	2.22×10^7	1.36×10^7
Late stationary (24 h)	1.51×10^8	1.39×10^8	2.94×10^8	1.41×10^8	2.48×10^8
Early decline (52 h)	1.35×10^8	8.59×10^7	1.69×10^8	1.02×10^8	9.11×10^7

maximum population in the stationary phase, and longer half-life for cultures in decline (Fig. 1). The results compare favorably with the doubling times (3) and growth curves (10) reported for other strains. To secure a healthy inoculum, growth was initiated with a culture in exponential phase, derived from a single stock of a clone of strain 14027, so that damaged cells would be eliminated through dilution. These precautions may account for the good growth responses. Except for their lesser dimensions, the forms typical of logarithmic phase growth under the above modified conditions (Fig. 2a,b,c) were similar to those in most rapidly dividing bacterial cultures.

The morphological types present during logarithmic growth persisted into the late stationary phase, when pleomorphic forms emerged (Fig. 3a to e). These suggested that growth and division had proceeded independently. Among the pleomorphic forms described were some considered by others (1, 9) as replicative, by mechanisms that included multiplication through the fragmentation of filaments (Fig. 4a), and single (Fig. 4b) or multiple (Fig. 4c,d) budding. The possibility that such forms might represent polymorphism rather than pleomorphism (i.e., a stage in a normal life cycle rather than involution forms) was considered unlikely because of their heterogeneity and rarity.

Of the methods available for isolating small viable cells from mycoplasma cultures, filtration should least affect morphology and viability. Conditions were determined which allowed the maximum number of viable cells to gravitate passively through membranes. Although both sonication and enzyme treatment increased the total number of CFU, only exposure to enzymes increased their filtrability through 450- and 220-nm membranes (Table 1). Pronase brought about the highest recovery of viable cells in the 220-nm filtrates (Table 3). The complex results obtained with this enzyme could be interpreted as follows. The CFU recovered in the filtrates exposed for 5 min are small cells, which lose viability on longer exposure to the enzyme. Further exposure of larger cells, by removing external layers, permits their passage through filters of lesser porosity. An alternative possibility, that the CFU recovered from cells exposed to Pronase for 20 to 30 min was due to smaller cells released from within larger ones, is not supported by thin-section preparations. The possible release of small cellular entities through dispersal of cell aggregates is a more plausible hypothesis.

Most of the cells in Fig. 4f are larger than the 100 to 250 nm diameter reported for elementary bodies. Although the particles illustrated in Fig. 4g are within that size range, they have rough surfaces, which again suggest a denudative process. Such an effect could cause loss in viability; but only about one-third of similarly treated cells in the 450-nm filtrate failed to grow, and an equivalent decrease in the number of CFU in the 220-nm filtrate would not alter the results significantly. Without such treatment, no viable cells passed the 220-nm filter and therefore would not have been isolated. Moreover, those which were isolated showed normal growth.

Previous filtration studies have used positive or negative pressure to speed the passage of mycoplasmas through membranes. Our approach has placed special emphasis upon the deformability of these cells, and assumes that a direct relationship obtains between the functional pore size of a given filter and the smallest dimension of any rigid particle passing through it (2, 20). In our experiments, presumably the extreme care taken in filtration and subsequent fixation resulted in little, if any, distortion to the cells.

The suggestion has been made that mycoplasmas are not completely fixed by glutaraldehyde treatment alone (16) and might undergo lysis or alteration on exposure to suspending fluids; but no supporting data have been provided for fixed organisms exposed to hypotonic conditions. Our findings imply that typical reproductive units are larger than was formerly believed. This is consistent with the demonstration (15) that, under both positive and negative pressure, prefixed cells are retained by filters through which nonfixed cells passed. Furthermore, the micrographs of logarithmic phase cells (Fig. 2a) indicate that replicating cells are usually at least 400 nm in diameter, an observation conforming with the opinion expressed by Morowitz, based on theoretical grounds, that structures of lesser diameter are unlikely to possess "the complexity and stability necessary to be biological self-replicating entities" (18).

The low viability of the small cells suggests that rather than representing a new stage in development, they are aberrant forms resulting from suboptimal cultural conditions. Complex life cycles are not recognized as occurring in bacteria, and *M. hominis* seems to be similar in this regard. Bacteria are, however, known to be pleomorphic under unfavorable growth conditions.

There are at least two plausible explanations for the presence of apparently acellular globules in old cultures of *M. hominis* (Fig. 3g). They may be small cells resulting from aberrant division, or vesicles spontaneously reas-

sembled from membrane fragments. If the former explanation applies, the very small forms may be mostly anucleate, with a rare unit having both a complete genome and the ribosomal complement necessary for self-replication, as in the "minicells" of *Escherichia coli*. On the other hand, the phenomenon of membrane reaggregation is known to occur with solubilized membranes of mycoplasma (7, 8, 22).

The question arises as to which of the cells in an aging culture of *M. hominis* are viable. The only morphological types present throughout growth are the spheres and rods characteristic of the logarithmic phase, and these would seem the most likely to be regenerative. Why certain cells retain normal morphology, while others in the same population apparently develop into markedly aberrant forms, cannot be determined from this work. However, one can speculate that the fate of a particular cell might depend on its stage in the division process when the limiting conditions developed.

A simplified scheme depicting the morphological changes exhibited by *M. hominis* 14027 during growth under optimal conditions is sketched in Fig. 5. This basic pattern was demonstrated alike by a type culture strain, a reputedly filamentous strain (17), and three clinical isolates—strong evidence that it represents the species behavior. The evidence presented here is apparently contrary to the concept of the "elementary body" as the essential reproductive unit in this species of *Mycoplasma*. The usual replicating cell for this microorganism has a diameter of about 400 nm, and population increase occurs through binary fission.

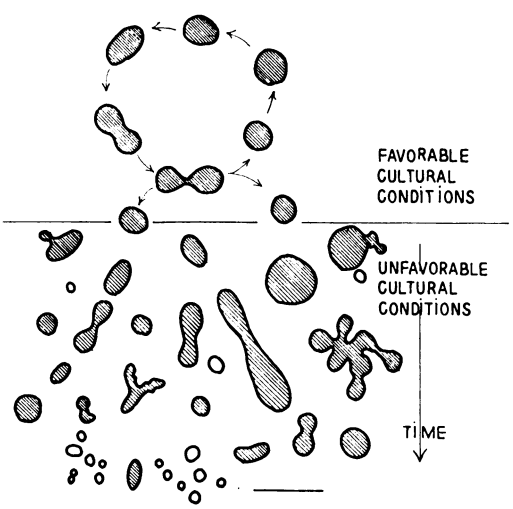

FIG. 5. *Morphological development postulated for M. hominis 14027 in batch cultures under optimal cultural conditions. The shaded cells are thought to have the potentiality for growth. The bar represents 1 μm.*

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