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Nuclear and Cell Division in *Bacillus subtilis*: Dormant Nucleoids in Stationary-Phase Cells and Their Activation

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The morphology of nucleoids and mesosomes of *Bacillus subtilis* in stationary- and lag-phase cultures was studied by making three-dimensional cell reconstructions in plastic of electron micrographs of serial sections. In cells from stationary cultures, the dormant nucleoids are frequently, but not always, spherical and the mesosomes are small and compact. It is suggested that the spherical nucleoids represent the resting stage in which replication and segregation have been completed. In cells from lag-phase cultures, the compact mesosomes develop into an elaborate system of tubes and wider sacs which become wrapped around the elongating nucleoids and invade the nucleoplasm in preparation for division.

Little is known at the ultrastructural level of the essential features of the bacterial nuclei (nucleoids) in phases of rest and replication. The morphology of nuclear division and cell division cannot well be understood without a thorough knowledge of the structure of the cell at rest. This is particularly so because of the important suggestions made about the way in which the bacterial cell can regulate its chromosome replication in relation to the generation time. With very long generation times the required slowing down of the chromosome replication is first achieved by increasing the interval between two rounds of replication, resulting in periods of nuclear rest interposed between periods of nuclear division (4, 5, 7, 12).

To gain insight into the changes in ultrastructure of bacterial nuclei and other cell structures during the cell cycle, an adequate study of the morphology of the cell at rest is essential. This prompted a study of the morphology of nucleoids and mesosomes in cells that are definitely at rest, i.e., in stationary-phase cells. The changes in morphology of nucleoids and mesosomes during regained activity were studied in cells that were stimulated to grow from the lag phase.

The current views on bacterial mesosomes have been reviewed by Ryter (20), Salton (23) and, more recently, by Burdett (2) and by Reusch and Burger (18). The function of the mesosome in bacilli is still obscure, since the chemical differences revealed so far between the mesosomal and cell membrane appear to be mainly, although not totally, of a quantitative nature (16, 26). Furthermore, some confusion exists as to the influence of the preparative method on the morphological structure, and even on the presence and the number of mesosomes in bacilli. It has been described, for example, that in the presence of low concentrations of magnesium or calcium ions, or in a medium of low ionic strength, the mesosomes assume the conformation of a membranous whorl, whereas vesicles in the mesosomes might be formed at high divalent cation content and at elevated osmolarity (3, 24). Therefore, some attention has to be given to the preparative method itself, and the presence and structure of mesosomes has to be first studied under widely varied circumstances.

This communication is part of an extensive study involving more than 25,000 electron micrographs. When the Ryter-Kellenberger method (22) is applied with a prefixation with osmium tetroxide in the culture medium, surprisingly reproducible results are obtained with cells from comparable physiological conditions (W. van Iterson and J. A. Aten, submitted for publication). So far, the only technique that can offer insight into the overall structure of the nucleoids and their spatial relationships to the other structures in the complete bacteria is the construction of three-dimensional models based on electron micrographs of serially sectioned cells (cf. 21). Detailed models have been constructed and pen-and-ink drawings of a few of these models accompany this paper.

It will be shown here that, when *B. subtilis* is

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cultivated far into the stationary phase in Spizizen's medium with glucose, acetate, or citrate as the carbon source, or when grown in comparatively rich tryptone medium, the cultures contain a certain number of cells with spherical nuclei and with small mesosomes. When, in fresh medium, these resting cells enter the lag phase of growth, the mesosomes develop into elaborate systems concomitant with the elongation of the nucleoids with which they are in close physical contact. Such coordinated behavior of nucleoids and mesosomes can hardly be fortuitous and must reflect an underlying functional interdependency.

MATERIALS AND METHODS

Organism. *B. subtilis* of the wild-type Marburg strain has been used, kindly supplied by C. F. Robinow.

Cultivation and media. Liquid cultures were grown in 250-ml Erlenmeyer flasks, with aeration by shaking, at 37 C in either Spizizen's minimal medium (1) with slight modification, or in tryptone medium. Spizizen's minimal medium includes (per 1 liter of water): K_2HPO_4, 14 g; KH_2PO_4, 6 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 2 g; trisodium citrate $\cdot 2H_2O$, 1 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; L-alanine, 0.050 g; L-tryptophan, 0.050 g; Casamino Acids (Difco), 0.5 g (pH 7.0). The carbon source was 5 g of glucose, acetate, succinate, or citrate. The tryptone medium contained (per 1 liter of water): tryptone (Difco); NaCl, 5 g; $MgCl_2 \cdot 6H_2O$, 2 g; $CaCl_2 \cdot 2H_2O$, 1.5 g (pH 7.0).

The stationary-phase cultures are from overnight growth. Stationary-phase cultures which were the last of three serial transfers were inoculated in 80 ml of fresh medium. Their growth was followed by optical density measurements at 620 nm with a Vitatron spectrophotometer.

The osmolarity of the media has been measured with an advanced clinical osmometer model 3A (Advanced Instruments Inc., Newton Highlands, Mass.). The ionic strength of Spizizen's medium with glucose was found to be 337 mosmol, and the supernatant of stationary-phase cultures was 352 mosmol; for tryptone medium these values were, respectively, 283 and 314 mosmol.

Electron microscopy. Bacteria were prefixed for 5 min in culture medium with 0.1% OsO_4, centrifuged, embedded at 46 C in 2% agar with Ryter-Kellenberger buffer (pH 6), fixed overnight, and treated further as described by Ryter and Kellenberger (22).

The specimens in Vestopal W were serially sectioned with glass knives on an LKB Ultratome, picked up on Formvar-covered copper grids, and stained with lead citrate according to Reynolds (19). The section thickness was 0.03 to 0.04 μm as calculated from the diameter of the cells.

In some experiments, $CaCl_2$ (0.01 M) was added to the 0.01 M $MgCl_2$ containing Ryter-Kellenberger buffer. The effect of a rapid prefixation has been studied by pouring an equal volume of culture into a 2% OsO_4 solution, giving a final concentration of 1% OsO_4.

A Philips EM 300 electron microscope was operated at 80 kV, and pictures of serial sections were studied at final magnifications of $\times$125,000 and $\times$195,000.

Three-dimensional models. The electron micrographs of the serial sections were copied on transparent sheets. The cell walls were drawn in black, the nucleoids in red, and the mesosomes in blue. The sheets were then mounted at a distance of 2.5 to 5 mm from each other, and the models were studied at various angles on an illuminated milk-glass plate.

RESULTS

Reproducibility of nucleoids and mesosome structure in relation to the fixation. Satisfactory results, reproducible in parallel experiments, were obtained with the standard method of Ryter and Kellenberger with 0.01 M magnesium acetate in the buffer. A check on greater rapidity of the prefixation and a supplementation with more cations during the treatments was not seen to have any further influence on the nucleoid or mesosome fine structure. However, prefixation with 0.1% OsO_4 was found to be essential in the procedure, as otherwise the morphology of the cell changes under modified physiological conditions, such as centrifugation (van Iterson and Aten, submitted for publication).

Mesosomes are present in the cell under widely varied circumstances. They are found in living cells as well as in cells that were dead at the time of their fixation. Therefore, it is evident that they are not produced as the living organism's response to the fixation process. Figure 1 shows membranous whorls in a cell killed in an experiment with actinomycin D (van Iterson and Aten, submitted for publication). Mesosomes have also been observed many times in cells in the beginning stages of autolysis when the culture medium runs short of amino acids (Fig. 2). The mesosomes in dead cells are more often of the membranous type (Fig. 1) than sacs filled with vesicles (Fig. 2). The location of mesosomes under various other conditions of growth will be further described below and in other reports (van Iterson and Aten, submitted for publication).

When the *B. subtilis* cells were rapidly killed by pouring a culture into fixation medium having twice the normal content of OsO_4 (i.e., 2%), no differences in mesosome structure were apparent, as compared with cells in the same physiological phase but prefixed according to the normal Ryter-Kellenberger procedure.

Spizizen's medium, based on citrate buffer, is unsuitable when the influence of divalent cations on the selective formation of vesicular or membranous mesosomes is studied. When cul-

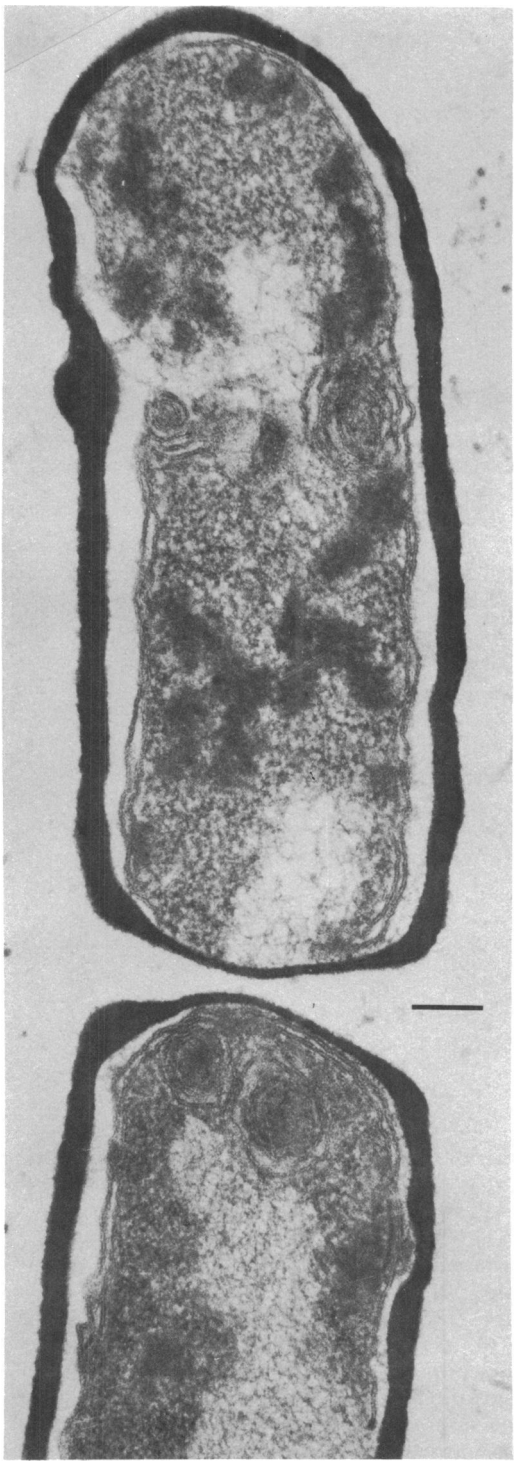


FIG. 1. *Electron micrograph of a section showing mesosomes in a dead cell as whorls of membrane at the same locations as in comparable living cells (submitted for publication). The scale marker, as on all other electron micrographs, indicates 0.1 μm.*

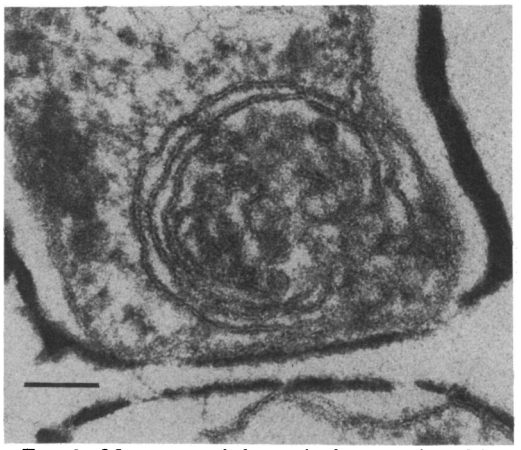


FIG. 2. *Mesosome of the vesicular type found in a dead cell from a culture short in amino acids.*

tures grown in Ca-containing tryptone medium were fixed in the fixation medium with 10 mM calcium added to the routine 10 mM magnesium, no striking differences in the structures of the mesosomes could be observed. The differences observed between the mesosomes of the cells grown in Spizizen's medium (poor in divalent cations) and in cells grown in tryptone medium are considered to be due to different physiological conditions of cells, and not to a cation effect, or merely to differences in ionic strength between the two media (see Discussion).

Cells in stationary phase. When cells in tryptone medium were transferred often enough to suppress spore formation, cultures could be obtained which, in stationary phase, contained predominantly spherical nucleoids of the type seen in Fig. 5. Cells with spherical nucleoids are also present in stationary-phase cultures of this *B. subtilis* strain in Spizizen's medium with 0.5% glucose, acetate, succinate, citrate, or glycerol, but the yields are then lower. Figure 3 is representative of spherical nuclei in a stationary-phase cell in Spizizen's medium with glucose, whereas in Fig. 4 the nucleoid is nonspherical in a similar cell. The fibrillar texture of the spherical resting nucleoid is compact and the fibrils are in whorls around centers in the nucleoid (Fig. 3). In the nonspherical nucleoids, the fibrils are also twisted and they are attached to small mesosomes in the periphery of the cell. These small, compact mesosomes in the stationary-phase cells are difficult to keep intact during the sectioning. One is always certain to find a single spherical nucleoid in each of the short double cells so often found in stationary-phase cultures (Fig. 3 and 5). The presence of two spherical nuclei in long cells arrested in

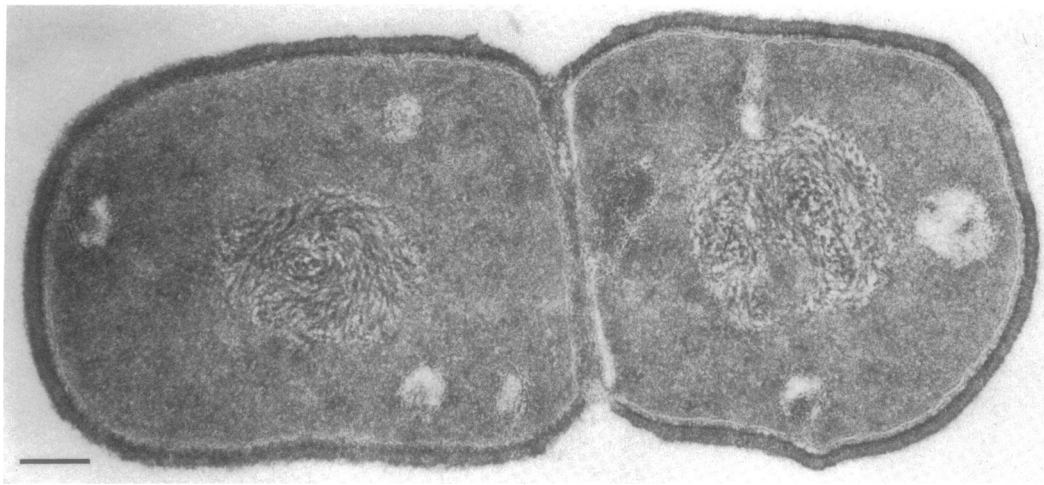


FIG. 3. *Stationary-phase cells from Spizizen's medium with glucose, with spherically condensed nucleoids and small condensed mesosomes.*

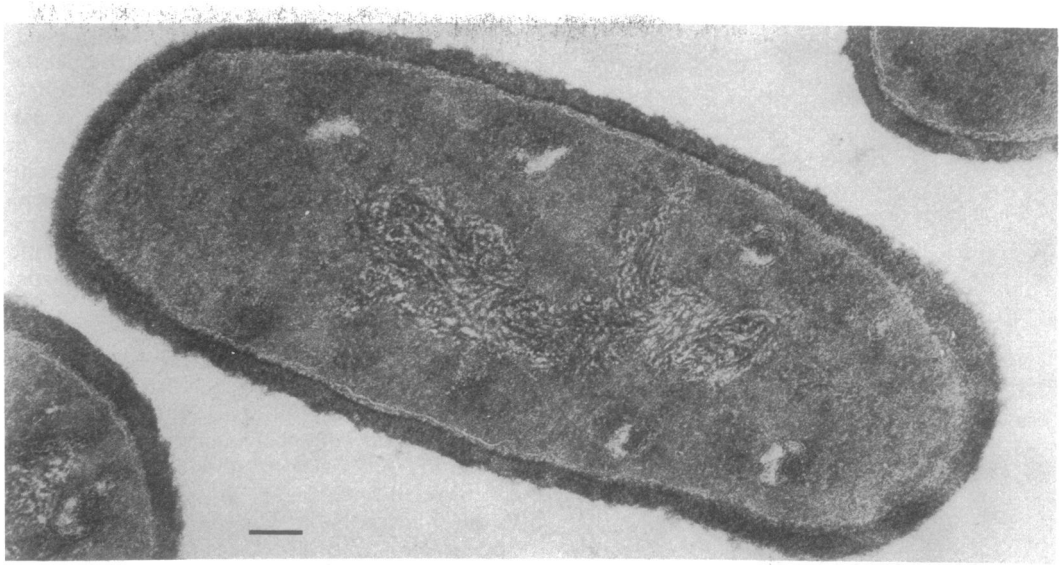


FIG. 4. *Cell from a culture in stationary phase in Spizizen's medium with glucose; note that the nuclear material is in an open instead of a spherical configuration. In the open, as opposed to the closed, configuration, many of the small mesosomes in the cell periphery remain in contact with the nucleoplasm.*

stationary phase apparently depended on whether a final nuclear division had recently taken place (Fig. 8a, b).

The limiting factor which causes the cell to enter the stationary phase in Spizizen's medium remains unknown. The aeration and the pH, which remains constant, have been checked. In tryptone medium the pH changed from 7 to 8.2. Lowering the amino acid concentration in the Spizizen's medium with glucose does not favorably affect the number of cells with spherical nuclei; it slows down the growth rate, but the cell yield remains eventually the same (in confirmation of Spizizen's findings [25]). Neither are more cells with spherical nuclei obtained by lowering or raising the concentration of glucose. A lowering of both glucose and Casamino Acids to one-tenth the normal concentration produces short chains in stationary cultures. Such cells appear to be "frozen" during septum formation at the end of the exponential phase of growth.

When glycerol is the carbon source in the Spizizen's medium, long chains of cells are present in stationary phase, some with spherical nuclei and many others with their nucleoplasm arranged in axial filaments.

Activation of stationary-phase cells. This phenomenon has been studied in tryptone medium with stationary-phase cells practically all containing spherical nuclei. From cultures in the Spizizen's medium the double cells have

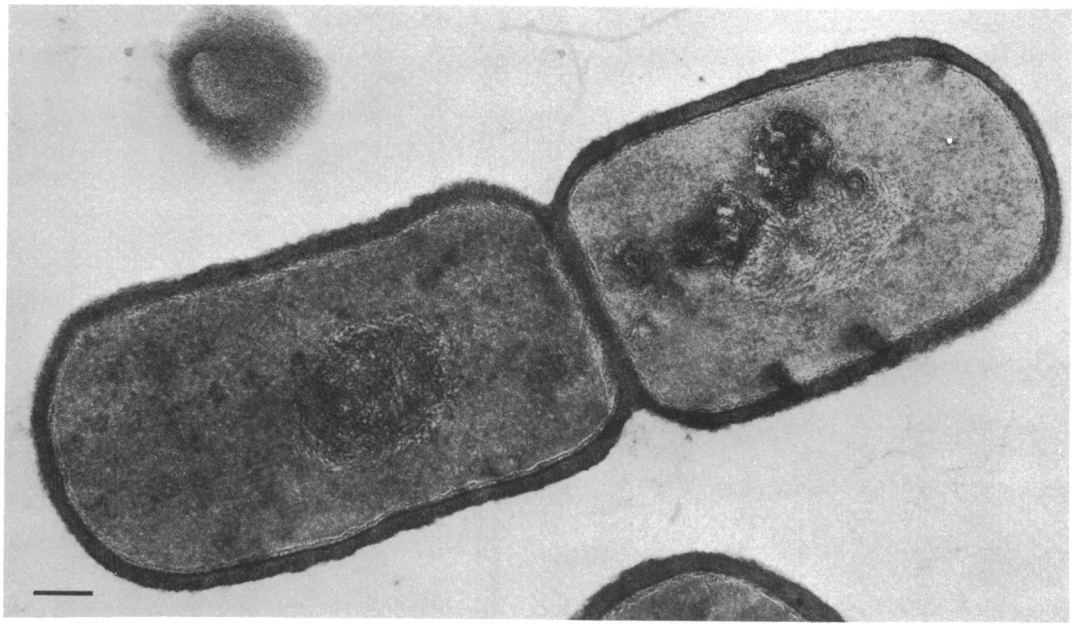


FIG. 5. *Stationary-phase cells from tryptone medium, with the nucleoids spherically condensed; one of them is seen in contact with a mesosome.*

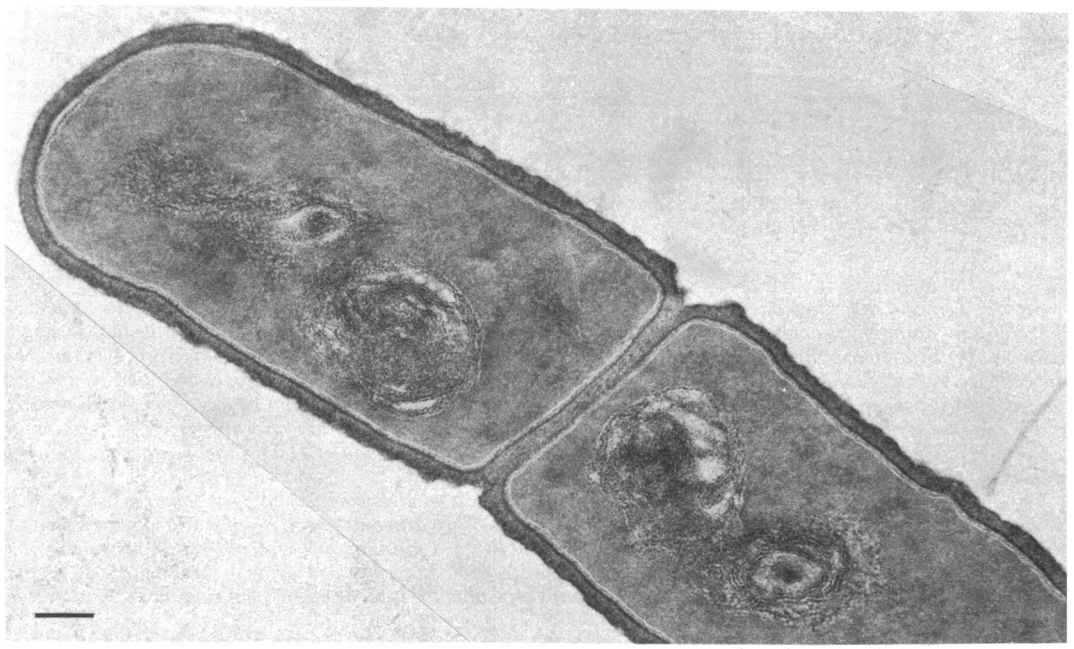


FIG. 6. *Cell from a 27-min lag-phase culture in tryptone medium. Note that the nucleoids and mesosomes grow out intermingled in axial orientation.*

been selected because of their spherical nuclei. The pen-and-ink drawings in Fig. 7a and 8a and b are made after some of the three-dimensional models of cells in stationary phase, whereas those in Fig. 7b through d and Fig. 8c are of lag-phase cells and should give an overall impression of the event of the activation of the cells in Spizizen's medium containing succi-

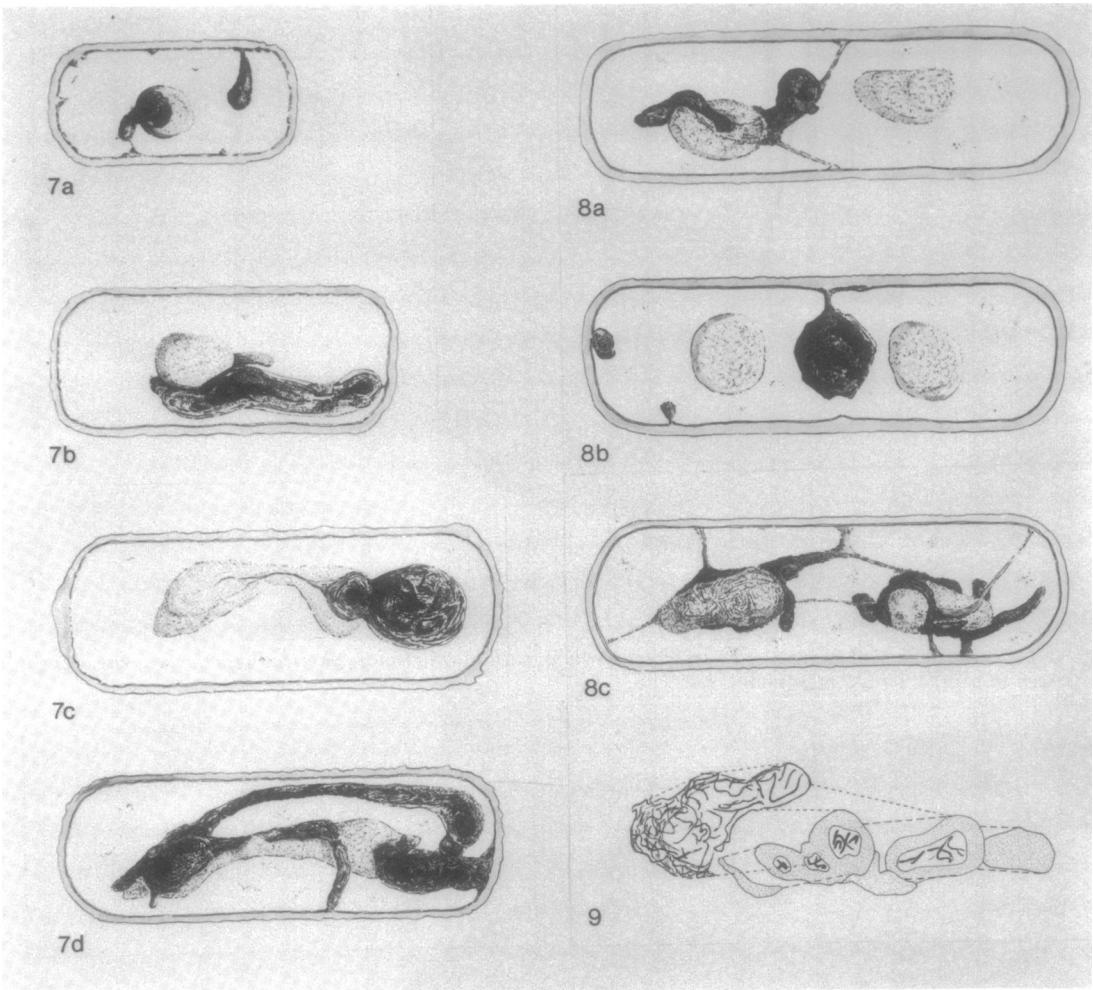


FIG. 7. *Pen-and-ink drawings of three-dimensional plastic models after serially sectioned cells grown in Spizizen's medium with succinate. The drawings have all been made to the same scale. The cells have a single nucleoid. (a) Stationary-phase cell. (b–d) Stages in development of nucleoid and mesosome during increase in the cell size, all selected from a 45-min-old culture. In (b) the enlarged mesosome becomes wrapped around the nucleoid. In (c) the mesosome (which conceals its contact with the cell membrane) consists of a vesicular part and a lamellar part invading the nuclear area, comparable to Fig. 10. In (d) an elaborate system of mesosomal tubes and widened sacs in close contact with the nucleoplasm is shown. Note tube contacting the cell membrane, more or less in the middle of the cell.*

FIG. 8. *Pen-and-ink drawings after three-dimensional plastic models from serially sectioned cells with two nucleoids (from Spizizen's medium with succinate). (a and b) Two different examples of stationary-phase cells: (a) from a 0-min culture; (b) from a 15-min-old culture. In (a) a mesosomal tube passes through one of the nucleoids. In (b) both condensed nucleoids are separate from the spherical central mesosome. (c) In the lag-phase cell the mesosome has begun to develop into an elaborate tubular system enveloping both nucleoids (from a 15-min culture in fresh medium).*

FIG. 9. *Nuclear fibrils were found to have penetrated deeply into a mesosome to which it is attached. As the lines indicate, this has been drawn as if the main part of the nucleoplasm had been removed sidewise from the sectioned mesosome.*

nate. Figures 7a through d represent uni-nucleate cells and Fig. 8a through c bi-nucleate cells. In the electron micrographs of the stationary- and early lag-phase cells from succinate medium, the structure of the mesosome is often very vague, and one is therefore surprised to find that, when drawings of series of electron micrographs on transparent sheets have been placed at about the right distance one over the other, the mesosome is usually a tubular system meandering through the cell. Figure 8a is an example in which the tubular mesosome does not curve around the nucleoid but passes right through it. In Fig. 8b the mesosome is of the rounded shape so commonly illustrated, but it is here detached from the two spherical nucleoids, whereas in other studies the mesosomes and the nucleoplasm are usually in contact.

When resting cells are activated, the mesosome starts to develop extensively. Again the three-dimensional reconstructions show that the vague areas in the single sections together constitute an expanding tube, which is often found to wind around the nucleoid (Fig. 7b). In fresh medium (in the succinate medium between 15 and 45 min) the mesosomes widen and spherical sacs filled with vesicles are found, whereas the part of the mesosome that penetrated deeply into the nucleoplasm is a whorl of membranes (Fig. 10). Such mesosomes that are "aroused" lose the amorphous electron opaqueness which they showed in the beginning of their development and become more structured. On further growth of the cell the mesosomes are seen to develop into a complicated system of narrow tubes with widened areas enveloping the nucleoids and invading them (Fig. 7d and 8c). The tubes approach and contact the cell membrane at several sites.

The most dramatic development of mesosomal material was found in lag-phase cells in tryptone medium (Fig. 6, 11, and 12). Figures 5, 6, and 11 together constitute more or less a sequence; it can be seen that, while the cells lengthen, the mesosomes and the nucleoplasm develop very strongly, orientated along the axis of the cell. The nucleoplasm and the elements of the mesosomal material are frequently so intermingled that they are often difficult to distinguish from each other.

The mesosome often originates on the cross wall and extends with the lengthening of the cell, thus preserving its contact with both the cross wall and the nucleoplasm (Fig. 12). As this cell was in fresh tryptone medium for only 27 min, its cytoplasm is as yet little differentiated in comparison with the structure on longer incubation (Fig. 11).

Structure of nucleoids. In stationary phase the relatively large resting nucleoid matches the small volume of the cell by the tight coiling of its strands (Fig. 3). During the activation of the dormant nucleoid the mesosomal tubes wind around the elongating nuclear area (Fig. 7 and 8). The previously condensed nucleoplasm (Fig. 3 and 5) loosens up as it is pervaded by the mesosomal material (Fig. 6, 11). When dividing the nucleoplasm is of open structure, contrary to the condensed state of the nucleoid at rest. Another distinction between the dormant and the activated nucleoid is that the first is usually only in rather superficial contact with

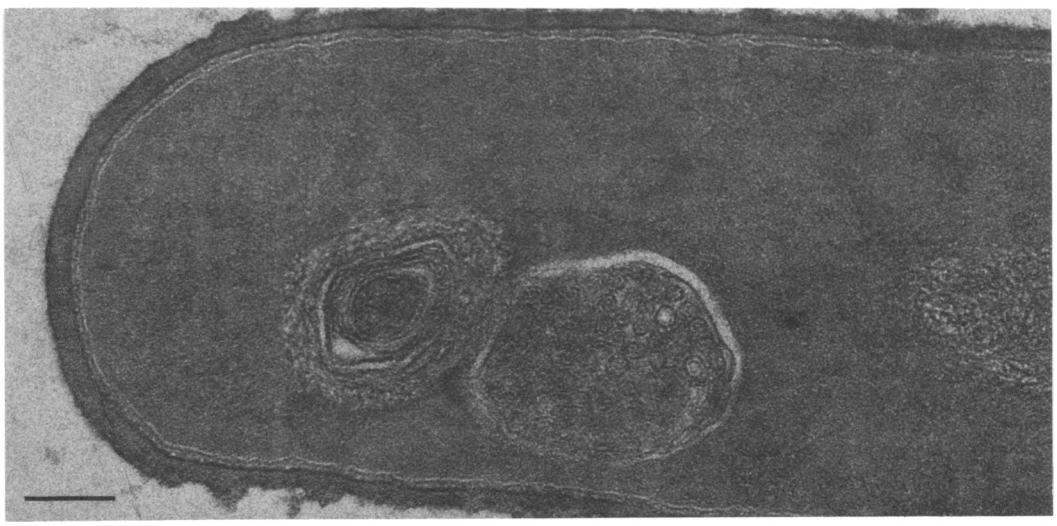
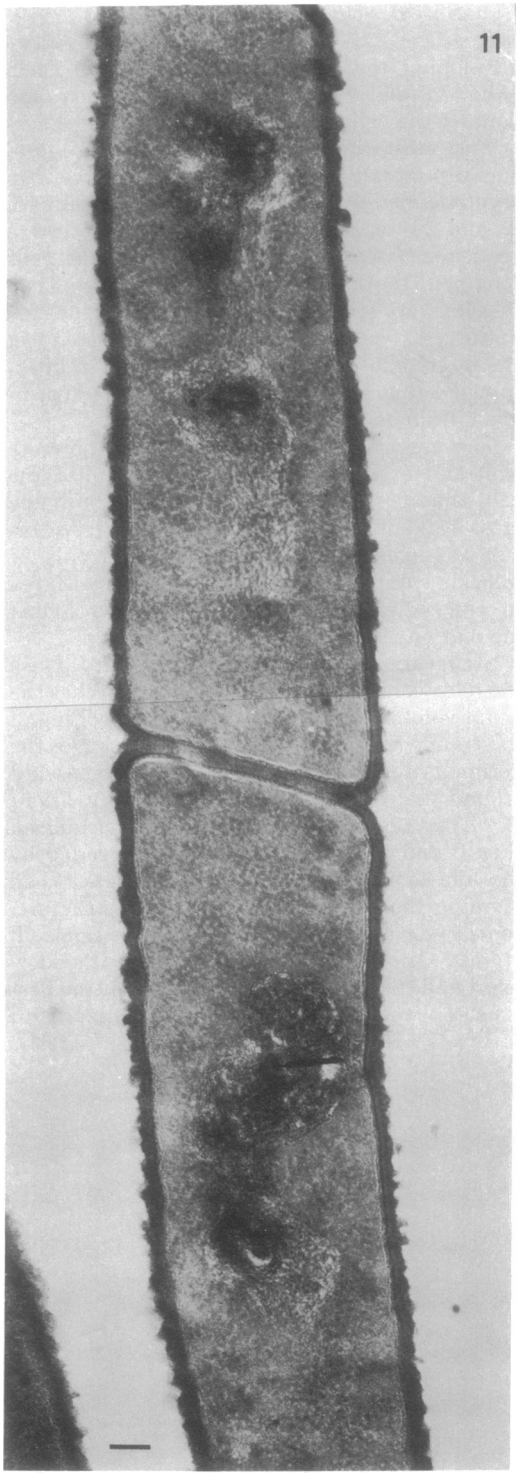


FIG. 10. *Cell from a 45-min lag-phase culture in Spizizen's medium with succinate. The mesosome has developed a vesicular part and a membranous part; the latter grows inside the nucleoplasm.*



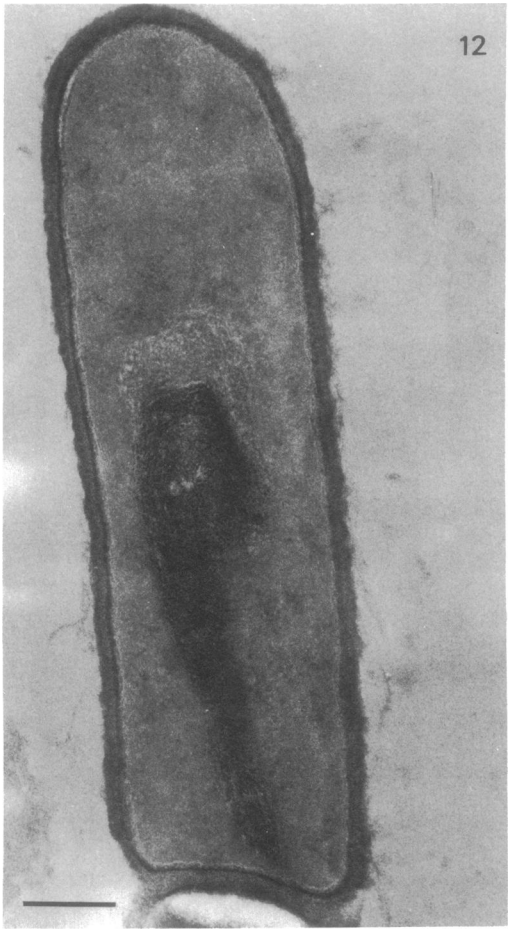


FIG. 11. *Cells taken from tryptone medium after 92 min. The cells have increased in length (cf. Fig. 6). Nucleoplasm and mesosomal material in preparation for nuclear division are intermingled.*

FIG. 12. *Cell activated from dormancy in tryptone medium after 27 min. The cytoplasm still has the glassy appearance of the little differentiated cell. The mesosome, on one side connected to the septum and on the other side to the nucleoplasm, has elongated in conformity with the lengthening of the cell.*

the condensed mesosomal material, whereas the activated nucleoplasm is intrinsically invaded by mesosomal material. Conversely, nuclear fibrils can be present in the mesosome, even in the resting cell (Fig. 9).

DISCUSSION

Apart from an early communication by Kellenberger (10) little relevant information has been published about the shape and fine structure of normal dormant nucleoids. In our stationary-phase cultures, a varying number of spherical nucleoids has been found and these are considered as nucleoids at rest.

We did not study the conditions affecting the yield of spherical nucleoids. In Spizizen's medium the glucose content is not a limiting factor, as a 10-fold decrease in its concentration lowered the growth rate but still resulted in considerable optical density, though the maximum was followed by a decline as described by Monod (14) and Koch (11). Changes in amino acid concentrations did not alter the yield of spherical nucleoids. In tryptone medium an almost 100% yield has occasionally been obtained.

The presence in the same culture of both spherical nucleoids and those in more open configuration (Fig. 3 and 4) may well be related to the stage in which replication is arrested. Yoshikawa et al. (29) reported that the chromosomes of the wild-type W23 strain of *B. subtilis* in stationary phase are in the completed form, but those of strain W168 remain in various stages of replication. Copeland (6) found that on amino acid starvation in the W23 strain about 89% of the chromosomes had not completed replication to the terminus and that the forks stopped in a nearly random fashion. On restoration of the conditions for chromosome replication, the replication forks continued from the loci where they had stopped. Different strains and cell lines of *B. subtilis* were found by Thomas and Copeland (27) to respond differently in their replication to leucine starvation, whereas with starvation for tryptophan the replication was completed to the terminus. It is therefore possible that in our wild-type Marburg strain of *B. subtilis* the spherically condensed nucleoids are those that on entering the stationary phase have completed their last round of replication up to the terminus, followed by a complete segregational condensation of the nuclear material, whereas those in more open configuration (Fig. 4) were arrested before the replication was complete. The spherical nucleoids which can be obtained by interruption of the normal cell cycle, i.e., by centrifugation (van Iterson and Aten, submitted for publication), can probably also be considered as nucleoids at rest. However, the spherical nucleoids induced by inhibiting macromolecular synthesis with antibiotics (van Iterson and Aten, submitted for publication) may well differ from the resting nucleoids described here.

Surmising that the spherical nuclei in the resting phase represent those that on attaining dormancy had replicated the chromosome to the terminus, it should be noted that part of the cells are arrested with two such nucleoids without a septum between them (Fig. 8), whereas in others a septum already separates them, resulting in a double cell or even separate cells (Fig. 7a). But the explanation that a spherical nucleoid equals a totally replicated chromosome is complicated by Johnston and Young's finding (9) of a variable deoxyribonucleic acid content in bacilli, which could be interpreted by assuming that a nucleoid consists of several genomes.

In their study on the effect of threonine or valine starvation on *Streptococcus faecalis*, Higgins and Shockman (8) observed a "pooling" of the nuclear material. As these nuclear "pools" were still arranged in the complex figures they observed in log-phase cells, this condensation of the nuclear material may not be quite comparable to the smooth dormant nucleoids obtained here in the stationary phase. On the other hand, the nucleoids observed by Remsen (17) and by Morioka et al. (15), by applying the freeze-etch technique to stationary-phase cells, are morphologically in fair agreement with those in this study. The freeze-etch technique, however, does not permit an analysis in three dimensions of the nucleoid with its mesosome connected to it, nor of the changes ensuing from the development in the lag phase.

In stationary-phase cultures the cells possess small, compact, often numerous, dormant mesosomes. Higgins and Shockman (8), on the other hand, showed that during valine starvation the mesosomal membranes of *S. faecalis* continue to accumulate slowly with the same morphology as in exponential-phase cells; during threonine starvation the accumulation stopped.

The most important novel feature emerging from the analysis of the three-dimensional models of cells in the lag period was the simultaneous expansion of mesosome and nucleoid. In cells grown in Spizizen's medium with succinate as carbon source, but probably also in other media, the mesosome develops into a single continuous system of tubes and wider sacs (Fig. 7 and 8). The inner surface is greatly

increased by the well-known membranes, vesicles, and tubules (2, 13, 20). The thin mesosomal tubes which envelop and invade the nucleoid have not been described before.

In some of the drawings (Fig. 7 and 8) mesosomal tubes can be seen to contact the cell membrane at various sites. These probably are the sites where future septa will develop for the first cell division (Fig. 7d and 8c) and also for the next (Fig. 8c), as will be discussed in later papers (van Iterson and Aten, submitted for publication).

According to Silva (24), membranous mesosomes are found under conditions of calcium deficiency, whereas in this study vesicular mesosomes in the cells grown in the calcium-poor Spizizen's medium abounded. In the calcium-richer tryptone medium, on the other hand, the mesosomes are either little differentiated or partly vesicular and partly lamellar, even when fixed with extra calcium ions. The structure of mesosomes in cells from Spizizen's medium, occurring as small compact organelles in the stationary phase, changing into sacs filled with vesicles in the lag phase, and even occurring as combinations of vesicles and membranes in the same mesosome (Fig. 10), all suggest a physiological variation of the same organelle.

The pen-and-ink drawings (Fig. 7 and 8) show that during the lag phase of growth the spherical nucleoids elongate while enveloped and invaded by the concomitantly developing mesosomal system. The communicating system of mesosomal tubes and sacs, as seen in the drawing of activated cells (Fig. 7d and 8c), is reminiscent of a miniature endoplasmic reticulum. Such a system may well be suited to conduct precursors and other materials to where they are needed or to remove broken-off materials and secretion products. The eukaryotic nucleus, after all, during part of the cell cycle is completely enveloped by a membrane system communicating with the endoplasmic reticulum. However, the present morphological work permits us only to conclude that, concomitantly with the elaboration of the nuclear material and in intimate physical contact with it, a mesosomal system of tubes and more elaborate structures develops in the cell as it is activated from dormancy. The morphologically close association of the mesosomes with the nuclear material, together with their coordinated development, are suggestive of a functional interdependency.

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