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Comparative Ultrastructure of the Thiobacilli

J. M. SHIVELY,[1] G. L. DECKER, AND J. W. GREENAWALT

Department of Physiological Chemistry, Johns Hopkins University Medical School, Baltimore, Maryland 21205

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The ultrastructure of seven *Thiobacillus* species was studied. The structure of their cell envelopes is similar, if not identical, to that found in other gram-negative bacteria. Obvious differences were noted in the middle layer of the cell envelope of the seven cultures. Polyhedral inclusion bodies were apparent in four of the organisms: *T. thioparus*, *T. neapolitanus*, *T. intermedius*, and *T. thiooxidans*. Lamellar bodies, similar to those present in certain photosynthetic bacteria were found in a few cells of *T. thioparus*. Structures resembling mesosomes were discovered in *T. dinitrificans*. A few cells of *T. intermedius* possessed paracrystalline bodies. Other inclusions, probably volutin and polysaccharide, were present in some of the cultures.

The organisms of the genus *Thiobacillus* are characterized by their ability to oxidize inorganic sulfur compounds as a source of energy. Individual species are distinguished on the basis of their metabolism, i.e., the inorganic sulfur compound(s) utilized, the end products produced, adaptation to heterotrophy, and anaerobic growth in the presence of nitrate. As a group, this genus is considered, taxonomically, to be related to other chemosynthetic autotrophs such as *Ferrobacillus*, *Nitrosocystis*, *Nitrosomonas*, and *Nitrobacter*. Because of the unusual nature of their energy-yielding metabolic processes, considerable interest has been generated in the ultrastructural organization of autotrophic organisms.

To date, three thiobacilli have been subjected to ultrastructural studies: *T. thiooxidans* (9), *T. novellus* (7, 16), and *Thiobacillus* A2 (14). These thiobacilli were found to possess structural organization in general similar to, if not identical with, that of other gram-negative bacteria; this is especially true with regard to the architecture of the cell envelope and the mode of cytokinesis. Complex cytomembrane systems like those found in *Nitrosocystis*, *Nitrosomonas*, and *Nitrobacter* (11) were not present in these thiobacilli. An electron-dense layer present in the cell envelope of heterotrophically grown cells of *T. novellus* was shown to be absent when the culture was grown autotrophically (16). In addition, the heterotrophically grown cells of *T. novellus* contained large polysaccharide inclusions. Kocur, Martinec, and Mazanec (7) demonstrated the presence of vacuoles in *T. novellus;* however, they postulated that these structures were artifacts resulting from plasmolysis of the cells during preparation for electron microscopy. Van Caeseele and Lees (16) later confirmed this postulation; vacuoles were not found in their preparations of *T. novellus*. *T. thiooxidans* was shown to contain moderately electron-dense inclusion bodies which were tentatively identified as stages in the development of volutin granules (9). Other unusual or distinguishing features were not observed in these thiobacilli.

Since the species of the genus *Thiobacillus* exhibit a variety of metabolic processes, it seemed appropriate to compare the ultrastructural organization of several of these species.

MATERIALS AND METHODS

Organisms and sources. The *Thiobacillus* species examined were *T. thiooxidans* ATCC 8085 [adapted to utilization of thiosulfate as described by Barton and Shively (1)], *T. thioparus* ATCC 8158 and *T. neopolitanus* (E. Johnson, Tulane University, New Orleans, La.), *T. intermedius* (S. Rittenberg, University of California, Los Angeles), *T. novellus* ATCC 8093, *Thiobacillus* A2 [a recent isolate possessing characteristics of *T. novellus* (14)], and *T. denitrificans* ATCC 25259 (B. Taylor and D. Hoare, University of Texas, Austin).

Methods of cultivation. *T. thiooxidans* was grown with thiosulfate added as the energy source, as previously reported (1). *T. denitrificans* was cultured by inoculating 20-ml screw-cap tubes containing 19.8 ml of medium with 0.2 ml of a 48-hr culture and incubating at 30 C. The medium consisted of (grams per liter of distilled water): $Na_2S_2O_3 \cdot 5H_2O$, 5.0; NH_4Cl, 1.0; KNO_3, 2.0; KH_2PO_4, 2.0; $NaHCO_3$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.8; $FeSO_4 \cdot 7H_2O$, 0.02; and trace metals (17),

[1] Public Health Service Postdoctoral Fellow on leave of absence from the Department of Microbiology, University of Nebraska, Lincoln.

1.0 ml. All other species were grown in 250-ml Erlenmeyer flasks containing 50 ml of the thiosulfate medium of Vishniac and Santer (17). For *T. intermedius*, the Vishniac-Santer medium was supplemented with yeast extract (0.1 g/liter). Flasks were inoculated with 0.5 ml of a 48-hr culture and incubated at 30 C on a rotary shaker (model G-10, New Brunswick Scientific Co., New Brunswick, N.J.) adjusted to 240 rev/min. Samples were routinely taken for electron microscopy while cultures were in the logarithmic growth phase.

Electron microscopy. All solutions used in the fixation (3% glutaraldehyde and 2% osmium tetroxide) and washing (0.2 M sucrose) of the cells were prepared in phosphate buffer (KH_2PO_4-K_2HPO_4, 0.065 M, *p*H 7.2) containing 4.5×10^{-4} M $CaCl_2$. Fixation, washing, and dehydration procedures were performed at 0 to 4 C.

Cells were harvested by centrifugation and suspended in a small quantity of the spent growth medium. Small samples of each cell suspension were added to Microfuge tubes (Beckman Instruments Inc., Fullerton, Calif.; total volume, 400 μliters) containing 300 μliters of 3% glutaraldehyde, and the tubes were immediately centrifuged for 1 to 2 min in a Beckman Microfuge operated at maximal speed. This procedure resulted in pellets about 0.5 mm thick. The supernatant fluid was removed without disturbing the sediments, and fresh glutaraldehyde was added directly to the pellets; fixation was continued for 2 hr. To insure the complete removal of the glutaraldehyde at the end of that time, the pellets were exposed to two changes of washing medium and then allowed to stand overnight in the second change of the washing solution. The washing medium was removed, and the cells were fixed, as pellets, with 2% osmium tetroxide for 2 hr. The double-fixed specimens were dehydrated with a series of increasing concentrations of acetone (50 to 100%) and embedded in Epon 812, essentially as described by Luft (8).

Thin sections were cut on an LKB Ultratome and were collected, unsupported, on 400-mesh grids. The sections were stained with uranyl acetate followed by lead citrate, and were examined in a Siemens Elmiskop I electron microscope at an acceleration voltage of 60 kv.

All of the cultures were also processed for electron microscopy by use of the procedure of Kellenberger, Ryter, and Séchaud (6). Since samples could be readily handled as small pellets after centrifugation and dehydration in the Microfuge tubes, dispersion in agar was unnecessary and was eliminated from the procedure.

Samples were also negatively stained by the drop method with the use of phosphotungstic acid (PTA), *p*H 6.8, at a final concentration of 1%.

RESULTS

In the course of this investigation, many sections from numerous (6 to 10) embedments of each species were examined thoroughly in an electron microscope. The distinctive features of each organism became so evident as a result of this study that each could be identified upon examination without prior knowledge of which species had been placed in the microscope. These detailed observations provide the basis for the present communication which compares the ultrastructure of the individual thiobacilli with each other as well as with that of gram-negative bacteria in general. Although relatively few cells of each species are shown in this paper, the micrographs have been carefully selected to illustrate the general ultrastructural features common to all of the species, and to emphasize the distinguishing or unusual structures observed in certain cells.

Cell envelope. The double-fixation procedure described above (Materials and Methods) yielded the best preservation of internal cellular structures; however, as observed also by Remsen and Lundgren (13), the cytoplasmic membrane was not clearly revealed with this method. Therefore, details of the cell envelope are illustrated in micrographs (Fig. 1a–7a) of cells fixed by Kellenberger's procedure.

Each species has a multilayered cell envelope characteristic of gram-negative bacteria (Fig. 1a–7a). This consists of an outer, dark-light-dark, unit membrane-like structure (OL), an electron-dense middle layer (ML), and the dark-light-dark cytoplasmic membrane (CM). The outer layer of the cell envelope and the cytoplasmic membrane are about 8 and 7 nm thick, respectively, for all species studied. The appearance of the middle layer varies considerably in different species; e.g., those of *T. thiooxidans* and *Thiobacillus* A2 are very prominent (Fig. 4a and 6a), those of *T. thioparus* and *T. intermedius* (Fig. 1a and 3a) are less obvious, and the middle layer of *T. novellus* is very diffuse or possibly absent (Fig. 7a). The external surfaces of the envelopes of all cultures except *T. novellus* are rippled or undulating in contour; cells of *T. novellus* are relatively smooth in outline.

Older cultures of *T. thioparus* (maximal stationary phase cells) contain a few cells in which a lamellar body is found (Fig. 1c and 1d); one end of the structure is in close proximity to the cytoplasmic membrane, with most of the structure extending into the cytoplasm at a sharp angle to the membrane. Each dense band of the array averages 9 nm in thickness.

Mesosomes, or at least what appear to be invaginations of the cytoplasmic membrane, were seen in *T. denitrificans* (Fig. 5 and 5b). In thin sections, these structures appear as electron-transparent areas showing tubular structures of varying complexity (Fig. 5). Negative stains demonstrate that these structures (easily penetrated by PTA) protrude into the cytoplasm from the periphery of the cell (Fig. 5b).

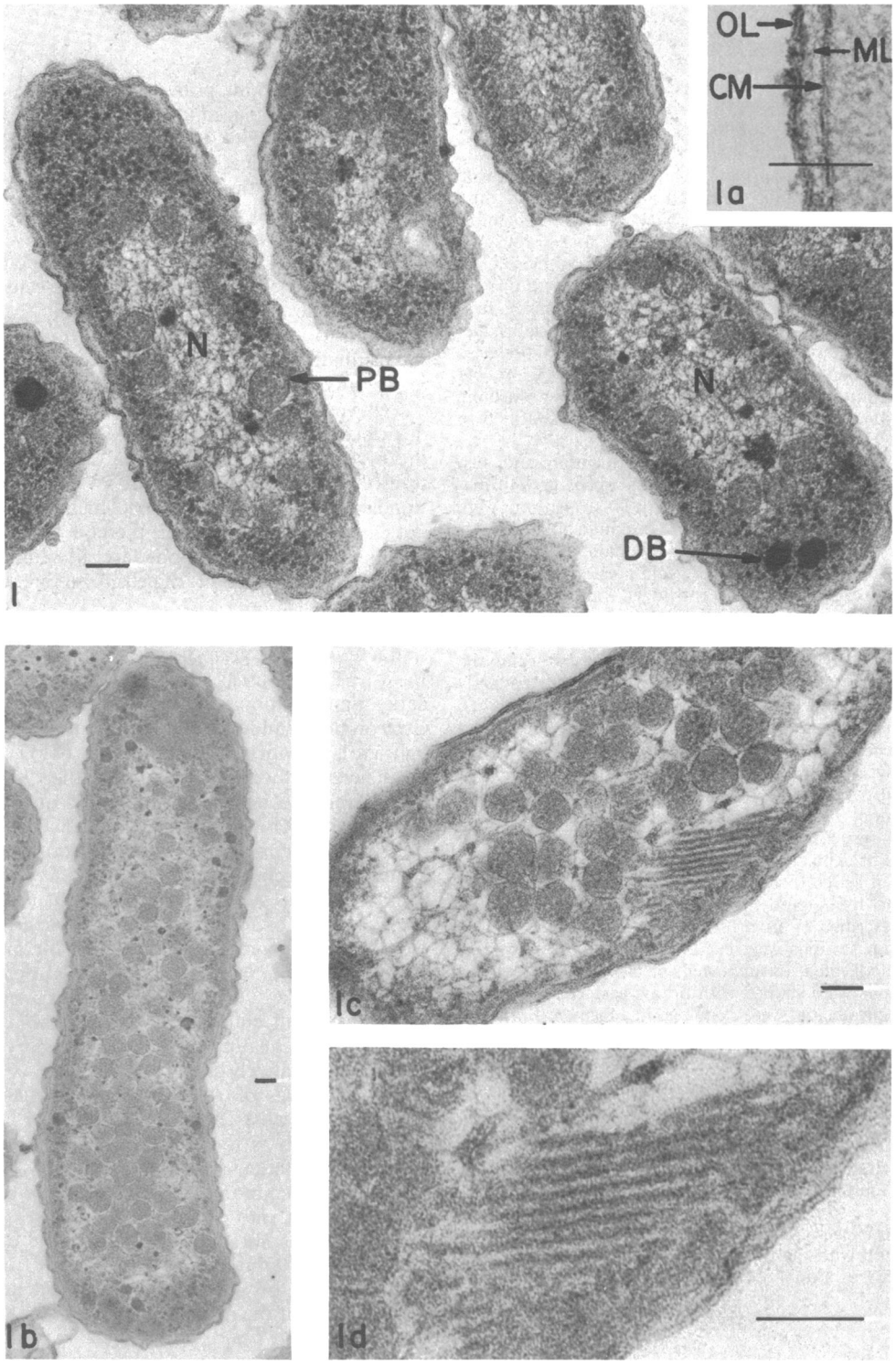

FIG. 1–1d. *Thin sections of T. thioparus showing: general cell structure (Fig. 1); cell envelope ultrastructure (Fig. 1a); a cell sampled during the maximal stationary growth phase possessing an increased number of polyhedral inclusion bodies (Fig. 1b); a cell sampled during the maximal stationary growth phase containing a lamellar body (Fig. 1c); and an enlargement of the lamellar body (Fig. 1d). Abbreviations: nuclear area (n), polyhedral inclusion body (PB), dense inclusion body (DB), outer layer of the cell envelope (OL), middle layer of the cell envelope (ML), and cytoplasmic membrane (CM). In all of the micrographs, the marker bar represents 100 nm.*

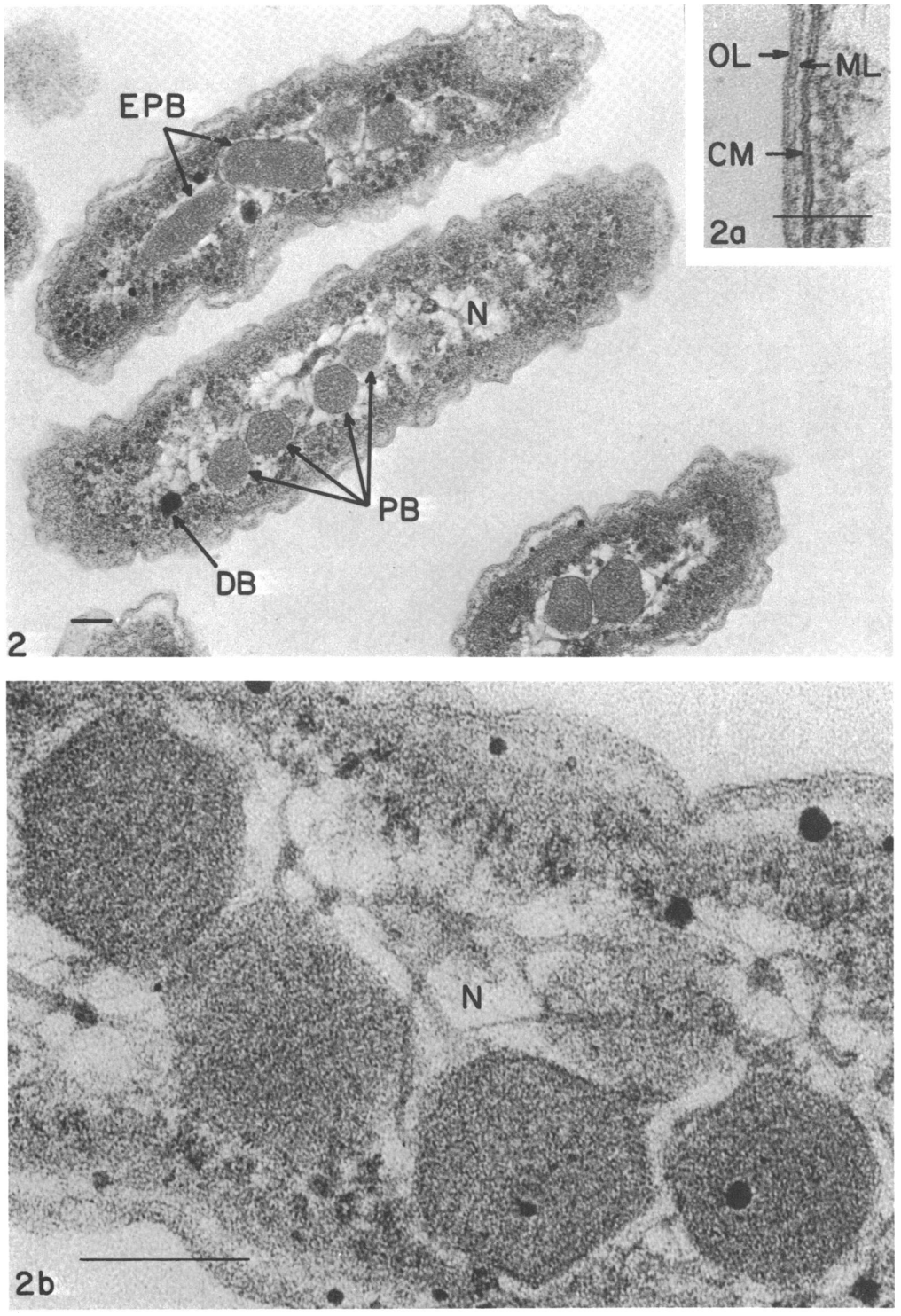

FIG. 2–2b. *Thin sections of T. neapolitanus showing: general cell structure (Fig. 2); cell envelope ultrastructure (Fig. 2a); and polyhedral inclusion bodies (Fig. 2b). Abbreviations: elongated polyhedral inclusion body (EPB).*

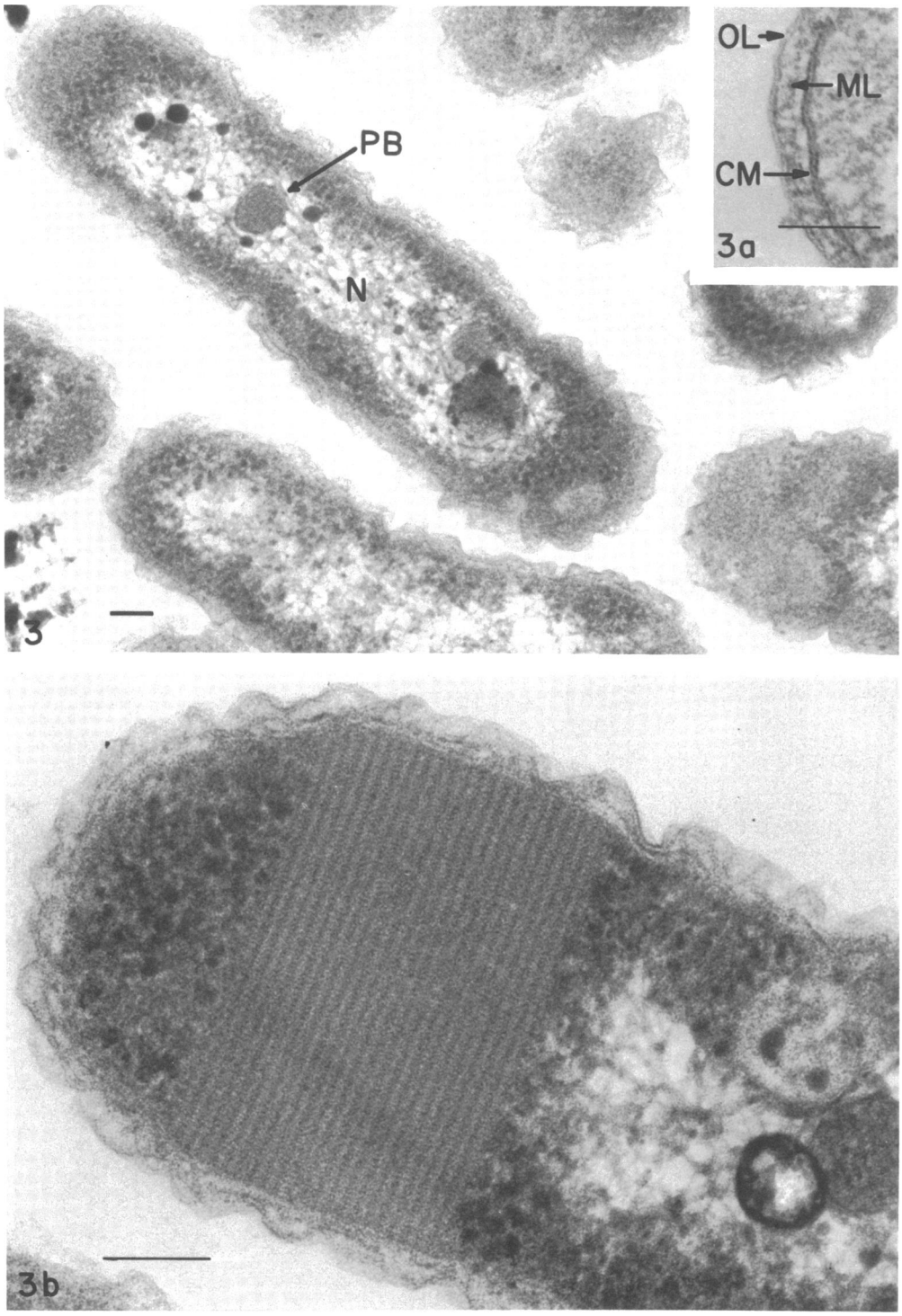

FIG. 3–3b. *Thin sections of T. intermedius showing: general cell structure (Fig. 3); cell envelope ultrastructure (Fig. 3a); and a paracrystalline inclusion (Fig. 3b).*

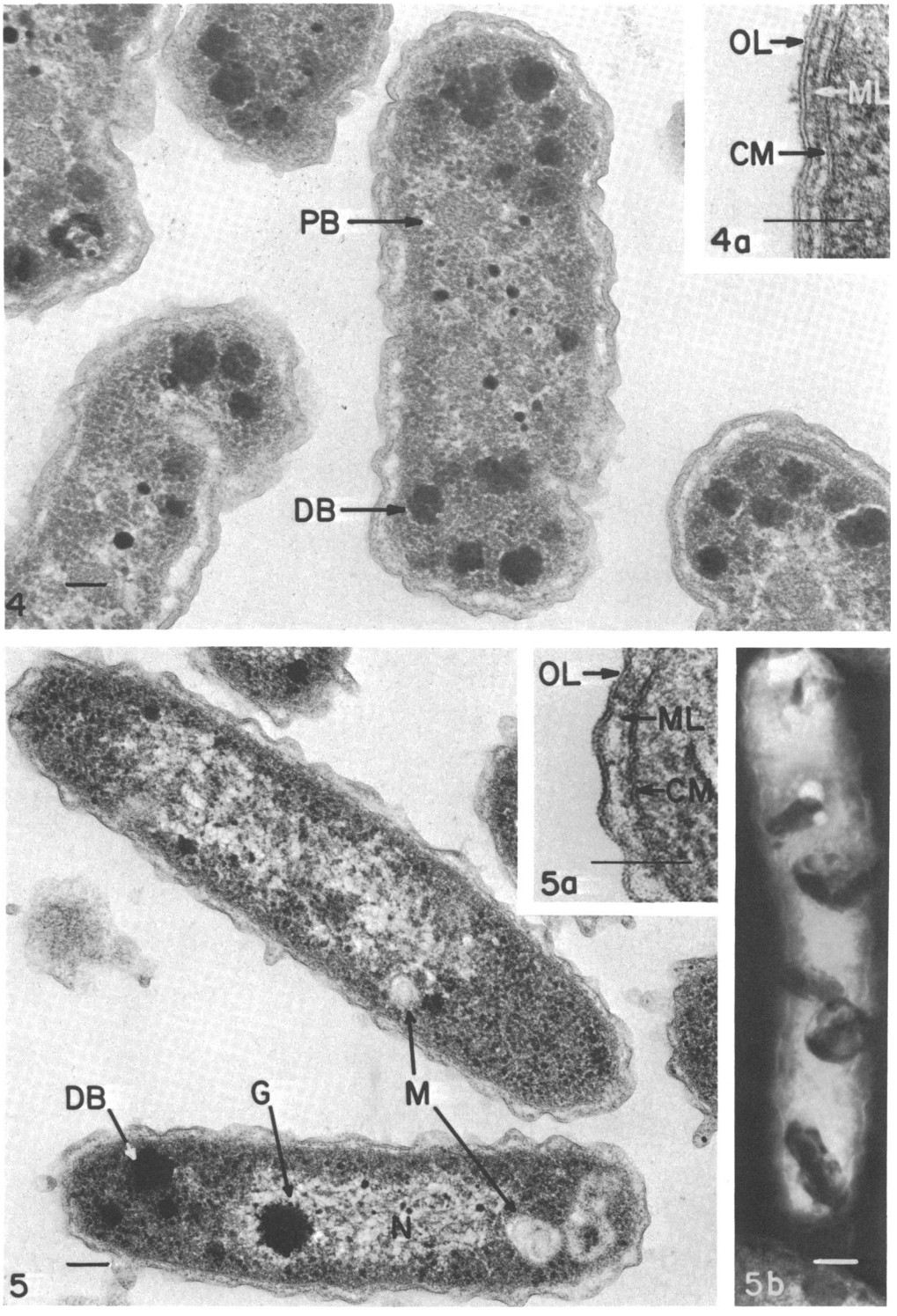

FIG. 4 and 4a. *Thin sections of T. thiooxidans showing: general cell structure (Fig. 4); and cell envelope ultrastructure (Fig. 4a).*

FIG. 5 and 5a. *Thin sections of T. denitrificans showing: general cell structure (Fig. 5); and cell envelope ultrastructure (Fig.* 5a). *Abbreviations: granule (G) and mesosome (M).*

FIG. 5b. *Negative stain (PTA) of T. denitrificans showing mesosomes.*

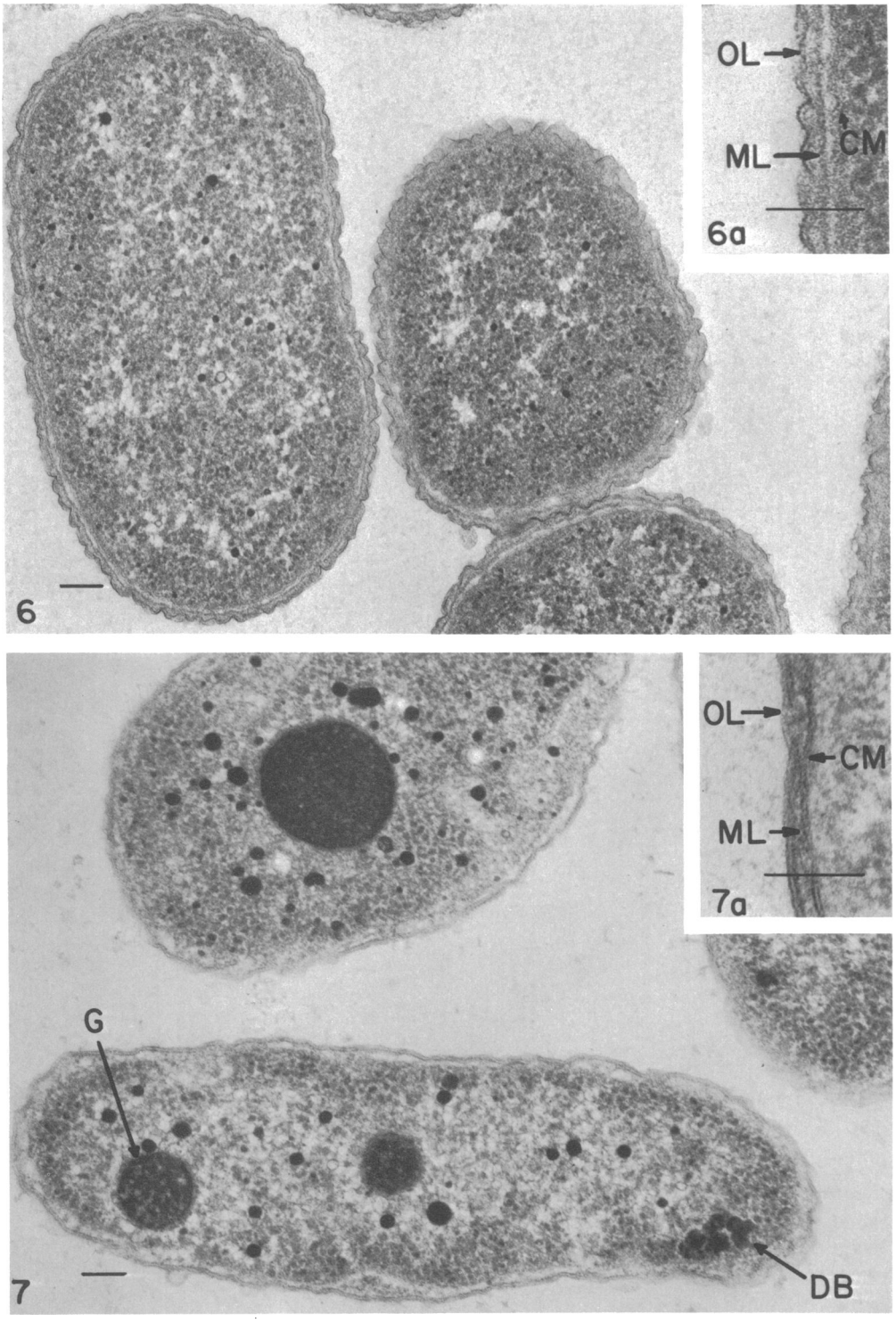

FIG. 6 and 6a. *Thin sections of Thiobacillus species A2 showing: general cell structure (Fig. 6); and cell envelope ultrastructure (Fig. 6a).*

FIG. 7 and 7a. *Thin sections of T. novellus showing: general cell structure (Fig. 7); and cell envelope ultrastructure (Fig. 7a).*

Cytoplasm and nucleoplasm. Ribosomes were clearly observed in all of the species examined and appeared well preserved. The nucleoplasm varies somewhat from one cell to another and from species to species. This difference, ranging from a compact nuclear region to extremely dispersed filaments, may result from differences in the stages of cytokinesis of individual cells, as well as from differences in response to the fixation and embedding techniques. Vacuoles were not observed in any of the species.

Polyhedral inclusion bodies (PB) were seen in *T. thioparus*, *T. neapolitanus*, *T. intermedius*, and *T. thiooxidans* (Fig. 1–4). These inclusions (average diameter, 100 nm) generally appear hexagonal in thin sections and are consistently found in association with the nuclear material. In contrast to cells sampled from the logarithmic growth phase, cells sampled during the maximal stationary phase of growth show increased numbers of these bodies; as many as 60 have been counted in a single cell in thin section (Fig. 1b). Low and intermediate magnifications (Fig. 1b, 1c, and 2) suggest that these inclusions possess a degree of substructure, perhaps including an outer layer; however, examination of thin sections at higher magnification did not reveal the organizational detail of these bodies (Fig. 2b). On rare occasions these inclusions appeared asymmetrical, being elongated (EPB) in one dimension (Fig. 2).

Irregularly shaped, densely staining bodies (DB) were commonly found in several of the species (Fig. 1, 2, 4, 5, and 7), and may represent polysaccharide (glycogen) accumulations. In addition, very electron-dense granules (G), probably volutin, can be seen associated with the nuclear material of *T. denitrificans* and *T. novellus* (Fig. 5 and 7).

An extremely large, paracrystalline structure was observed in the polar region of a few cells of *T. intermedius* (Fig. 3b). These appear as alternating dark and light bands measuring about 7.5 and 5.0 nm thick, respectively. The dark and light bands of these bodies are oriented perpendicular or at a slight angle to the longitudinal axis of the cell.

DISCUSSION

The rippled appearance of the outer surface of the cell envelope is a prominent feature of most of the thiobacilli. This observation has been previously recorded for *T. thiooxidans* (9), for *Thiobacillus* A2, and for a closely related organsim, *F. ferrooxidans* (13). To what extent this effect may be due to the osmotic environment to which the cells are exposed during fixation is not clear. However, in some instances it appears to be an authentic characteristic of the cells, e.g., *T. denitrificans* was studied with the use of a number of preparatory techniques and always showed the undulating surface. An understanding of the response of the various species to changes in the osmotic environment and the effects of these environmental changes on cell morphology and ultrastructure require additional study.

The ultrastructural organization of the cell envelope appears to be the same as that reported for other gram-negative bacteria (3, 10), although, as mentioned earlier, obvious morphological differences occur in the middle layer of the seven species. This layer, which is composed at least partially of mucopeptide, contributes rigidity to the cell (3). It is possible that some of the observed structural differences of the middle layer are due in part to our fixation and staining procedures; however, the absence or very diffuse nature of the electron-dense middle layer of the cell envelope in autotrophically grown cells of *T. novellus* has also been recorded by Van Caeseele and Lees (16). In addition, we have noted that many *T. novellus* cells show pleomorphism, indicating a lack of rigidity. It would be of interest to determine whether the structural differences can be correlated with differences in chemical composition of envelope components.

The nature, function, and biological significance of the polyhedral inclusion remain open to speculation. These bodies already demonstrated in *F. ferrooxidans* (4, 18), *T. thiooxidans* (9), and *N. agilis* (11) seem to be peculiar to autotrophic bacteria. The inclusions observed in each of the cultures may or may not be identical, but they do appear very similar morphologically. Preliminary experiments indicate that these inclusions accumulate as the cultures age. This observation argues against the probability that they are pre-volutin granules as suggested by Mahoney and Edwards (9). The size and shape of these inclusions and their consistent association with the nuclear region support the theory that they represent some stage in the development of a bacteriophage, i.e., prophage. The fact that on rare occasions they appear elongated raises some doubt about this interpretation. Furthermore, attempts to induce virus production and release by treating cells with ultraviolet light have thus far been unsuccessful. These structures may, in fact, represent the storage of some, as yet unidentified, material. Further work is clearly needed to answer these questions.

The lamellar bodies of *T. thioparus* are somewhat similar in appearance to the photosynthetic

apparatus of *Rhodospirillum molischianum* (5). The electron-dense bands of *T. thioparus*, however, appear to be considerably thicker than those found in the photosynthetic bacterium. The functional role of these structures is an intriguing prospect for future studies.

Factors affecting the formation, structure, location, and number of mesosomes in *T. denitrificans* have not been extensively studied at this time. We have seen, however, that these structures vary in appearance from simple invaginations to complex tubular arrays and show a definite similarity to those found in the gram-negative bacterium *Caulobacter crescentus* (2). The mesosomes in *T. denitrificans* are obviously more numerous than if, as single structures, their formation and function were restricted to nuclear division or cell division, or to both. In this regard, *C. crescentus* grown under oxygen-limiting conditions was reported to contain numerous mesosomal structures (2). In the present study, *T. denitrificans* was grown under conditions in which the oxygen level of the medium was greatly reduced; these cells also contained many mesosomes. Experiments designed to investigate the regulation of mesosome structure, function, and formation in *T. denitrificans* grown under different conditions are now in progress.

The chemical composition and function of the paracrystalline inclusions of *T. intermedius* are at present unknown, although the structural appearance is reminiscent of urate oxidase deposits observed in mammalian cells (15) and the proteinaceous toxin deposits of *Bacillus thuringiensis* (12). The regularity in the structure of the *T. intermedius* inclusions suggests that these may also contain a high concentration of a fairly homogeneous material, possibly protein. Prospects for further study into the nature of this material in *T. intermedius* are dimmed by the apparent infrequent occurrence of these inclusions in the cells.

Our micrographs and the one published by Taylor and Hoare (14) show the same structural appearance for *Thiobacillus* A2. This new isolate possesses the metabolic characteristics of *T. novellus*, but differs structurally from *T. novellus* in that the former is a different shape (shorter, wider), shows a very prominent middle layer of the cell envelope, and does not contain the large volutin granules.

The relationship of cell ultrastructure to the nutritive content of the growth medium needs further investigation. Van Caeseele and Lees (16) showed obvious differences (envelope structure and inclusions) between autotrophically (thiosulfate) and heterotrophically (glucose) grown cells of *T. novellus*. In the course of our studies, *T. novellus* and *Thiobacillus* A2 were grown heterotrophically in nutrient broth (no glucose); these cells had the same ultrastructural appearance as those grown autotrophically. With regard to nutritional requirements, it should also be noted that the thiosulfate-utilizing *T. thiooxidans* used in our investigation appears to be ultrastructurally identical to the sulfur-utilizing culture used by Mahoney and Edwards (9).

It is apparent from the results of the present study that considerable research remains to be done on the relationship of structure to function in the thiobacilli.

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