ELECTRON MICROSCOPE STUDY OF THE RELATIONSHIP BETWEEN MESOSOME LOSS AND THE STABLE L STATE (OR PROTOPLAST STATE) IN *BACILLUS SUBTILIS*

ANTOINETTE RYTER AND OTTO E. LANDMAN

Institut Pasteur, Paris, France, and Department of Biology, Georgetown University, Washington, D.C.

Received for publication 16 March 1964

ABSTRACT

RYTER, ANTOINETTE (Institut Pasteur, Paris, France), AND OTTO E. LANDMAN. An electron microscope study of the relationship between mesosome loss and the stable L-state (or protoplast state) in *Bacillus subtilis*. *J. Bacteriol.* 88:457-467. 1964.—In a prior publication, it was postulated that inability of protoplasts to restart cell-wall synthesis and cell division and the inability of stable mass-conversion L forms to return to the bacillary state were both equivalent and both due to the interruption of a membrane-associated reaction sequence. It was further postulated that this reaction sequence might reside in the mesosome. In the present publication, it is shown by means of electron microscopy of thin sections that protoplasts and L forms do not contain mesosomes. The sequence of events leading to loss of the mesosomes during protoplasting is as follows. Soon after lysozyme addition, the mesosomes are extruded from the cell interior into the space between cell wall and cytoplasmic membrane. Mesosome fragments in the form of small vesicles gather at the poles of the cells and are released, along with intact protoplasts, when the wall fragments. (Sudden shift of bacilli to hypertonic environment also causes extrusion and fragmentation of mesosomes, but this damage is later repaired.) In intact bacilli, mesosomes are in contact with both the peripheral membrane and nuclear material. Upon extrusion of the mesosomes, a direct attachment between nuclear material and cytoplasmic membrane is observed. Deoxyribonucleic acid (DNA)-membrane attachment may play a role in the control of DNA replication. *Bacillus subtilis* L-colonies consist of irregularly shaped bodies of varying sizes, bounded only by a membrane. Many of the smaller bodies do not contain nuclear material, and many of the large ones appear inviable. Division is accomplished by a disorganized-appearing constriction process. There are no septa.

When a suspension of *Bacillus subtilis* is treated with lysozyme in osmotically stabilized media,

the cells are quantitatively converted to protoplasts. All of these protoplasts can be recovered as L-colony forming elements on chemically defined soft agar media. They can be propagated in the L state indefinitely or can be mass-reverted to the bacillary state by altering ingredients of the medium. Mass-reversion is induced by 2% agar media and, most efficiently, by 15 to 30% gelatin (Landman and Halle, 1963).

We have postulated that the conversion of bacteria to the heritable L state, which occurs concurrently with cell-wall removal, is due to the interruption of a self-sustaining (feedback) process. Reversion is thought to be due to reinitiation of the feedback process by a priming activity.

What are the cellular functions controlled by this feedback process? It is quite generally conceded that cells in the L state have lost the ability to make cell wall. Further, it is clear that such cells have suffered severe damage to their division mechanism. This damage is manifest in the inability of protoplasts and (many) stable L forms to divide in liquid media. Even on soft agar media, multiplication in the L state is extremely slow. Further, the wide variations in the size of the L bodies and the marked local differences in L-body morphology within each colony all indicate that the normal division process which controls the apportionment of protoplasm between daughter cells is not functioning.

In searching to understand the molecular basis of the stability of L forms (or of the inability of protoplasts in suspension to resume bacillary form), we are thus led to seek a feedback process involved in both septation and cell-wall formation.

Past studies have provided clues concerning the nature of the priming activity which reinitiates the feedback mechanism. Important among these clues is the observation that reagents which alter the physical consistency of the medium (namely, hard agar and gelatin) and even purely

mechanical stimuli (Landman, *unpublished data*) are highly effective in inducing reversion. These findings, among others, indicate that priming occurs at the cell periphery, perhaps at the cell membrane. What is the nature of the presumed priming stimulus at the cell periphery? Recent studies on the behavior of mesosomes have suggested the possibility that priming might be attributable to this membranous organelle itself. In particular, electron microscopic observations have led various workers to postulate that mesosomes play a vital role in cell-wall synthesis (Chapman and Hillier, 1953; Glauert, 1962; Ohye and Murrell, 1962) and in septum formation (Fitz-James, 1960; Imaeda and Ogura, 1963; Van Itersen, 1961). Strong support for the presumed role of mesosomes in septum formation was provided recently by Ryter and Jacob (1963), who showed, through superposition of transparent electron micrographs of serial sections of whole bacteria, that all nascent septa are connected to mesosomes.

Most importantly, Fitz-James (1960) indicated that mesosomes might be lost in the course of the conversion of bacilli to protoplasts in *B. megaterium*. If the mesosomes themselves were indeed responsible for the priming function, presence and absence of mesosomes should regularly be correlated with presence and absence of septation-and-wall-formation activity. The present collaboration was initiated to investigate this postulated correlation by studying the behavior of mesosomes during protoplast formation, and by tracing their subsequent fate in the L state.

MATERIALS AND METHODS

Strains. The Marburg strain of *B. subtilis* (strain 168; see Spizizen, 1958) was the principal strain employed. Certain of the experiments were also performed with a lysine mutant of strain ATCC 9945A of *B. licheniformis* (Thorne, 1962).

Media. The standard chemically defined medium (D-medium) used in these experiments has the following composition: glucose, 2 g per liter; L-tryptophan, 0.02 g per liter; NH_4NO_3 , 1 g per liter; K_2HPO_4 , 3.5 g per liter; KH_2PO_4 , 1.5 g per liter; gelatin, 20 g per liter; D-methionine, 0.6 g per liter; MgCl_2 , 0.0005 M; sodium succinate (pH 7.0), 0.5 M; agar, 7 g per liter. This medium permits efficient propagation of the L form of *B. subtilis*. The D-methionine strongly retards the reversion of L forms to the bacillary

state (Landman and Halle, 1963). Acid-hydrolyzed casein (0.2%) was added to this medium for growing L forms of *B. licheniformis*. D-medium may be prepared in batches and melted at 100 to 115°C for use as needed. The complex medium (Co-medium) used for routine growth of *B. subtilis* was 0.8% Difco nutrient broth with 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% KCl, and 0.001 M MnCl_2 .

Procedures. For electron microscopy, agar blocks containing intact L colonies were excised and fixed, without prefixation, according to the method of Kellenberger, Ryter, and Séchaud (1958). After overnight fixation in osmium tetroxide, the agar blocks were treated with 1% uranyl acetate for 2 hr and were then dehydrated and embedded in Vestopal (Ryter and Kellenberger, 1958).

To follow the formation of protoplasts, it was important to stop lysozyme action rapidly and to take samples at rather frequent intervals. At first, a procedure was followed in which fixative was added to cultures in liquid protoplasting medium (Landman and Halle, 1963) at a fixed time prior to the centrifugation of samples. This procedure often resulted in lysis. A new method was therefore developed. The bacteria were grown in Co-medium to late log phase and harvested by centrifugation. The pellet was then taken up in a few drops of melted D-medium containing 2 mg/ml of lysozyme (at 45°C). Drops of this suspension were then deposited on petri dishes of solid D-medium plus lysozyme, and these dishes were incubated at 37°C. Examination of sample droplets in an optical microscope showed that the conversion of bacilli to protoplasts was 50% complete in 60 min and virtually complete in 90 min. For electron microscopy, sample droplets were removed from incubation every 5 or 10 min, and were fixed in the manner outlined above for L colonies.

RESULTS

L forms. The following major features were noted. The bodies contained in *B. subtilis* (Fig. 1) and *B. licheniformis* (Fig. 4) L colonies are bounded by a cell membrane only. No cell wall or residual cell-wall material has been observed.

The L bodies do not contain mesosomes. Approximately 400 thin sections which traverse the nucleus were examined. In none of them were mesosomes or structures resembling mesosomes found. In sections of control bacillary populations

of both *B. subtilis* and *B. licheniformis*, mesosomes can be seen in 50% or more of the bacteria. (Fig. 5, 6, and 7).

The L colonies contain three types of bodies (see Fig. 1 and 4): (i) very small spherical bodies which appear to be devoid of nuclear material; (ii) medium-sized bodies, containing dense cytoplasm apparently very rich in ribosomes. These bodies are quite variable in shape and sometimes exhibit constrictions suggestive of cell division. The constrictions are irregularly placed and are sometimes observed to cut off cytoplasmic fragments (Fig. 2 and 3). We suppose that the small anucleate spherical bodies which have been described originate in this way. Each medium-sized L body generally appears to contain only a single nuclear region which is usually located near the center of the cell. Occasionally, however, cells with elongated nuclei are observed in which the nucleus sometimes seems to make contact with the cell membrane (Fig. 3). These nuclei characteristically display their constituent fibrils in parallel array along the long axis of the nucleus. (iii) A third class of elements found in *B. subtilis* and *B. licheniformis* L colonies are very large bodies (Fig. 1 and 4). The cytoplasm of these cells is generally much less dense than that of the smaller bodies and is often bounded by projections reminiscent of pseudopods. The large nucleus contains very sparse filaments. These cells appear to be on their way toward lysis and are probably not viable.

The proportion of the three cell types varies from one colony to the next. This variability appears to be related to the age of the colony but probably also involves other unknown physiological factors.

Bacillary forms. Bacillary colonies growing on D-medium (Fig. 5 and 7) consist of bacteria which exhibit typical *B. subtilis* or *B. licheniformis* cytology (Fig. 6). Their cytoplasm is very dense; it contains filamentous areas which correspond to the nucleus, and lamellar or tubular structures which are the mesosomes. The mesosomes are generally located near the nucleus or near the septa. Despite the high osmolarity of D-medium, there is no sign of plasmolysis. It should be noted, however, that older colonies of *B. licheniformis* on D-medium contain many empty and sparsely filled cells.

Protoplast formation. The first cytological change which becomes apparent when bacteria are embedded in lysozyme-containing D-medium

is the displacement of intracytoplasmic mesosomes toward the cell periphery (Fig. 9). This process is complete in 10 to 15 min. Intracytoplasmic mesosomes are no longer visible after this; instead, numerous small, round vesicles can now be seen in the area between the cytoplasmic membrane and the cell wall (Fig. 8). Once plasmolysis has progressed, these vesicles become localized at the cell extremities (30 min; Fig. 11 and 12).

If the bacterial pellet (from the low tonicity medium) is embedded in lysozyme-free D-medium, plasmolysis and the extrusion of mesosomes towards the cell periphery are also observed, but both processes occur at a slower rate.

In many cells with two distinct nuclear masses, fusion of the two masses occurs concurrently with the movement of the mesosomes toward the cell periphery. A single large nuclear body is formed which is often spherical in shape (Fitz-James, 1958; Fig. 12, 13, and 15). Whether nuclear fusion occurs depends on the state of division of the cell at the time of transfer to D-medium: in bacteria which have already started to form a septum, the nuclei remain separate and the membrane completes closure of the septum (Fig. 11 and 12) while the synthesis of the cell wall is interrupted.

In a few sections of plasmolyzed cells, a point-to-point attachment between nucleus and membrane is observed (arrows in Fig. 8 and 12). A more detailed electron microscopic study of this phenomenon (Ryter and Jacob, 1963) suggested that these point-to-point attachments are the regular result of the extrusion of nucleus-linked mesosomes. It is inferred that the nucleus is pulled to the cell periphery as the mesosome is everted.

Occasionally, small anucleate spheres are formed during plasmolysis (Fig. 13 and 14). Upon cell-wall rupture, these bodies are released into the medium.

At 30 to 45 min after lysozyme addition, breakage of the cell wall occurs. This rupture may take the form of a single tear in the polar region of the cell (Fig. 12 and 15) or may be manifested in numerous areas (Fig. 13). At the time of rupture, no general thinning of the cell wall is observed, and the walls retain their characteristic elongated shape even after large gaps have become visible. The protoplasts disengage little by little from the debris and assume spherical shape only after they are free from it (Fig.

FIG. 1. *L* colony of *Bacillus subtilis*. The cells are bounded by a membrane only. They never contain mesosomes. Three major cell types are observed: (a) small spheres, generally devoid of DNA; (b) medium-sized bodies of varied shapes, usually exhibiting large nuclei; and (c) very large bodies with sparsely distributed nuclear and cytoplasmic material, probably inviable. Magnification, 20,000 $\times$.

FIG. 2 and 3. Formation of anucleate cytoplasmic fragments by constriction. In Fig. 3, the nucleus is in contact with the membrane (arrow). Magnification 60,000 $\times$.

FIG. 4. L forms of *Bacillus licheniformis*. They show the same characteristics as the *B. subtilis* L forms: they lack walls and mesosomes and comprise the same variety of cell types. Magnification, 23,000 $\times$.

FIG. 5. *Bacillus subtilis* bacilli, grown on D-medium. These cells are cytologically identical to bacilli grown on Co-medium. Their walls show no abnormalities and there are no signs of plasmolysis. Mesosomes (m) are observed near the nucleus and near the septa. Magnification, 45,000 $\times$.

FIG. 6. *Bacillus licheniformis* bacilli grown on Co-medium. *B. licheniformis* generally resembles *B. subtilis* but can be distinguished by its smaller cell size and by its tendency to form long chains. Magnification, 50,000 $\times$.

FIG. 7. *Bacillus licheniformis* grown on D-medium. These cells are indistinguishable from *B. subtilis* grown on this medium. Magnification 45,000 $\times$.

FIG. 8. *Bacillus subtilis* embedded in D-medium plus lysozyme for 10 min. The mesosomes (m) have already been extruded from the cytoplasm and now appear in the form of rounded vesicles in the area between wall and membrane. A link between nucleus and membrane is visible (arrow): Magnification, 70,000 $\times$.

FIG. 9. Mesosome (m) apparently on its way toward extrusion. Magnification, 100,000 $\times$.

FIG. 10. Release of mesosome fragments into the medium during cell-wall rupture. Magnification, 50,000 $\times$.

FIG. 11. Plasmolyzed *Bacillus subtilis* bacilli (30 min after transfer from Co-medium to D-medium without lysozyme). Mesosome fragments have collected at the cell extremities. Crosswall formation has been interrupted but the cells show completed membranes. Each cell contains a single nuclear region. Magnification, 16,000 $\times$.

FIG. 12. *Bacillus subtilis* embedded in D-medium plus lysozyme for 30 min. Plasmolysis and mesosome fragmentation are at the same stage as in Fig. 11. In addition, cell-wall breakage is observed at the pole of the lower cell. In the upper cell, the nucleus is in contact with the cell membrane (arrow). Magnification, 70,000 $\times$.

FIG. 13. Cell-wall breakage in several areas. A small spherical body, apparently devoid of nuclear material, is being released. Magnification, 50,000 $\times$.

FIG. 14. Small spherical bodies released into the medium after wall breakage. Magnification 54,000 $\times$.

FIG. 15. View of cell-wall breakage. Magnification, 40,000 $\times$.

FIG. 16. Release of protoplast from cell-wall debris. The protoplast becomes fully spherical only after being freed completely from the debris. Magnification, 60,000 $\times$.

16). During the rupture of the wall and the disengagement of the protoplast, the mesosome fragments accumulated at the cell termini are liberated into the medium (Fig. 10). It is not clear whether some of these fragments still hang together, but evidently they are not attached to the protoplasts.

DISCUSSION

The electron microscopic observations outlined above present a clear correlation between septation-and-wall-forming capacity and the presence of mesosomes. Thus, protoplasts and L forms of *B. subtilis* and L forms of *B. licheniformis*, which all lack the ability to septate and make wall, are devoid of mesosomes, whereas their bacillary forms exhibit mesosomes prominently. One important exception to this correlation was noted, however: when bacilli were transferred from low-salt Co-medium to lysozyme-free, high-salt D-medium, plasmolysis caused extrusion of the mesosomes and, presumably, their destruction. Yet, such cells, which retain an intact cell wall, can evidently regenerate mesosomes, since all of them give rise to bacillary colonies composed of normal, mesosome-containing cells. It appears that, in *B. subtilis*, the postulated feedback sequence

is not severed by the destruction of the mesosome. Persistent loss of wall-and-septation priming only occurs when the wall is destroyed (and mesosome loss follows in the wake of wall destruction). Although the removal of the wall is thus seen to play a key role in the interruption of the feedback sequence, it does not necessarily follow that re-establishment of this sequence during reversion is triggered by cell-wall accretion. Our data on the stimulation of reversion by physical agents have suggested the alternative view that membrane invagination may be the primary event in reversion, and that invagination, in turn, is followed by wall-and-septation priming. It is hoped that an electron microscopic study of reversion may provide an answer to the presently unsolved question: which comes first in reversion, the mesosome or a wall fragment?

Whatever the ultimate answer to the question of primer primacy, it is clear that protoplasts and L forms can live and multiply without mesosomes. Our pictures show, however, that cell division (the organized process of apportioning nuclear and cytoplasmic material between sister cells) is very seriously disturbed in the L-colonies. There are no septa. Instead, an irregular constriction process subdivides the protoplasm, cutting off bits of nucleate and anucleate material of various sizes and shapes. The disorganized mode of division probably accounts for both the low growth rate and poor viability of L colonies. Division disorganization already becomes manifest during plasmolysis when, occasionally, anucleate fragments are pinched off. Our observations of this phenomenon support the findings of Weibull and Beckman (1960), who described the presence of small spherical bodies devoid of deoxyribonucleic acid (DNA) in their experiments with *B. megaterium*.

The ease with which cytoplasmic fragments are cut off during plasmolysis and L-form growth, and the efficiency with which viable protoplasts with intact cell membranes are reconstituted after mesosome eversion, all point to the conclusion that the cell membrane is exceedingly plastic and flexible. The behavior of mesosomes during growth also supports this conclusion (Ryter and Jacob, 1963). Further, we believe that this plasticity may account for the difference between the shapes of the L bodies in our thin sections and those ordinarily observed in L-colony squashes or suspensions under a light microscope. Since, in the present work, the L colonies were fixed for sectioning in situ without prior disturbance, we suppose that the irregular shapes seen in the electron micrographs are more truly representative of the natural state of the L forms than the spherical bodies normally seen in a light microscope. We infer that originally irregularly shaped bodies assume spherical shape when they are manipulated.

In the experimental section, we have repeatedly referred to points of attachment between the nucleus and the cell membrane. These observations assume significance in the light of the recent hypothesis that the replication of the bacterial chromosome is triggered at a point of contact with the cell membrane and that DNA replication is coordinated with septation at this site (Jacob and Brenner, 1963; Jacob, Brenner, and Cuzins, 1963). In the bacillary state, contact

between nucleus and membrane is implicit in the regularly observed association between mesosome and nucleus (Ryter and Jacob, 1963). This connection is made manifest after eversion of the mesosomes when nucleus-to-membrane bridges can occasionally be directly observed. It is documented, also, by the observations made with a light microscope by Fitz-James (1958), that the nucleus in recently formed protoplasts of *B. megaterium* occupies a position at the cell periphery.

In principle, observations on the persistence of nucleus-to-membrane bridges in dividing L colonies could provide an answer to the question whether a membrane attachment point is an obligatory requirement for bacterial "chromosome" duplication. Unfortunately, such an investigation faces numerous difficulties, both in the areas of technique and of interpretation, and it has, therefore, not yet been attempted.

In this paper, the relationship of two phenomena in *B. subtilis*—the mass-conversion stable L (or protoplast) state, and the behavior of mesosomes—has been conceptually and experimentally explored. It may be asked whether the relationships which have been delineated exist elsewhere in the microbial world. This question cannot be answered with certainty for any particular case until loss of priming function has been correlated with wall-and-mesosome loss. We feel, however, that L-colony formation (generally in response to penicillin) is so similar in a wide variety of bacterial species that the underlying mechanisms must be fairly similar (Landman and Halle, 1963). This view has recently received support by the discovery of mesosomes in bacterial species, especially gram-negative species, which had previously been thought to lack these structures. Thus, mesosomes have been observed in *Escherichia coli* (Vanderwinkel and Murray, 1962; Kaye and Chapman, 1963; Ryter, unpublished data), in *Agrobacterium tumefaciens* (Ryter and Manigault, in press), in *Moraxella* (Ryter and Piéchaud, 1963), in *Treponema* (Ryter and Pillot, 1963), and in *Fusobacterium polymorphum* (Takagi, Ueyama, and Ueda, 1963). In sum, it appears that mesosomes, much like L-colony formation potential, are found quite generally throughout the bacterial world.

ACKNOWLEDGMENTS

The propagation of *B. licheniformis* protoplasts in the L state was first accomplished by D. Mattheis and C. G. Leonard. We wish to

express our appreciation to these workers for permission to study this material in an electron microscope.

LITERATURE CITED

- CHAPMAN, G. B., AND J. HILLIER. 1953. Electron microscopy of ultra-thin sections of bacteria. I. Cellular division in *Bacillus cereus*. *J. Bacteriol.* **56**:362-373.
- FITZ-JAMES, P. C. 1958. Studies on the morphology and nucleic acid content of protoplasts of *Bacillus megaterium*. *J. Bacteriol.* **75**:369-382.
- FITZ-JAMES, P. C. 1960. Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. *J. Biophys. Biochem. Cytol.* **8**:507-528.
- GLAUERT, A. M. 1962. The fine structure of bacteria. *Brit. Med. Bull.* **18**:245-250.
- IMAEDA, T., AND M. OGURA. 1963. Formation of intracytoplasmic membrane system of *Mycobacteria* related to cell division. *J. Bacteriol.* **85**:150-163.
- JACOB, F., AND S. BRENNER. 1963. Sur la régulation de la synthèse du DNA chez les bactéries: l'hypothèse du réplicon. *Compt. Rend.* **256**:298-300.
- JACOB, F., S. BRENNER, AND F. CUZIN. 1963. On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symp. Quant. Biol.* **28**:329-348.
- KAYE, J. J., AND G. B. CHAPMAN. 1963. Cytological aspects of antimicrobial antibiotics. III. Cytologically distinguishable stages in antibiotic action of colistin sulfate on *Escherichia coli*. *J. Bacteriol.* **86**:536-453.
- KELLENBERGER, E., A. RYTER, AND J. SÉCHAUD. 1958. Electron microscope study of DNA-containing plasmids. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *J. Biophys. Biochem. Cytol.* **4**:671-678.
- LANDMAN, O. E., AND S. HALLE. 1963. Enzymically and physically induced inheritance changes in *Bacillus subtilis*. *J. Mol. Biol.* **7**:721-738.
- OHYE, D. F., AND W. G. MURRELL. 1962. Formation and structure of the spore of *Bacillus coagulans*. *J. Cell Biol.* **14**:111-123.
- RYTER, A., AND F. JACOB. 1963. Étude au microscope électronique des relations entre mesosomes et noyaux chez *Bacillus subtilis*. *Compt. Rend.* **257**:3030-3063.
- RYTER, A., AND E. KELLENBERGER. 1958. L'inclusion au polyester pour l'ultramicrotomie. *J. Ultrastruct. Res.* **2**:200.
- RYTER, A., AND M. PIÉCHAUD. 1963. Étude au microscope électronique de quelques souches de *Moraxella*. *Ann. Inst. Pasteur* **105**:1071-1079.

- RYTER, A., AND J. PILLOT. 1963. Étude au microscope électronique de la structure externe et interne du *Tréponème* Reiter. Ann. Inst. Pasteur **104**:496-501.
- SPIZIZEN, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S. **44**:1072-1078.
- TAKAGI, A., K. UYAMA, AND M. UEDA. 1963. Reduction of tetrazolium salts to formazans in *Fusobacterium polymorphum*. J. Gen. Appl. Microbiol. **9**:287-298.
- THORNE, C. B. 1962. Transduction in *Bacillus subtilis*. J. Bacteriol. **83**:106-111.
- VANDERWINKEL, E., AND R. G. E. MURRAY. 1962. Organelles intracytoplasmiques bactériens et sites d'activité oxydoréductrices. J. Ultrastruct. Res. **7**:185-199.
- VAN ITERSON, W. 1961. Some features of a remarkable organelle in *Bacillus subtilis*. J. Biophys. Biochem. Cytol. **9**:183-192.
- WEIBULL, C., AND H. BECKMAN. 1960. Metabolism of small bodies isolated from a stable *Proteus* L-form. Nature **188**:428-429.