Analysis of the Life Cycle of *Mycoplasma gallisepticum*¹

HAROLD J. MOROWITZ AND JACK MANILOFF²

Department of Molecular Biophysics, Yale University, New Haven, Connecticut

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ABSTRACT

MOROWITZ, HAROLD J. (Yale University, New Haven, Conn.), AND JACK MANILOFF. Analysis of the life cycle of *Mycoplasma gallisepticum*. J. Bacteriol. **91**: 1638–1644. 1966.—A series of electron microscope observations on *Mycoplasma gallisepticum* strain A5969 have been made by use of thin-section techniques and negative staining. The methods presented a consistent picture of a postdivision cell, which contains a fibrillar nuclear region, surrounding ribosomal region, highly organized bleb at one end of the cell, granular infrableb region, and bounding unit membrane. Cell division commenced with the appearance of a second infrableb area at the end of the cell opposite the original bleb. A new bleb grew in this area, and the cell then elongated. The nuclear material segregated into two parts separated by a band of ribosomes. A constriction appeared, in this central ribosome-packed area, leading to the formation of two daughter cells. The following was noted: the cells were very small (volume, 5×10^{-14} cm³); each cell was highly structured and strongly ordered; and the replication appeared to be a very precisely programmed series of events.

From the point of view of cellular ultrastructure, cell replication is the time sequence of structures, starting right after one cell division and terminating after the next cell division. For a total view of the process, these structures must then be correlated with the sequence of chemical reactions occurring in the cell over the same time cycle.

Mycoplasma gallisepticum strain A5969 [an avian pleuropneumonia-like organism (PPLO)] has proven to be ideally suited for the study of cellular ultrastructure during replication for several reasons. (i) The cell's fine structural elements have been catalogued and contain several easily identifiable microstructures which can be conveniently studied by thin-section electron microscopy (1, 5). (ii) The cell contains a very well-defined polar body (the bleb), so that a reference point exists for the orientation of all other structures. (iii) The cell appears to be extremely well ordered with respect to its sub-

structures. (iv) The extremely small size of the cell (volume, 5×10^{-14} cm³) makes it an interesting model system for self-replication.

In this study, log-phase cultures, containing cells distributed over all stages of the life cycle, were examined in thin section and in negatively stained preparations of whole fixed cells. To study the time sequence, it was necessary to order the stages. This was done by ordering these pictures by analogy with the studies of the reproductive processes of many other cell types, and by obtaining, from independent experiments, the time sequence of morphological transitions throughout the replication cycle. This latter sequence was determined by the periodic examination (of fixed, negatively stained samples) of collections of single cells growing on electron microscope grids. The cell's overall morphological cycle was thus correlated with the fine structure observed in the thin-section studies.

MATERIALS AND METHODS

Organism. These studies were carried out with *M. gallisepticum* A5969, which was obtained from M. E. Tourtellotte of the University of Connecticut. These cultures were originally obtained from H. Van Roekel of the University of Massachusetts, who isolated them from a chicken with chronic respiratory disease.

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² Present address: Chemistry Department, Metcalf Research Laboratory, Brown University, Providence, R.I.

Medium. The cells were grown on medium prepared as follows: Tryptose (Difco), 20 g; NaCl, 5 g; tris(hydroxymethyl)aminomethane (Tris), 5 g; and water, 1,000 ml. The pH was adjusted to 8.0 with HCl, and the broth was autoclaved, supplemented with 50 ml of sterile 20% glucose and 10 ml of PPLO Serum Fraction (Difco), and inoculated. A 10-ml inoculum from a 24-hr culture was used per liter of medium, and the culture was incubated at 37 C.

For growth on solid medium, 10 g of agar was added per liter of Tryptose Broth (Difco), prior to sterilization. As the broth-agar mixture cooled, it was supplemented with glucose and serum as above, and petri plates were poured.

Preparation for ultrastructure study. By use of a 1:100 ratio of inoculum to medium, PPLO A5969 were found to exhibit a typical bacterial type of growth curve: a lag phase of about 3 hr, an exponential or logarithmic phase to 40 hr, and a stationary phase to at least 70 hr. For studies of exponential-phase cells, a 22- to 26-hr culture was used.

In these studies, broth culture samples were taken at 37 C and poured into an equal volume of cold (4 to 6 C) fixative [12.5% glutaraldehyde in 0.2 M sodium cacodylate, final pH about 7.2 (5)]. After 1 hr at 4 to 6 C, either a drop of the suspension was put on an electron microscope grid for negative staining, or the cells were harvested by centrifugation ($14,350 \times g$ for 10 min), washed overnight in 0.1 M cacodylate buffer at 4 to 6 C, and the pellets were prepared for thin-section electron microscopy.

Electron microscopy. The procedure for preparation of the fixed pellets for thin-section electron microscopy (dehydration, Epon embedding, thin sectioning, and uranyl acetate staining) has been previously described (4). The negative-staining procedure (with 2% phosphotungstic acid, pH 7.0) has also been described elsewhere and has been shown to preserve cellular morphology (5). The grids, containing thin sections or negatively stained suspensions, were examined with an RCA EMU-3B electron microscope.

Growth on grids. Carbon-shadowed, Formvar-coated 200-mesh stainless-steel electron microscope grids were placed in a petri dish on a nutrient agar surface. On each grid was placed one drop (about 0.05 ml) of inoculum, which was obtained by filtering a log-phase culture through a $0.45\text{-}\mu$ (pore size) Millipore filter (to disperse cell aggregates). The petri dishes were then incubated at 37 C, and, at 10-min intervals, grids were removed. The grids were immediately immersed in 6.25% glutaraldehyde, at 4 to 6 C, for 1 hr. They were then negatively stained, as described above.

RESULTS

Figure 1 shows cells that were taken from a culture growing in liquid media, filtered through a Millipore filter (pore size, $0.45\text{ }\mu$), fixed, negatively stained, and examined in the electron microscope. The pictures have been ordered to give a continuous picture of the life cycle, based on the fundamental aspects of cell division found

throughout nature: formation of a predivision cell which contains the components of two identical, but unseparated, daughter cells, followed by division and subsequent repetition of the process.

A random sample of 467 cells was counted, of which 33 (7%) had two blebs. This indicated that, of the cell's 210-min generation time, approximately 7%, or about 15 min, is spent in two bleb stages in broth culture. Of the 33 two-bleb cells, 8 had an identifiable constriction, indicating that the actual division process occurred approximately during the last 4 min of the life cycle.

A time series was obtained for cells grown on grids and sampled at 10-min intervals. Selected pictures are shown in Fig. 2. At zero-time (Fig. 2a), most of the cells had the appearance of a somewhat teardrop-shaped structure with a bleb at one end. During incubation the cells elongated, and, by 10 min, a second bleb formed at the opposite end of the cell from the first bleb (Fig. 2b and c). A constriction then formed midway between the blebs (Fig. 2d) at about 50 to 60 min. Two cell microcolonies were seen at 120 min, and by 140 min most cells had divided (Fig. 2e, f, and g). Three cell microcolonies began to appear at 200 min, and most cells had reached this stage by 240 min (Fig. 2h and i). In a sample grown for 18 hr, at least 90% of the cells formed microcolonies.

The time sequence of events of cellular replication on grids was in agreement with that proposed for cells in liquid culture. The differences in the duration of the events in the two experiments could be expected to arise owing to the different environmental conditions in each case.

Some of the two-bleb cells, seen by thin-section electron microscopy, are shown in Fig. 3. The blebs showed a characteristic fine structure. Between each bleb and the center of the cell (containing the nuclear material and the ribosomes) was a granular region of moderate density, the infrable region. These cells appeared to be either quite narrow (about $0.20\text{ }\mu$; Fig. 3d) or quite broad (about 0.50 to $0.60\text{ }\mu$; Fig. 3a) and were about $1.4\text{ }\mu$ long. Some of the two-bleb cells showed a constriction midway between the blebs (Fig. 3e). In these cells, a band of ribosomes was seen separating the two nuclear areas. Although this may be an artifact due to the plane of section, this ribosomal region was seen in most cells having a noticeable constriction and, hence, was considered to be a part of the morphology.

DISCUSSION

The purpose of this study has been to concentrate on the detailed changes of subcellular

FIG. 1. Filtered cells, glutaraldehyde-fixed and negatively stained. The cells have been ordered to give a continuous picture of the life cycle. (a, b, and c) Cells having one bleb (bl), with the infrable region (ir) and the periphery of the large section (arrows) most heavily penetrated by the stain. (d and e) Elongated cells having two blebs. (f, g, h, and i) Cells showing increasing stages in the formation of a constriction. $\times 36,400$.

FIG. 2. Cells growing on grids, removed at various times, glutaraldehyde-fixed, and negatively stained. Cells show the time sequence of changes in cell shape and morphology during replication. (a) Zero-time, single teardrop-shaped cell having one bleb; (b and c) 30 and 60 min, respectively, cells having two blebs; (d) 90 min, a constriction has begun midway between the blebs; (e and f) 120 and 150 min, respectively, two-cell microcolonies; (g) 180 min, one cell of the two-cell microcolony can be seen to have formed a constriction, midway between the two blebs; (h and i) 210 and 240 min, respectively, three-cell microcolonies. $\times 36,400$.

FIG. 3. Thin-section electron micrographs of cells, glutaraldehyde-fixed, Epon-embedded, and uranyl acetate-stained. Each cell is bounded by a "unit" membrane and contains two blebs, two infrableb regions, a fibrillar nuclear material (n), and ribosome-filled regions (r). The cell in (e) shows a suggestion of a constriction in the central, ribosome-packed part of the cell. $\times 72,800$.

morphology during the replication process. The primary replicative act under the growth conditions studied has been a rather precise symmetrical binary fission. In concentrating on the single replicative process, we did not observe the elementary cells, platycytes, or exoblasts reported in *M. gallisepticum* by Shifrine, Pangborn, and Adler (6), or the wide variety of forms reported by Dutta, Dierks, and Pomeroy (2). All of the forms reported by these authors have been observed by us in previous work prior to our introduction of rapid glutaraldehyde fixation and our concentration on single cells rather than extended colonies on solid medium. A detailed discussion of the reasons for believing that these previously observed forms present a less accurate representation of the living cell has been given by Maniloff (Ph.D. Thesis, Yale Univ., New Haven, Conn., 1964) and Maniloff, Morowitz, and Barnett (5). These differences in technique make it unrewarding to go into detailed comparison of the binary fission scheme reported here with the life cycles postulated in the previous studies. We consider that the very good agreement between the results of negative staining and the thin-section work constitutes strong evidence in favor of the validity of the replicative scheme being proposed. This is not to deny the possibility of alternate modes of replication in *M. gallisepticum* under conditions of growth other than those reported here.

Before discussing the replicative mechanism, we shall briefly catalogue the ultrastructures in a single, one-bleb, postdivision cell (1, 5).

Surface membrane. This is a membranous structure, 110 Å thick, which bounds the cell. Whereas we have previously reported the appearance of a unit membrane, Stoeckenius (*personal communication*) has suggested that the micrographs might be interpreted as showing a very thin wall-like structure attached to a unit membrane.

Nuclear region. The unbounded area in the center of the cell contains the cell's deoxyribonucleic acid and shows 30 Å fibrils oriented parallel to the long axis of the cell.

Ribosomes. The ribosomes, with properties similar to those of other organisms (140 Å in diameter, 70S sedimentation coefficient, and ribonucleic acid-protein ratio of 0.68), fill the intracytoplasmic space between the nuclear material and the membrane.

Bleb. A highly structured polar body, 800 by 1,300 Å, forms the narrow end of the cell. A granular infrable region separates the bleb from the rest of the cell. Neither the bleb nor the infrable region contains structural nucleic acids, as determined by the use of a nucleic acid-

specific strain in thin-section electron microscopy.

The bleb appears to be a unique feature of *M. gallisepticum*. It has not been observed in a wide variety of other *Mycoplasma* species fixed and stained by exactly the same technique. This structure has not been reported in bacteria. In addition to blebs, another unique feature has been found in *M. gallisepticum*: ordered cylindrical arrays of ribosomes. These, also, do not appear in other *Mycoplasma* species which have been identically treated. These features lend support to the suggestion by Klieneberger-Nobel (3) that *M. gallisepticum* be treated as taxonomically distinct from the genus *Mycoplasma*.

The events in a single replicative cycle can now be ordered. A single postdivision cell is a teardrop-shaped entity with a bleb-infrable structure at the small end, a nuclear region in the center, and a ribosome-packed region between the nuclear region and the large end of the cell. The structural changes begin with the formation of a second infrable region usually at the pole of the cell opposite the original bleb. This is followed by the formation of a second bleb protruding from the newly formed infrable region.

The order of infrable region formation, followed by bleb formation, is suggested by the pictures of negatively stained cells. Cells growing in liquid media showed an area behind the bleb, identified as the infrable region from thin-section studies, that was penetrated by the stain. Some of these cells showed a second penetrated area at the end of the cell opposite the bleb (Fig. 1a and b). From the similarity of the staining characteristics, it is suggested that this second area also contains an infrable region, and it seems reasonable to assume that the second bleb is made after the formation of a second infrable region.

During the process of bleb formation, the nuclear region grows and divides into two areas as the cell elongates. The intranuclear zone becomes filled with ribosomes, and the entire structure appears as two symmetrical teardrops connected at the large end but aligned on a common axis. A constriction then appears, midway between the blebs, leading to the formation of two daughter cells, each now having one bleb. The constriction takes place in the central ribosome-packed area.

M. gallisepticum thus presents an example of a cell where the replicative apparatus appears to be programmed in a detailed way with respect to the cell's organelles. The cell interior is completely structured with organized entities so that no part of the interior can be regarded as a homogeneous

phase in a thermodynamic sense. The production of a second set of organelles appears to take place at specific times and places, leading to the replicative process discussed above.

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LITERATURE CITED

1. DOMERMUTH, C. H., M. H. NIELSEN, E. A. FREUNDT, AND A. BIRCH-ANDERSEN. 1964. Ultrastructure of *Mycoplasma* species. *J. Bacteriol.* **88**:727-744.
2. DUTTA, S. K., R. E. DIERKS, AND B. S. POMEROY. 1965. Electron microscopic studies of the morphology and the stages of development of *Mycoplasma gallisepticum*. *Avian Diseases* **9**:241-251.
3. KLIENEBERGER-NOBEL, E. 1962. Pleuropneumonia-like organisms, (PPLO): Mycoplasmataceae, p. 69-73. Academic Press, Inc., New York.
4. MANILOFF, J., H. J. MOROWITZ, AND R. J. BARNETT. 1965. Studies of the ultrastructure and ribosomal arrangements of the pleuropneumonia-like organism. *J. Cell Biol.* **25**:139-151.
5. MANILOFF, J., H. J. MOROWITZ, AND R. J. BARNETT. 1965. Ultrastructure and ribosomes of *Mycoplasma gallisepticum*. *J. Bacteriol.* **90**:193-204.
6. SHIFRINE, M., J. PANGBORN, AND H. E. ADLER. 1962. Colonial growth of *Mycoplasma gallisepticum* observed with the electron microscope. *J. Bacteriol.* **83**:187-192.