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Structural Changes in *Stigmatella aurantiaca* During Myxospore Induction

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Suspension cultures of *Stigmatella aurantiaca* (*Chondromyces aurantiacus*) were induced to form myxospores by addition of glycerol to the growing culture. The cells were fixed at various stages during conversion, thin sections were prepared, and changes in fine structure were studied. Vegetative cells are quite similar in their ultrastructure to *Myxococcus xanthus*. During transformation into myxospores, three important cytological changes were observed. Granules of storage material, probably polysaccharide and polyphosphate, accumulated; a 200 to 300-μm thick capsule was laid down, and the outer triple layer of the cell wall became locally folded. These cell wall folds were often densely packed and lay in pockets formed by the cytoplasmic membrane. We have suggested the possibility that the cell may store in these folds wall material which has become superfluous by the decrease in surface area during conversion.

Under certain conditions, myxobacteria go through a cycle of cellular morphogenesis, leading from slender, flexible vegetative rods to short, rigid, optically refractile myxospores. The myxospores may eventually germinate to vegetative cells. Conversion into myxospores is usually connected with the process of fruiting body formation. Under such conditions, it is difficult to study details of the conversion because of sampling and timing problems. In spite of these limitations, however, it was possible to work out the fine structure of *Myxococcus xanthus* during myxospore formation and germination (8, 10).

Dworkin and Gibson (2) described a method for inducing myxospore formation in liquid cultures of *M. xanthus*. Harvested cells were suspended in a Casitone-$MgSO_4$ medium containing ing 0.5 M glycerol as an inducer. Under optimal conditions, a 100% conversion into myxospores was obtained within 120 min.

In the course of our investigations on *Stigmatella aurantiaca*, it became necessary to examine the cytology of this organism. In addition, this represented an opportunity to extend the study of ultrastructure during morphogenesis to myxobacteria other than *M. xanthus*. We took advantage of the glycerol induction technique to examine myxospore formation in *S. aurantiaca*.

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MATERIALS AND METHODS

Organism. Several strains of *S. aurantiaca* Berkeley and Curtis (*Chondromyces aurantiacus* Thaxter) were isolated in 1966 from rotting wood and bark collected in and around Minneapolis, Minn. After cloning, one strain, cro Cl 1, was chosen for this study. Details about the organism will be published elsewhere (H. Reichenbach and M. Dworkin, *in preparation*).

The organism was grown in a modified CT medium (1): Casitone (Difco), 1.0%; $MgSO_4$, 0.15%. Cultures were grown in 250-ml Erlenmeyer flasks containing 25 ml of medium. They were shaken at 30 C on a reciprocal water bath shaker. Under these conditions, the generation time was about 7 to 8 hr, and the cells grew in a dispersed fashion.

Myxospore formation was induced by adding glycerol to a final concentration of 0.5 M directly to the growing culture. Induced cultures were shaken at 30 C.

Electron microscopy. Three different fixation techniques were used: (i) osmium tetroxide fixation according to Ryter and Kellenberger (6); (ii) fixation with picric acid and formaldehyde, usually followed by osmium tetroxide fixation (7); and (iii) fixation with 1 to 5% glutaraldehyde for 30 min at room temperature or at 4 C. The aldehyde was either added directly to the culture or to a buffered cell suspension. Glutaraldehyde fixation was always followed by osmium tetroxide fixation.

Fixation was completed in all cases by treatment with 0.5% uranyl acetate in distilled water for 2 hr at room temperature. The samples were embedded in Epon 812 (3) and sectioned on a Porter-Blum MT-2 ultramicrotome with a DuPont diamond knife. The sections were stained with lead citrate (5). All obser-

vations were made with an RCA electron microscope, model EMU-3G, operated at 100 kv.

RESULTS

The vegetative cell. Vegetative cells of *S. aurantiaca* are very sensitive to changes in their chemical environment. For satisfactory fixation, buffer concentrations had to be about 0.03 M. Higher concentrations led to distortion and disruption of the cell, lower concentrations to vacuolization of the cytoplasm. Addition of glycerol at a concentration of 0.5 M to the growing culture a few minutes before fixation stabilized the cells and improved their preservation considerably without changing their fine structure in any obvious way.

With the different fixation techniques, different results were obtained. Osmium tetroxide preserved the cell wall, cytoplasmic membrane, and nuclear material well, but fixation of the cytoplasm was not optimal. The cell wall was usually interrupted by many small protrusions. With picric acid-formaldehyde, cell wall and cytoplasmic membrane were well preserved, with very few protrusions, if any. Cytoplasm and ribosomes seemed to be well fixed, but the nuclear material formed dense areas with little resolution of the fine structure. Glutaraldehyde gave similar results, although in general, preservation of the cells was inferior to that with picric acid-formaldehyde.

The vegetative cells of *S. aurantiaca* are slender, flexible rods, slightly tapering toward the cell poles, 5 to 8 μm long and 0.7 to 0.8 μm wide. Their fine structure resembles that of other gram-negative bacteria and does not differ in any essential detail from that of *M. xanthus* (9, 10). The characteristic tapering at the cell poles was lost during the fixation process; the cell tips appeared blunt and often slightly inflated. The cytoplasm in this area was more or less vacuolated and seemed to be disorganized (Fig. 1).

At the cell surface, there was a cytoplasmic membrane with the usual triple-layered organization and a well-defined cell wall. The latter consisted of an outer triple layer and a third dense monolayer in the periplasm (Fig. 1). The third wall layer was visible only in well-preserved cells, but then could often be followed over long distances. The periplasmic space was of uniform width and clearly contained some material besides the third wall layer. The outer triple layer had the same dimensions and appearance as the cytoplasmic membrane. Often, it was locally disrupted, leaving small gaps or forming short protrusions. But this seems to be a fixation artifact, as well-preserved cells, especially after fixation with picric acid-formaldehyde, showed an

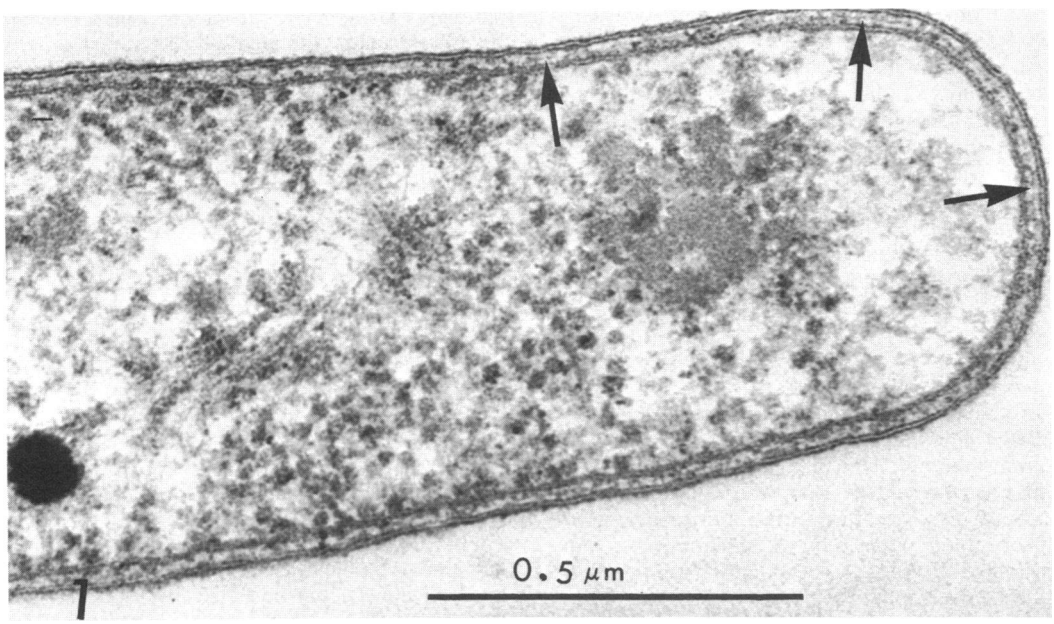

FIG. 1. *Cell pole of a vegetative cell. The tip of the cell has become rounded, and the cytoplasm in this area vacuolated during fixation. In the periplasm, a third dense wall layer is visible (arrows). The dense body is probably polyphosphate. Fibrillar nuclear material, ribosomes, and areas filled with an unknown finely granular material can be seen. Picric acid-formaldehyde fixation with glutaraldehyde prefixation.* × *99,500.*

uninterrupted outer triple layer (Fig. 1). Occasionally, cells were found with the outer wall layer locally detached from the cytoplasmic membrane and considerably distended, resulting in a rather large sphere connected only at its base with the cell. It is not unlikely that the formation of such spheres was due to an osmotic effect which would have required, however, that the outer wall layer was semipermeable.

In the cell interior, irregularly shaped areas of nuclear material showing the usual fibrillar structure were found. In the cytoplasm, there were numerous well-defined ribosomes, often arranged in strands and coils as demonstrated for *M. xanthus* (9). Often areas of a finely granular material were observed, possibly representing precipitated ribonucleoprotein. Many cells contained small granules of a very dense material, which may be polyphosphate, as occurs in *M. xanthus* (11).

The myxospore. The myxospores of *S. aurantiaca* are short, fat, refractile rods, 1.5 to 3.5 μm long and 1.1 to 1.8 μm wide. They are more stable than vegetative cells and were easily fixed with osmium tetroxide. After fixation with glutaraldehyde or picric acid-formaldehyde, structural details were less clearly resolved.

Mature glycerol myxospores were surrounded by a thin capsule, 20 to 30 nm wide, which appeared as a homogeneous band with a granular fine structure and fairly sharp outlines (Fig. 2). Occasionally, a slight indication of a layering could be seen. The outer triple layer of the cell wall and the cytoplasmic membrane were clearly observable, but the third dense wall layer could never be demonstrated. This may be due to the fact, however, that good fixation of myxospores was possible only with osmium tetroxide, a technique which did not preserve the third wall layer in vegetative cells either.

In almost every spore section, there were areas in which the triple layer of the cell wall was heavily folded and formed tight packs of either lamellar or vesicular organization. The capsule was not influenced by the wall folding, but ran smoothly over the folded area. The cytoplasmic membrane was, however, pushed inward, thus forming a pocket in which the wall folds were located. That the folds are really formed by the cell wall and not by the cytoplasmic membrane was clearly demonstrated in cases in which cell wall and cytoplasmic membrane were separated artificially by a gap. In myxospores, neither cell wall nor cytoplasmic membrane was as smooth as in vegetative cells, but always appeared undulating or ruffled.

Within the myxospore, the most conspicuous structural detail was relatively electron transparent granules, probably polysaccharide. These granules had a diameter of 60 to 120 nm, were densely coated with ribosome-like particles, and were always present in large numbers. Also present were some electron-dense granules which appeared to be polyphosphate. The rest of the cell consisted primarily of ribosomes and areas of fibrillar nuclear material.

Fine structure during conversion. During the first 30 min after induction with glycerol, no changes in shape or structure of the vegetative cell could be observed. At 30 min, the cell poles were no longer tapered, the first morphological sign of the conversion. About 45 min after induction, the cells had acquired the size and shape of myxospores, although they were not yet optically refractile. Thin sections revealed a very thin capsular layer, less than 5 nm wide (Fig. 3). There were already, at this point, many cell wall folds, usually located in pockets of the cytoplasmic membrane as in mature spores (Fig. 4, 5). There was not yet any substantial accumulation of storage material.

Comparison of induced myxospores with fruiting body myxospores. Although the fine structure of the fruiting body will be presented in another paper (H. Voelz and H. Reichenbach, *in preparation*), it may briefly be mentioned here that induced and fruiting body myxospores have essentially the same structure. The differences are merely quantitative in nature. In fruiting body myxospores, there seemed to be a slightly heavier capsule, the wall foldings appeared less pronounced, the spores contained more and larger polyphosphate granules, and perhaps also more polysaccharide material.

DISCUSSION

Electron microscopic study of *S. aurantiaca* revealed three changes during conversion of the vegetative cell into a myxospore: (i) the appearance of large amounts of granules which may represent storage material; (ii) the formation of a capsule; and (iii) a heavy folding of the outer triple layer of the cell wall.

In contrast to vegetative cells, which contain at most a few granules of polyphosphate, myxospores are always packed with granular material. Most of it is of relatively low electron density and may be polysaccharide, but there are usually also some granules of high electron density, presumably polyphosphate. This is true for fruiting body as well as for induced myxospores. It seems likely that the granular material plays some important role within the converting or germinating cell. Perhaps, compounds of low molecular weight are made osmotically ineffective by polymerization; also, metabolites or energy sources essential for germination may be stored. The "polysaccharide"

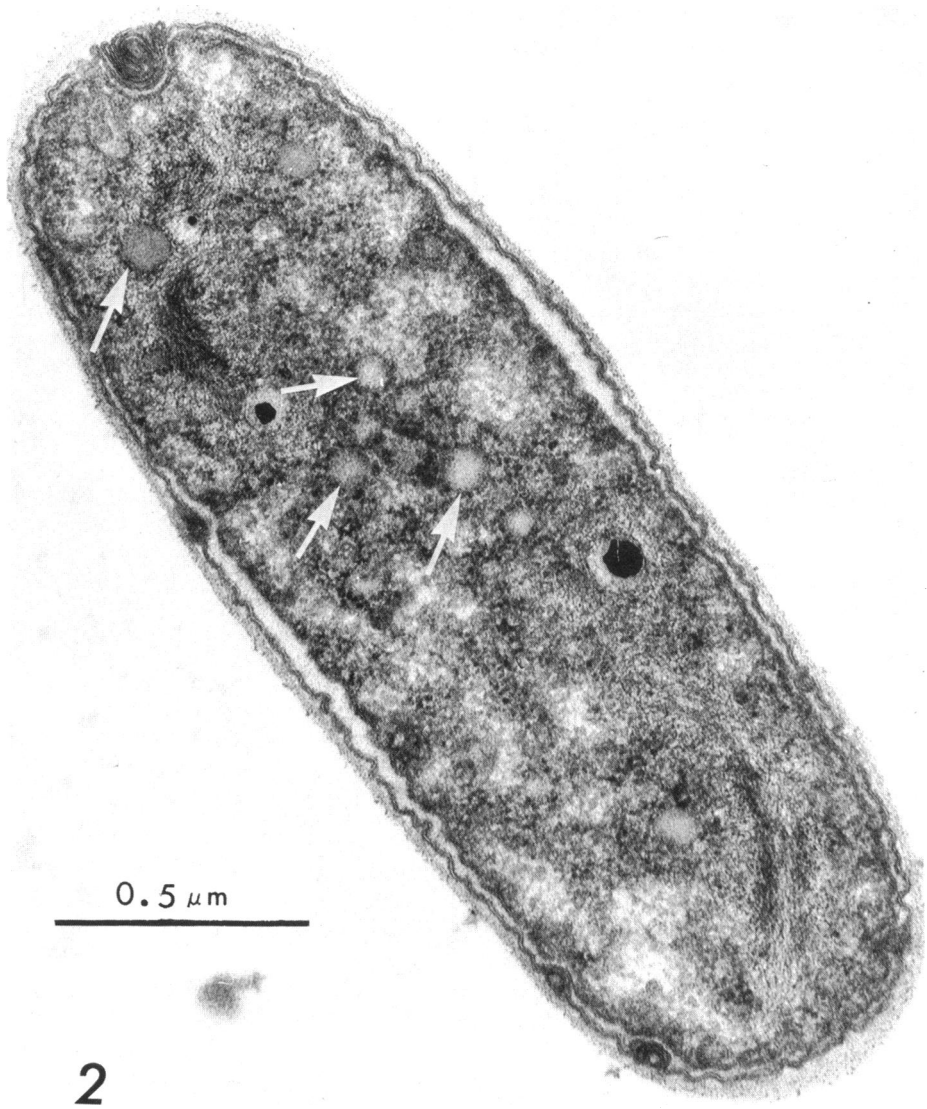

FIG. 2. *Mature myxospore. In several areas, the triple layer of the cell wall forms densely packed folds. Capsule, nuclear material, ribosomes, dense "polyphosphate," and less dense "polysaccharide" (arrows) granules are well differentiated. Ryter-Kellenberger fixation.* × *62,000.*

granules appeared late in the conversion process, after the shape change was accomplished. They, but not the polyphosphate granules, were densely coated with ribosome-like bodies. The meaning of this observation is not understood.

The presence of a capsule around the myxospore of *S. aurantiaca* was entirely unexpected, since light microscopic observations did not indicate such a structure. No capsule could be seen after staining with nigrosin or crystal violet-$CuSO_4$. The myxospore itself could easily be stained with the usual bacteriological dyes. No empty spore case was seen after germination. Only staining with alcian blue clearly differentiated a surface layer. This stain, however, is thought to be bound specifically to the peptidoglycan layer of the cell wall (4).

The thickness of the capsule of *S. aurantiaca* (20 to 30 nm) is much less than that of *M. xanthus* myxospores (8), if fruiting body myxospores are compared. This may relate to the fact that *S. aurantiaca* myxospores are borne in a tough-

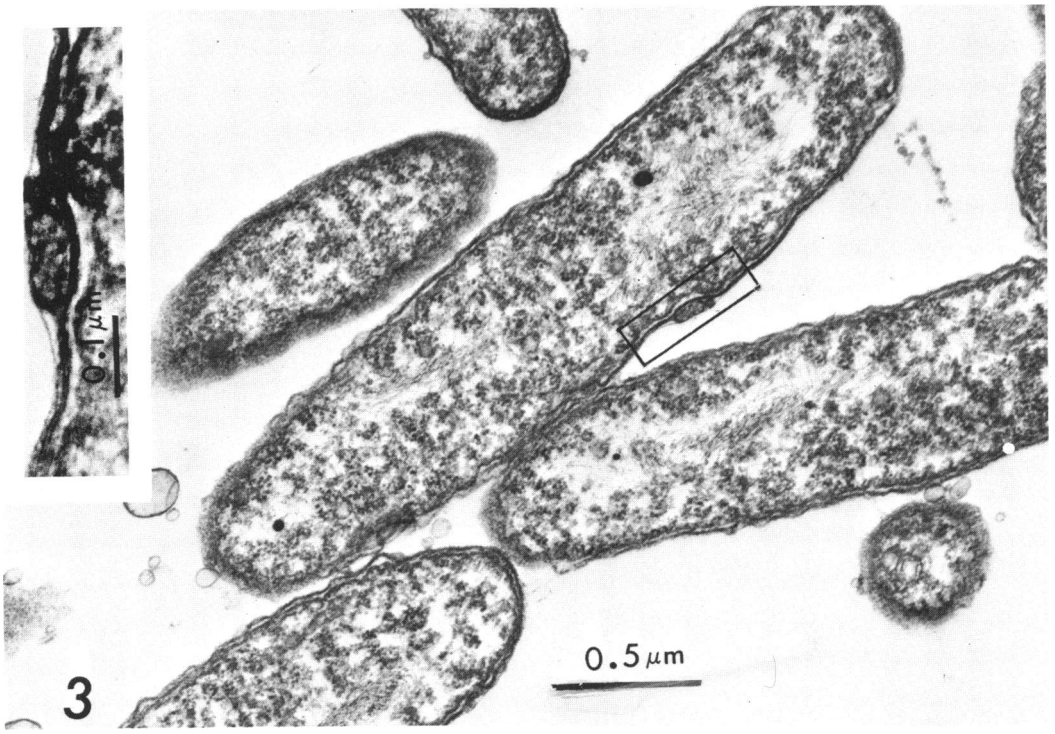

FIG. 3. *Converting cell, 45 min after induction. The cells have acquired the shape and size of myxospores. There are cell-wall folds and a very thin capsular layer. Ryter-Kellenberger fixation.* × *33,900; inset* × *103,000.*

walled fruiting body cyst, whereas *Myxococcus* spores are relatively unprotected.

The most interesting structural detail of the *S. aurantiaca* myxospore is the folding of the cell wall. It seems unlikely that these folds are simply artifacts because (i) they can be demonstrated with three different fixation techniques; (ii) they are connected with the triple layer of the cell wall and do not differ from it in their fine structure; (iii) the triple-layered cytoplasmic membrane is never folded, although it would have been subjected to similar artificial changes as the triple layer of the cell wall; and (iv) the folds are neither found in vegetative cells nor spheroplasts, but only in myxospores or converting cells. The question remains then as to the function of the wall folding.

We have never observed wall folding in vegetative cells or in glycerol-induced cells prior to the shape change; it is likely, therefore, that the folding is somehow connected with the conversion process. The following hypothesis seems attractive to us. When the cell shortens and thickens, its surface area decreases, and some wall material becomes superfluous. Rather than being excised and disposed of, the surplus wall area is simply folded up, possibly to be used again when the cell surface expands during germination.

There is no doubt that the cell wall has somehow to be reorganized during conversion. Perhaps, the shape change is even controlled to a large extent in the cell wall region. Thus, the cell wall folding may play an even more active role in the conversion process. Unfortunately, it has not yet been possible to follow the fate of the third wall layer, supposedly the "rigid" peptidoglycan layer, during spore formation. Further clarification will have to come from chemical and physiological approaches.

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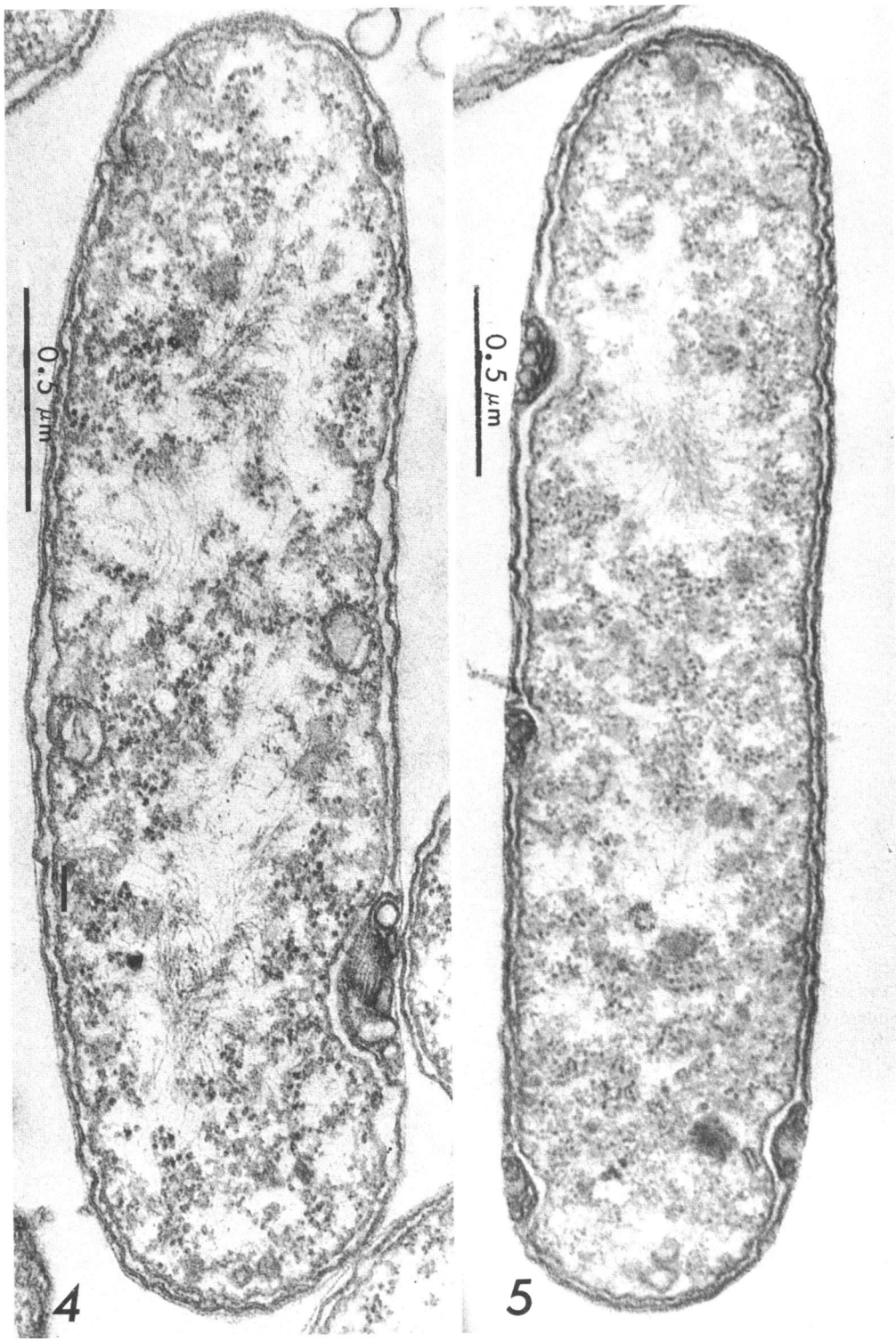

FIG. 4. *Converting cell, 60 min after induction. Cell wall foldings located in pockets formed by the cytoplasmic membrane. Ryter-Kellenberger fixation.* × *66,000.*

FIG. 5. *Converting cell, 70 min after induction. The capsule is still very thin. No well-defined granules of storage material have yet appeared. Ryter-Kellenberger fixation.* × *56,300.*

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