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NOTES

Effect of Preparatory Techniques on the Gross Morphology of *Mycoplasma hominis*

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A method is presented whereby cells of *Mycoplasma hominis* can be prepared with minimal distortion for electron microscopy. After the addition of glutaraldehyde to broth cultures, incubation is continued for 1 h. The cells are then collected by centrifugation, washed in distilled water, and used for negative-contrast preparations.

Disagreement on the gross morphology of organisms included in the family *Mycoplasmataceae* may partly reflect the heterogeneity of its members, but contributing factors are imperfect understanding of their growth requirements and their liability to deformity during preparation for microscopy. Various physical and chemical conditions, including gravitational force, osmotic change, and dehydration, have been implicated as distorting agents (1–5, 7, 11). Despite such wide recognition of the plasticity of these cells, no procedure was established for preparing these "soft-skinned" organisms with minimal distortion for electron microscopy. As an essential preliminary to a morphological study of the growth of *Mycoplasma hominis*, cells of strain ATCC 14027 were exposed to different conditions during their collection and preparation by the negative-contrast technique. (This work is based on a thesis submitted by J. Robertson to the faculty of graduate studies, McGill University, in partial fullfillment of the Ph.D. degree requirements.)

To ensure that the proportion of cells in the stock culture which were irreparably damaged by freezing and thawing would be negligible in the final population, the cultures were prepared as follows. Stock (1 ml) was added to 9 ml of broth and incubated overnight at 37 C; 1 ml of this culture was added to 9 ml of fresh broth and, when nephelometry readings indicated that exponential growth was well advanced, 0.5 ml was used to inoculate 49.5 ml of broth. This test culture was incubated until midway through the logarithmic phase. The broth medium used for this study (pH 7.3; 384 mosmol/kg) had been developed to furnish optimal growth of *M. hominis* through short generation time, high cell yield, and a short rate of decline (9). Silicone-coated glassware was used throughout to prevent cell adhesion to containers used for cell culture or collection.

To examine the effect of pre-fixation on morphology, a culture was divided into two parts. One part received 10% by volume of 5.0% (vol/vol) glutaraldehyde in Sorenson buffer (pH 7.4: 920 mosmol/kg) and was incubated at 37 C, while the other part was placed at 4 C. After 1 h the cells of both samples were collected at 3,090 $\times$ *g* for 15 min at 4 C and negative-contrast preparations were made with 1% (wt/vol) phosphotungstic acid adjusted to pH 7.4 with NaOH (34 mosmol/kg). Many of the fixed cells were obscured by a layer of darkly stained material which was apparently removed by washing them twice in Dulbecco phosphate-buffered saline (PBS) (pH 7.2; 270 mosmol/kg). Clumping caused by PBS was circumvented by washing in deionized, glass-distilled water instead of PBS. This caused no morphological change.

Figure 1 contains photomicrographs of the cells collected (a) with and (b) without pre-fixation, respectively. The fixed cells were spheres of 0.35- to 0.50-μm diameter and rod- or dumbbell-shaped forms up to 1.5 μm in length. The nonfixed cells (in PBS) were extremely pleomorphic, generally larger, and sometimes ghost-like. Preparations made from suspensions after overnight storage at 4 C showed the fixed cells (in water) were unchanged in size and shape, whereas the nonfixed cells were even larger and more pleomorphic than before refrigeration. The morphology of the fixed cells did not alter during 1 year of storage at 4 C.

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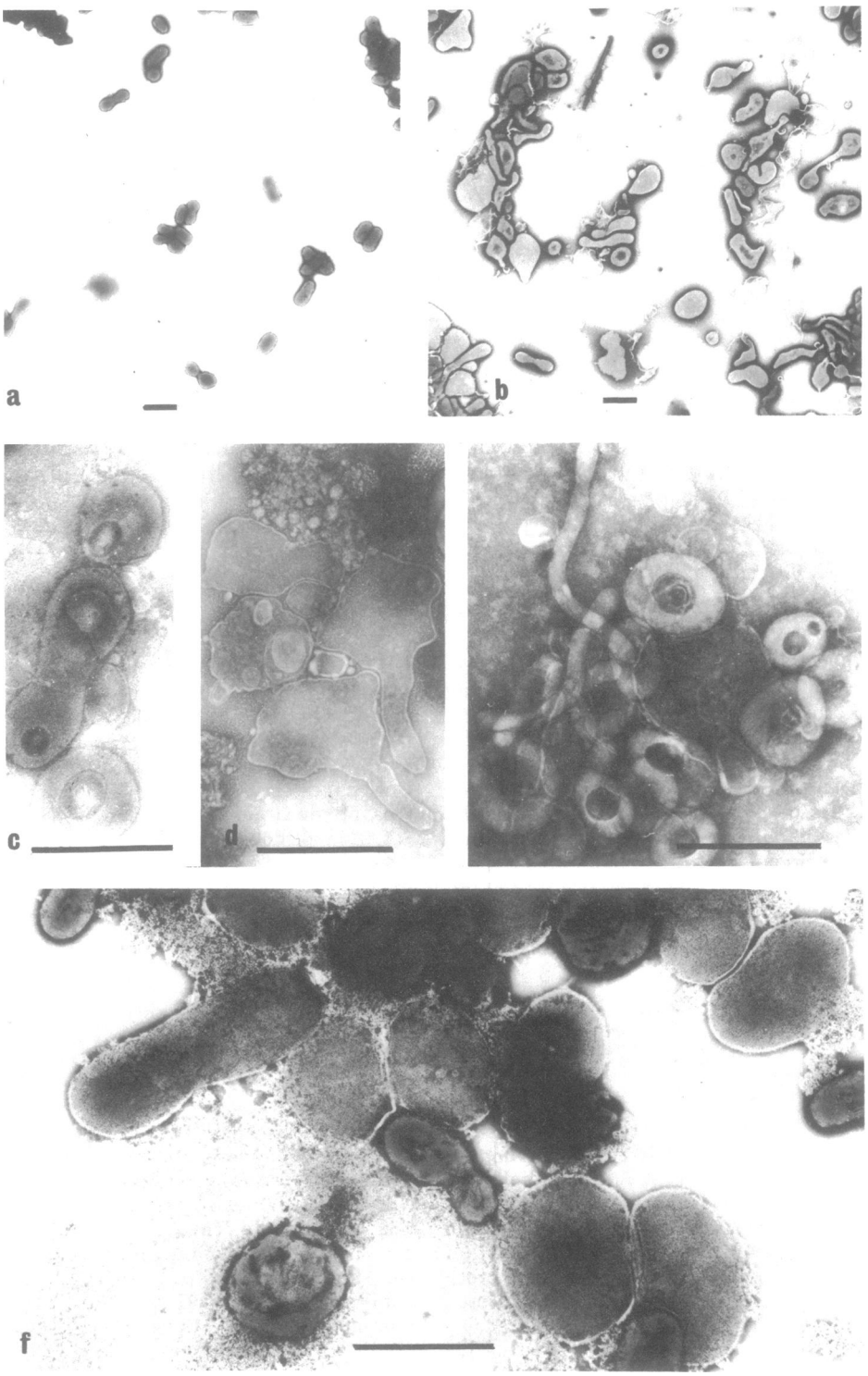

FIG. 1. *Photomicrographs of negative-contrast preparation of M. hominis cells in logarithmic phage growth. Cells collected at 3,090 × g for 15 min (a) with and (b) without pre-fixation (phosphotungstic acid). Nonfixed cells suspended in (c) broth and (d) iso-osmolal PBS; and (e) nonfixed cells collected at 17,750 × g after 15 min and suspended in broth (iso-osmolal ammonium molybdate). (f) Cells incompletely fixed in 0.25% glutaraldehyde (phosphotungstic acid). Grids were examined in a Philips EM 300 electron microscope at 60 kV. The bars represent 1 μm.*

Similar morphological types were seen in cells preserved in final fixative concentrations of 0.025, 0.5, or 2.5%, but with certain lots of glutaraldehyde used at the lowest concentration, half of the cells had at least double the expected dimensions (Fig. 1f). Such swollen forms were considered to be partially fixed because, unlike nonfixed cells, they retained their shape under hypotonic conditions. Because cells fixed at 4, 23, or 37 C appeared identical, the procedure was carried out at the 37 C incubation temperature.

To find the effects of gravitational force on *M. hominis* morphology, a logarithmic phase culture was divided into five parts. The cells in one portion were fixed and collected as described above; those from the other portions were collected without pre-fixation, three after 15 min at 3,090 × *g*, 8,900 × *g*, and 17,750 × *g*, respectively, and the last after 30 min at 3,090 × *g*. The nonfixed cells were gently suspended in broth, and negative-contrast preparations were made in ammonium molybdate (pH 7.4; 384 mosmol/kg). The fixed cells displayed the same morphological types as Fig. 1a. The appearance of nonfixed cells clearly depended on conditions of centrifugation. Cells collected for 15 min at 3,090 × *g* (Fig. 1c) were similar to fixed cells, but those in all other preparations were definitely more pleomorphic, the degree of morphological variation increasing with both the speed and the duration of centrifugation. Cells sedimented at 17,750 × *g* are shown in Fig. 1e. The usual forms (Fig. 1a,c) predominated, but filamentous structures and ghost forms were also present. Although these pliable microorganisms might be expected to respond to such treatment by undergoing morphological changes such as those reported for *Mycoplasma gallisepticum* (5), this tendency seems to have been generally overlooked by workers studying this family. The present study confirms that centrifugation can cause distortion and, furthermore, shows that the degree of pleomorphism, including filament formation, is roughly proportionate to the centrifugal force applied. Since filamentous forms appeared only when nonfixed cells were subjected to high-speed centrifugation, they may be considered artefacts, and reproduction occurring through their fragmentation was excluded as a usual means of replication by this organism.

The suspension of nonfixed cells collected at the lowest gravitational force used (Fig. 1c) was added to an equal volume of PBS brought to isoosmolality with the broth by the addition of 3.0 M NaCl. Negative-contrast preparations revealed that particles which in broth suspension had resembled the fixed cells, were now decidedly pleomorphic, a response due either to the salt used to modify the buffer or to the buffer itself. In addition to having the same hydrogen ion concentration and osmolality as the growth medium, the contrasting agent (ammonium molybdate) penetrated material obscuring the nonfixed cells in phosphotungstic acid preparations, thereby allowing the washing steps, a possible source of distortion, to be omitted. When mycoplasma cells are prepared for electron microscopy, attention is usually paid to the hydrogen ion concentrations of media, suspending fluids, and solutions used for achieving contrast. With certain exceptions (1, 3, 4) the osmotic environment of these cells is seldom considered. The osmolality of the broth medium used for this study (384 mosmol/kg) exceeds the 117- to 156- mosmol/kg range calculable from estimates of the internal osmotic pressure of certain *Mycoplasma* species (6, 10); but the excellent growth obtained from this medium suggests that its osmolality is appropriate for the present purpose.

Cells fixed in the fashion described have been used by us for several years with confidence that their morphology would remain constant on exposure to high-speed centrifugation, suspending fluids, contrasting agents, or storage in the cold, all factors which altered the morphology of nonfixed cells. Under standardized conditions of growth and fixation, the morphological types present in a given growth phase have been consistent. These are considered representative of *M. hominis* morphology in exponential growth. Although preparations of minimally distorted cells can be obtained without fixation, the low gravitational force used in their collection (3,090 × *g* for 15 min) removed only 93% of the colony-forming units in the culture and might not be representative. To ensure collection of over 99% of the colony-forming units including any small cells (elementary bodies) which might be present, a force of about 17,750 × *g* for 15 min was required, but distortion then occurred in the absence of prior fixation. In preparations of fixed cells collected at 17,750 × *g*, no new forms could be found. It is unlikely, therefore, that cell types other than those shown here are present during exponential growth of *M. hominis*. Some reduction in pleomorphism has been reported after fixation procedures at later stages than that described here, but these claims (2, 5, 7, 11) seem to have been unconvincing.

In addition to the usefulness in the study of the gross morphology of mycoplasmas, this prefixation procedure serves as the first step in preparing thin sections for studies of mycoplasma ultrastructure (8).

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