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Ultrastructure of Methanotrophic Yeasts

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The cellular structure of two yeast strains capable of growth on methane was investigated by electron microscopy. Microbodies were observed in cells of *Sporobolomyces roseus* strain Y and *Rhodotorula glutinis* strain CY when grown on methane but rarely when grown on glucose. The size of the microbodies and the number observed per cell in a thin section did not increase with culture age. No crystalline organization was observed within these organelles. Similar microbodies were also observed in cells of *R. glutinis* CY grown on hexadecane. The plasma membranes of both methane and hexadecane-grown cells exhibited increased invagination compared to that of glucose-grown cells. Catalase activity was detected in the microbodies of alkane-grown cells by using 3,3'-diaminobenzidine as a cytochemical stain. The data presented suggest that microbodies, and the catalase contained within them, play a role in eucaryotic methane metabolism.

Complex arrays of intracytoplasmic membranes have been observed in the cytoplasm of all bacteria that utilize methane (3, 14, 26). The type of membrane arrangement was used to characterize these methanotrophic microorganisms (3). Patt et al. (13) observed that intracytoplasmic membranes were present in cells of the facultative methane oxidizer *Methylobacterium organophilum* only when cultures were grown on methane, reinforcing the implication that the intracytoplasmic membranes were intimately involved in bacterial methane metabolism. Most facultative methanol-oxidizing bacteria do not possess these membranes (1), except *Hyphomicrobium* spp., which have well-developed internal membrane systems (2).

Methanol-utilizing yeasts exhibit subcellular localization of some enzymes involved in methanol oxidation. Cytochemical staining techniques as well as isolation and biochemical characterization of the microbodies from cells of methanol-grown cultures have shown that these organelles contain alcohol oxidase and catalase, the first two enzymes of the methanol oxidation pathway (4, 5, 16, 23, 25).

Recently, Wolf and Hanson (27) reported the first isolation and characterization of eucaryotes capable of growth on methane. Two of the methane-utilizing yeasts, identified as strains of *Sporobolomyces roseus* and *Rhodotorula glutinis* (H. J. Wolf and R. S. Hanson, submitted for publication), were examined by electron microscopy to determine if these methylotrophic microorganisms show ultrastructural modifications associated with growth on methane.

MATERIALS AND METHODS

Organisms and growth conditions. Two methanotrophic yeasts, *S. roseus* strain Y and *R. glutinis* strain CY, were used in this study. Both are facultative methanotrophs, and *R. glutinis* CY is capable of using hexadecane as a carbon and energy source for growth (27). The organisms were grown on several substrates on agar plates or in liquid culture, using the basal medium previously described (27) supplemented with 0.005% (wt/vol) yeast extract for *R. glutinis* and with the described amino acid and vitamin supplements for *S. roseus*. Glucose, acetate, ethanol, and hexadecane were filter sterilized and then added to the growth medium to a final concentration of 0.5, 0.1, 0.1, and 0.2%, respectively. Cultures grown methanotrophically were incubated under an atmosphere of 65% methane-15% CO_2-20% air. All cultures were incubated at 20°C without shaking.

Cells were harvested by centrifugation in the middle of the exponential phase of growth unless otherwise indicated, washed once with cold 0.05 M sodium phosphate buffer (pH 7.0), and washed again with cold 0.07 M cacodylate buffer (pH 7.3).

Electron microscopy. Cells of the yeast strains were fixed in 2.5% glutaraldehyde in 0.07 M cacodylate buffer (pH 7.3) for 1.5 h on ice, washed in the same buffer several times, and embedded in 2% Ionagar (Oxoid Ltd., London, England). One-millimeter blocks of the embedded cells were then postfixed in 1% OsO_4 in the cacodylate buffer for 14 to 15 h, washed several times, and stained with 1.5% aqueous uranyl acetate for 1.5 h. After dehydration with a graded series of cold ethanol, all preparations were embedded in Spurr epoxy resin-lower-viscosity mixture (19), using a 12-h incubation at each infiltration step. Thin sections were cut with a diamond knife on a MT-2 Sorvall microtome, placed on carbon-coated 200-mesh copper grids, and stained with 2% uranyl acetate for 30 min followed by Reynolds' lead citrate (15) for 15 min. Specimens were examined and photographed with a Zeiss EM9S electron microscope.

To estimate the number of microbodies per cell per section, cells from three different specimen preparations were examined. The numbers of microbodies

present in at least 150 cells of each preparation were determined. The average of these determinations was used as the value for the number of microbodies present in a cell section.

Cytochemical staining technique. To determine if catalase activity was localized in the yeast cells, cells that had been fixed with glutaraldehyde were treated with 3,3'-diaminobenzidine (7). After the glutaraldehyde fixation, cells were washed twice in the cacodylate buffer and once in the assay buffer. The reaction mixture consisted of 9.8 ml of 50 mM 2-amino-2-methyl-1,3-propanediol buffer (pH 9.5; Sigma Chemical Co., St. Louis, Mo.), 20 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co.), 0.2 ml of 1% H_2O_2, and 0.1 to 0.2 g (wet weight) of cells. The mixture was incubated at 25°C for 90 min in a stoppered test tube and then put on ice to stop the reaction. The cells were washed once in the assay buffer, once in the cacodylate buffer used for the fixation, and then postfixed by the procedure described above employing OsO_4 and uranyl acetate. The cells were embedded in epoxy resin and sectioned as described. Thin sections were not stained with uranyl acetate or lead citrate or both before examination.

Control experiments were run, using 3-amino-1*H*-1,2,4-triazole as an inhibitor of catalase activity. Glutaraldehyde-fixed cells were preincubated for 30 min with this compound before being stained in the reaction mixture containing aminotriazole.

RESULTS

Specimen preparation. The development of a new fixation procedure was required to examine the structure of *S. roseus* Y grown on methane or acetate and *R. glutinis* CY grown on methane, glucose, ethanol, or hexadecane. The glutaraldehyde-potassium permanganate fixation used to confirm the eucaryotic nature of the methanotrophic yeasts (27) gave poor preservation of cell structure when the organisms were grown on these substrates. Common results included cells with a very coarsely textured ground substance with areas devoid of fixed material, indistinct cellular structure, and nuclear and mitochondrial membranes in negative image. The glutaraldehyde-osmium tetroxide fixation described in this paper resulted in very good preservation of most cellular structures. However, vacuoles or inclusions and their membranes were often poorly preserved, resulting in white areas in the micrographs (Fig. 1b, 2b). An incubation of about 10 h was required to get noticeable fixation by the osmium tetroxide; 14 to 15 h provided optimum preservation of the cells. Sodium phosphate buffer (70 mM, pH 7.2) could be substituted for the cacodylate buffer; however, the sections obtained exhibited less contrast and were less sharp in detail when phosphate buffer was used.

The long incubation times during infiltration of the embedding resin could be shortened slightly by carrying out the final steps in 100% resin under vacuum. However, this procedure resulted in an increase in the amount of cell tearing that occurred during sectioning. Embedding using the low-viscosity resin and the shorter incubation times recommended by Spurr (19) worked poorly for these specimens.

Structural comparison of methane- and glucose-grown cells. The electron micrographs in Fig. 1 and 2 demonstrate the typical structures of cells of *R. glutinis* CY and *S. roseus* Y grown on methane and glucose. Methane-grown cells of both yeasts were smaller than glucose-grown cells, but retained the same basic cell shape.

Microbodies were observed in cells of both yeast strains when grown on methane (Fig. 1b, 2b). The organelles contained a homogeneous matrix bounded by a single-unit membrane (Fig. 3). The size of the microbodies did not appear to vary with culture age, nor did the number of microbodies observed per cell section appear to change. There was, however, great variability in the number of microbodies per cell (0 to 8) in a single section. *R. glutinis* CY had an average of 1 to 2 microbodies per cell section; *S. roseus* Y had 3 to 4 microbodies per cell section. Very few (one per section) or no microbodies were observed in glucose-, acetate-, or ethanol-grown cells of either organism.

Some other differences were observed in the structure of cells grown on methane and that of cells grown on glucose. Inclusions or vacuoles were observed in many cells of methane-grown cultures while the culture was still actively growing. These structures did not appear in glucose-grown cells until the culture neared the end of the exponential phase of growth. Both *S. roseus* and *R. glutinis* had fewer mitochondria and ribosomes when grown methanotrophically; ribosomes were often localized around the mitochondria. The plasma membranes of methane-grown cells appeared to be more invaginated than those observed in glucose-grown cells. Small structural elaborations, consisting of vesicles or membranous layers continuous with the plasma membrane, were sometimes observed between the cell wall and the plasmalemma in methane-grown cells (Fig. 1b). These structures are similar to plasmalemmasomes (9).

Structure of *R. glutinis* CY grown on hexadecane. The cellular organization of *R. glutinis* grown on hexadecane was examined to determine if structural similarities existed in cells of the organism grown on a gaseous alkane and those grown on a longer, aliphatic hydrocarbon. The cells were similar in size to cells grown on glucose (Fig. 4a). Hexadecane-grown cells contained fewer ribosomes and mitochondria

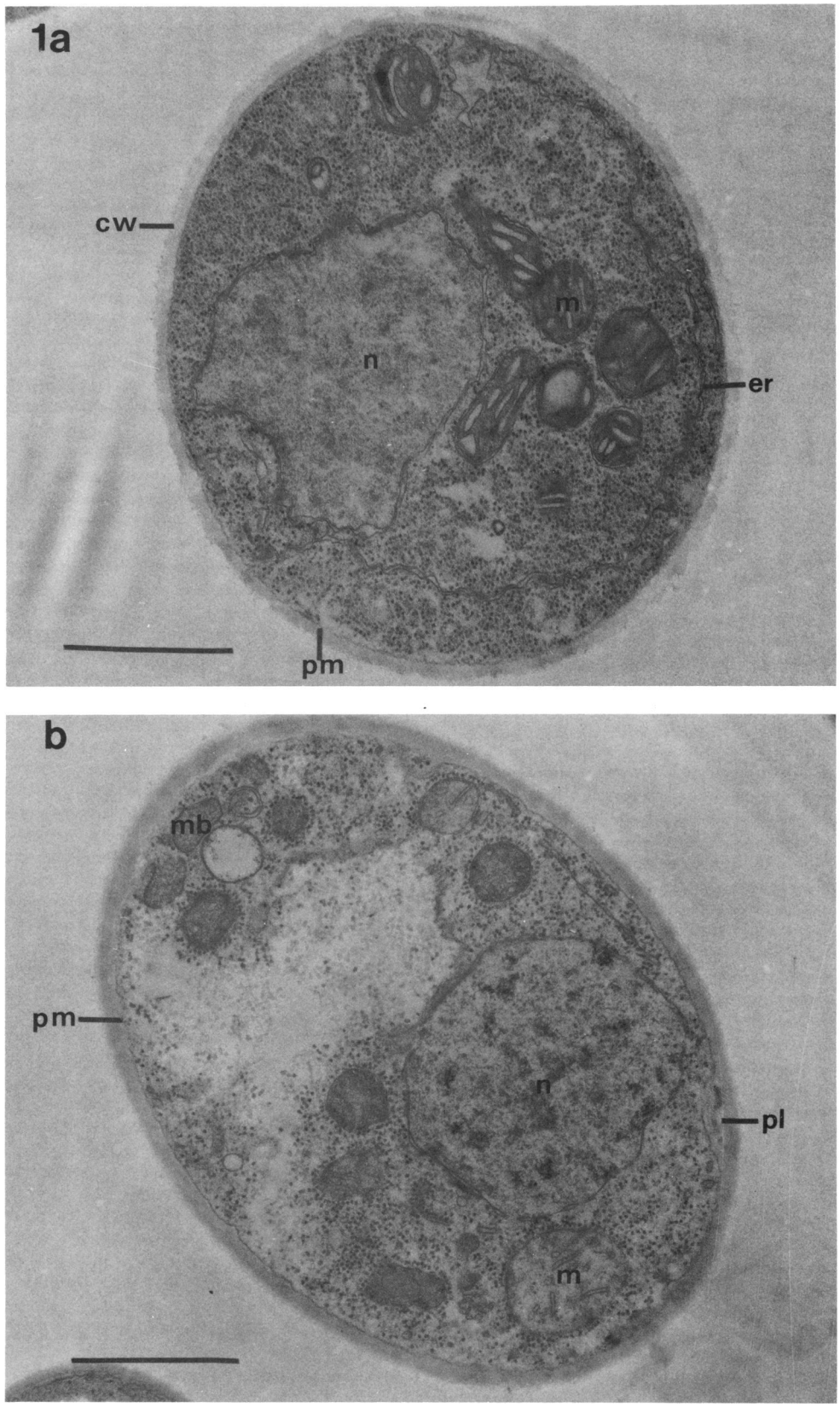

FIG. 1. *Comparison of the ultrastructure of R. glutinis grown on (a) glucose, (b) methane. Microbodies occur in the cytoplasm of the methane-grown cell. Fewer ribosomes and mitochondria occur in methane-grown cells. Nucleus (n), endoplasmic reticulum (er), mitochondrion (m), plasma membrane (pm), cell wall (cw), microbody (mb), plasmalemmasome (pl). Cells were fixed with glutaraldehyde-osmium tetroxide. The bars represent 1 μm.*

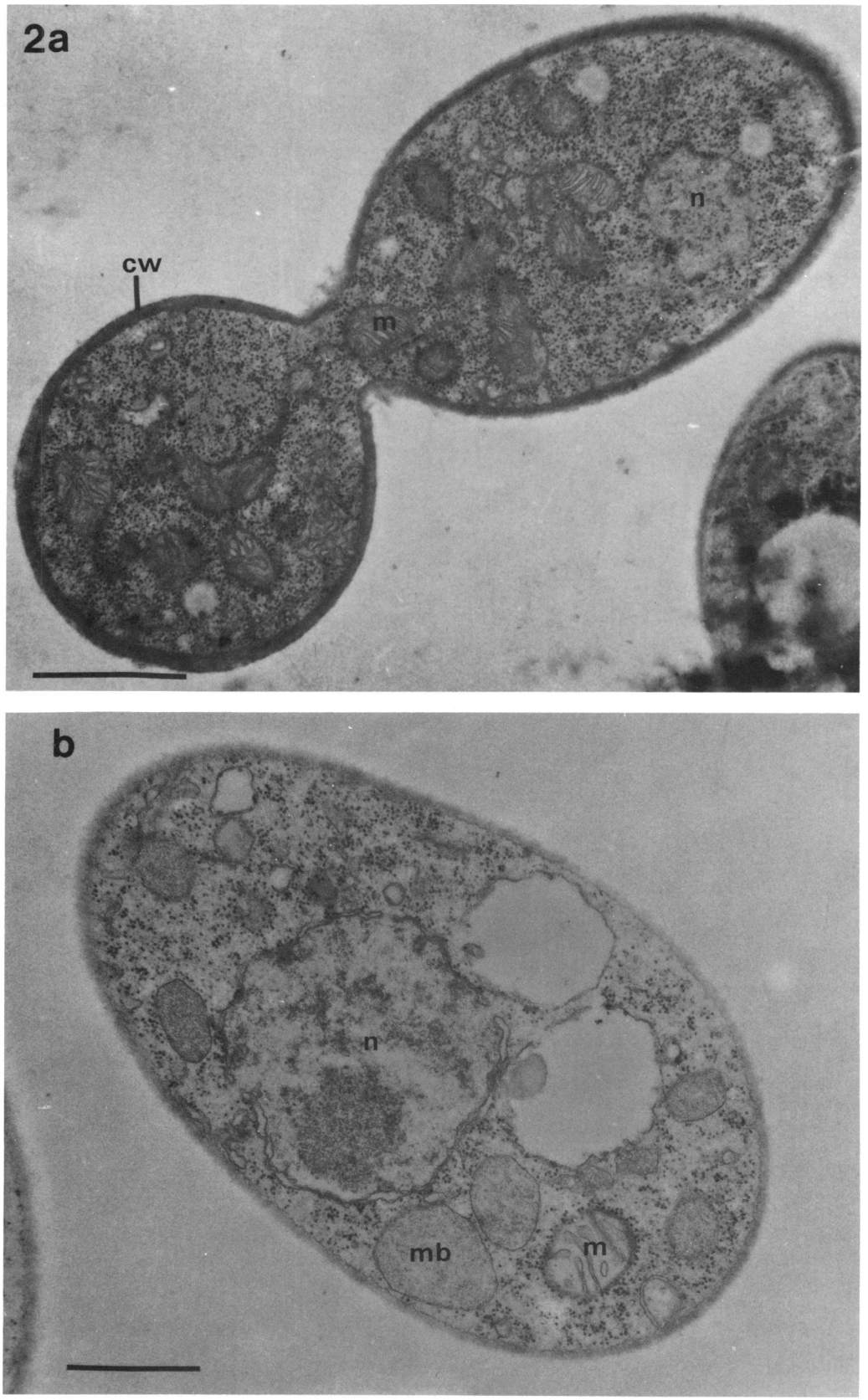

FIG. 2. *Thin sections of S. roseus grown on (a) glucose and (b) methane. Several microbodies (mb) can be seen in the methane-grown cells. Bar represents 1 μm.*

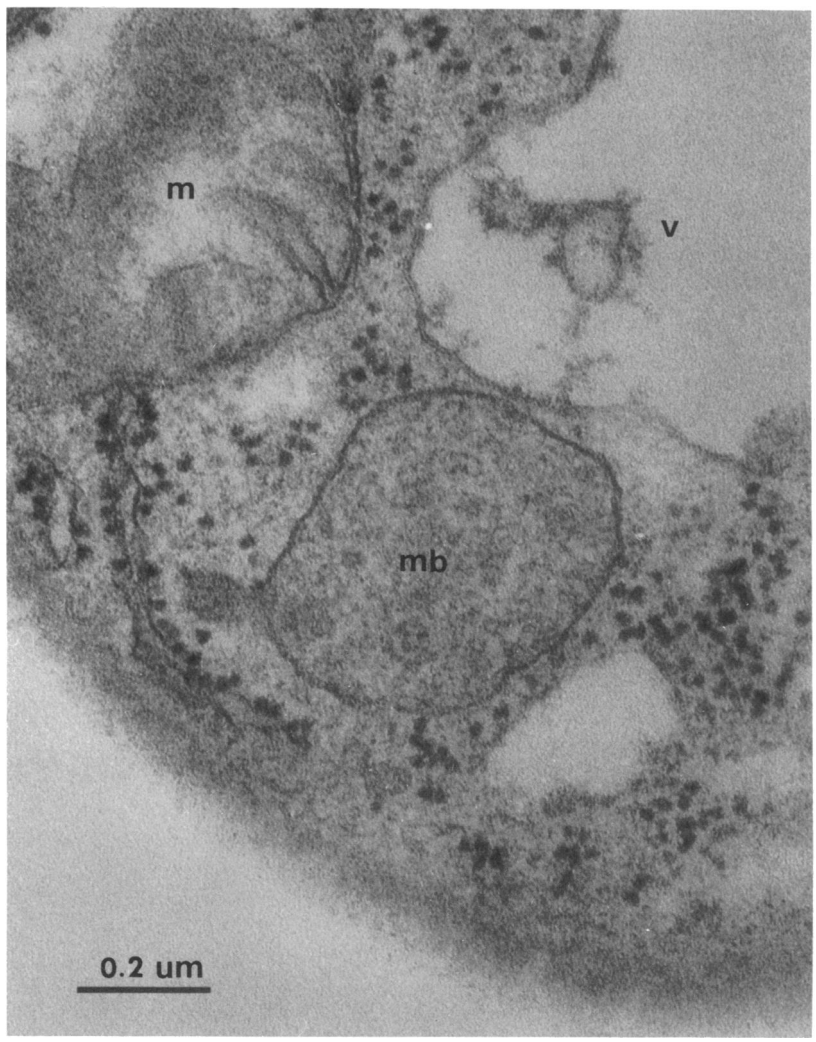

FIG. 3. *Section through a microbody observed in a methane-grown cell of S. roseus. No distinct organization occurs within the organelle. Bar, 0.2 μm.*

than methane-grown cells. The microbodies observed were similar in size and shape to those found in methane-grown cells of the organism; no change in size was observed during culture growth. However, the number of microbodies observed per cell section did change from a few when the culture was harvested during the middle of the exponential phase of growth to several per cell section in cells from a stationary-phase culture. The plasma membrane of cells grown on hexadecane was highly involuted. The plasmalemma also formed complex structures composed of numerous concentric membranous layers which occasionally contained discrete vesicles (Fig. 4b). The membrane layers were continuous with the plasma membrane. The structures (plasmalemmasomes) occurred in approximately one-half of the cell sections in cells from cultures harvested in the middle of the exponential phase of growth; fewer were observed in sections of cells of early-exponential- or stationary-phase cultures. Changes in the osmolality of the fixation mixtures or in the buffers and their concentrations did not affect the appearance of the structures.

Localization of catalase activity. The microbodies in methane-grown cells of both yeasts showed heavy accumulation of the 3,3′-diaminobenzidine reaction products resulting from catalase activity (Fig. 5a, 6a). No reaction products were deposited in the microbodies when aminotriazole, a catalase inhibitor, was included in the staining procedure. The reaction product was distributed throughout the microbody, al-

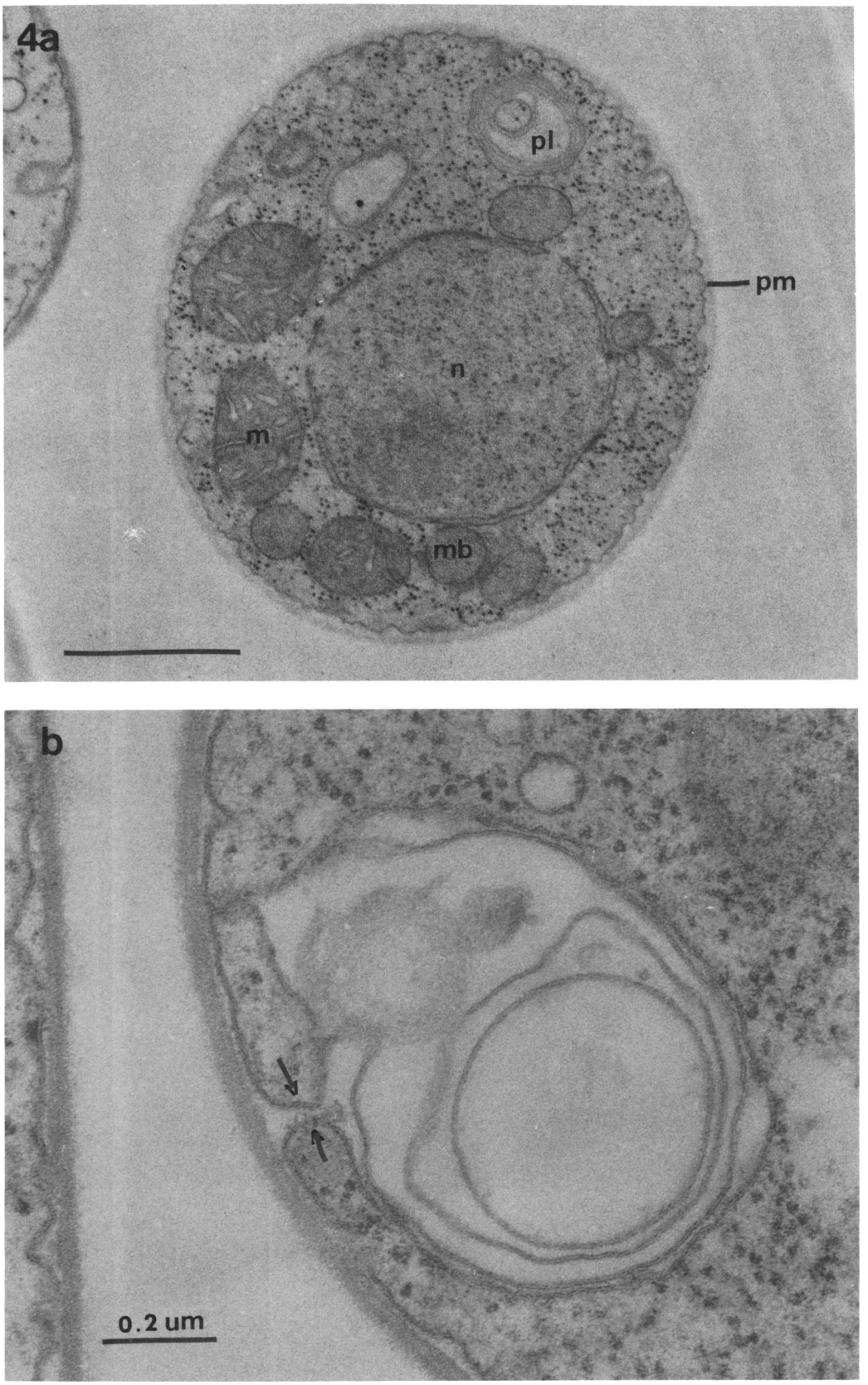

FIG. 4. *(a) Thin section of a cell of R. glutinis from a late-exponential-phase culture grown on hexadecane with microbodies. The plasma membrane is highly invaginated, and a plasmalemmasome is present. Bar, 1 μm. (b) Section of a portion of a hexadecane-grown cell showing the continuity of the plasma membrane with a plasmalemmasome (arrow). Bar, 0.2 μm.*

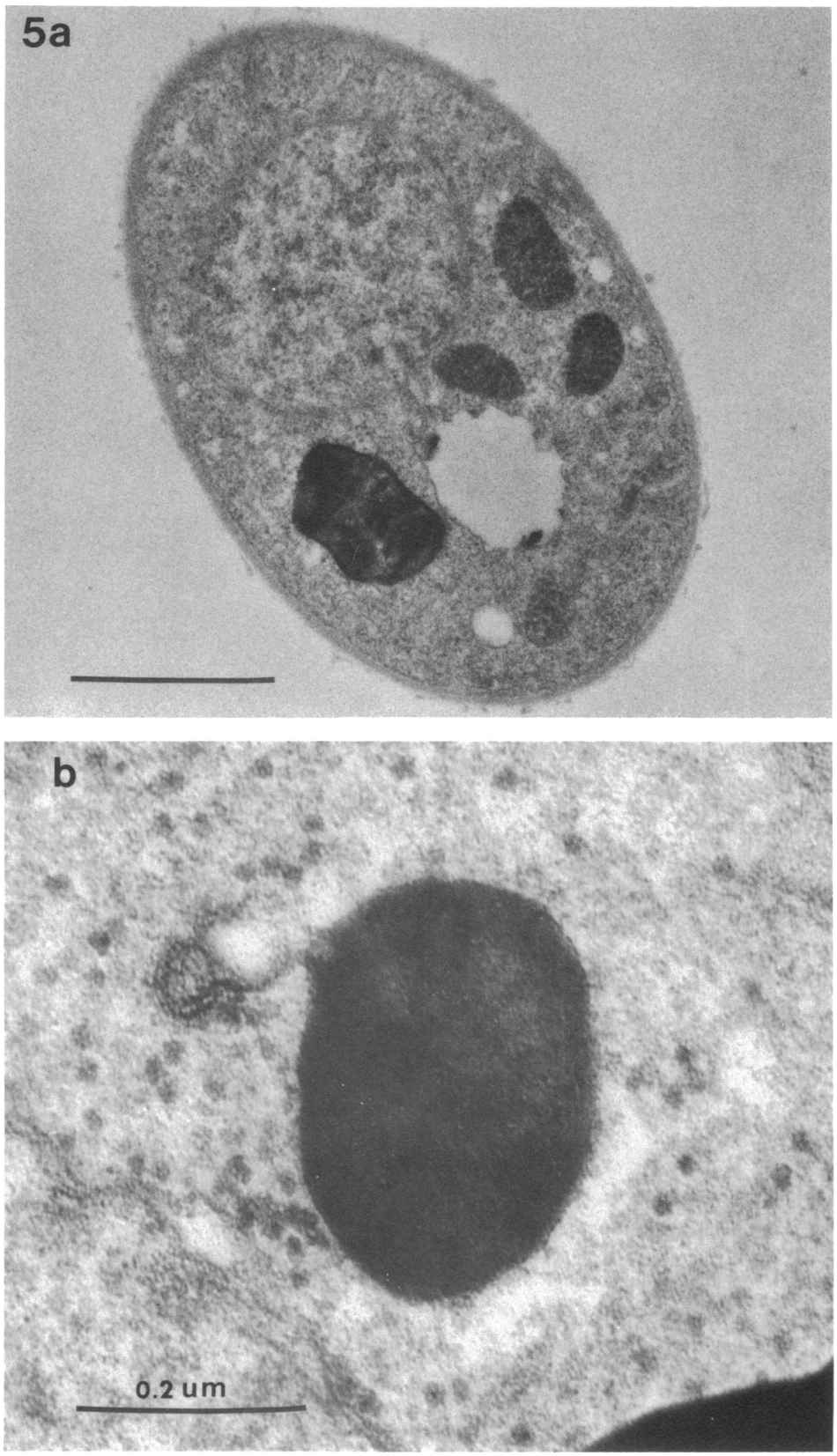

FIG. 5. *(a) Section of a cell of methane-grown S. roseus stained for catalase activity with diaminobenzidine. Bar, 1 μm. (b) Higher magnification of a microbody in a DAB-treated cell demonstrating a lack of crystalline structure within the organelle. Bar, 0.2 μm.*

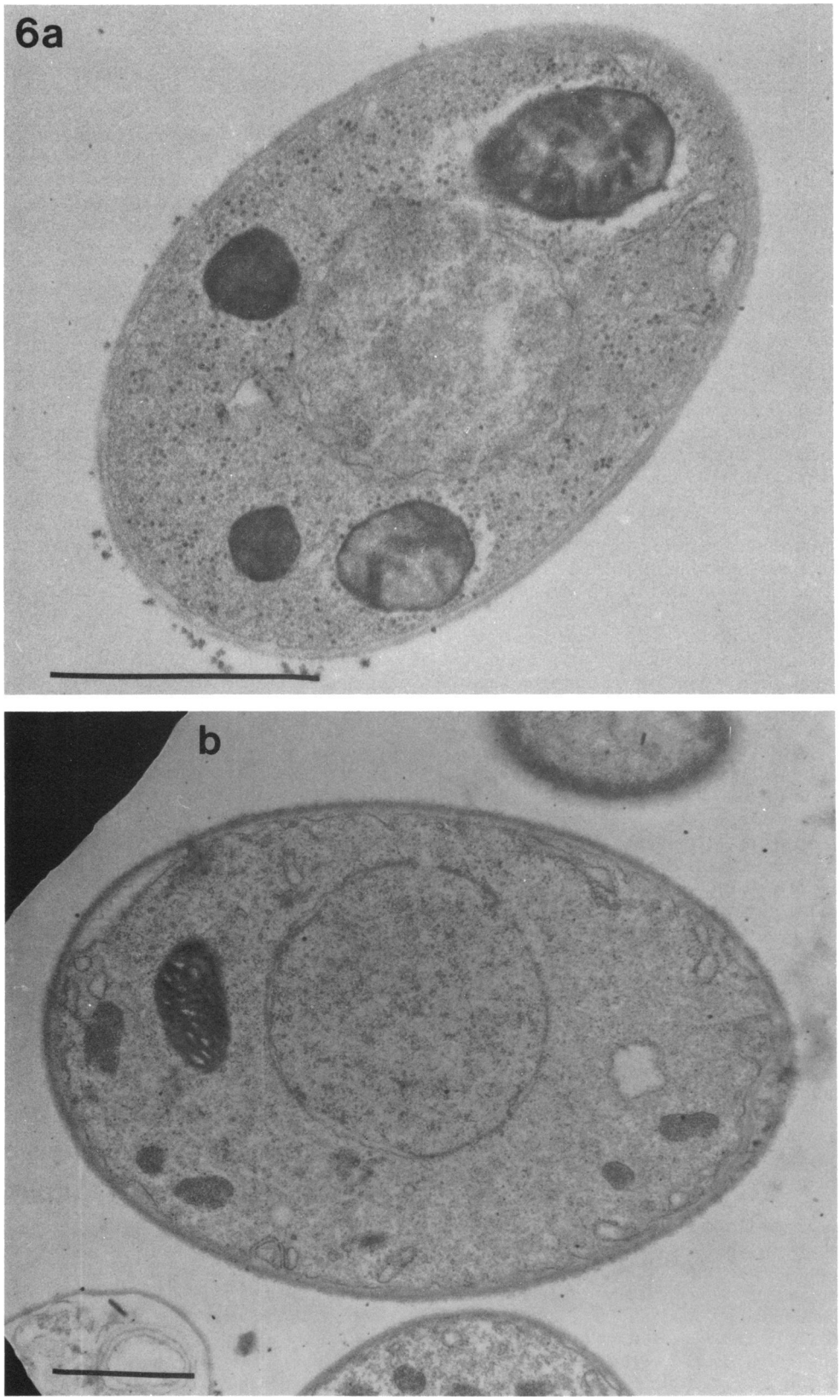

FIG. 6. *Diaminobenzidine-treated cells of R. glutinis grown on (a) methane, (b) hexadecane. Bar, 1 μm.*

though some less-electron-dense areas were visible in the matrix (Fig. 5b). No crystalloid inclusions were observed after cytochemical staining followed by the osmium tetroxide fixation. The DAB reaction products also accumulated in the cristae of the mitochondria; this was apparently due to peroxidase activity. The microbodies in cells of *R. glutinis* grown on hexadecane were also stained by accumulation of the DAB reaction products (Fig. 6b). Homogeneous deposition of stain within the microbodies was observed. However, the relative amount of reaction product accumulated in the microbodies of hexadecane-grown cells compared to the amount deposited in the mitochondria present in those cells was much less than the amount of product found in microbodies of methane-grown cells relative to the stain in the mitochondria.

DISCUSSION

Many methylotrophic microorganisms possess structural components that are specifically involved in their growth on one-carbon compounds. Investigation of the structure of the methane-utilizing yeasts has demonstrated that these microorganisms also modify their cellular organization when grown methanotrophically. These methanotrophs did not exhibit the complex arrays of internal membranes similar to those believed to be involved in methane oxidation in bacteria. This does not preclude the possibility of other membranes, such as the mitochondrial or the cytoplasmic membranes, playing a role in methane oxidation in the yeasts. Microbodies were observed in cells of both *S. roseus* Y and *R. glutinis* CY when methane was used as a growth substrate. The scarcity of these organelles in cells from glucose-, ethanol-, or acetate-grown cultures suggests these microbodies play a role during growth of yeasts on methane.

Microbodies found in several methanol-utilizing yeasts represent subcellular localization of some enzymes involved in the oxidation of methanol (21). The microbodies of methanol-utilizing yeasts were fairly large (0.4 to 1.5 μm in diameter) and were clustered within the cytoplasm with flat surfaces between them (17, 20, 22, 24). Microbody formation was induced when *Kloeckera* sp. 2201 began methylotrophic growth (20); the organelles increased in size with increasing culture age but did not change in the number observed per cell section. Ultrastructural studies, using cytochemical staining techniques, and biochemical studies, involving microbody isolation, have demonstrated the presence of catalase and alcohol oxidase within the microbody (4, 16, 23, 25). The crystalline structure of the microbody, seen after either a glutaraldehyde-osmium tetroxide fixation or after staining with diaminobenzidine followed by an osmium fixation, may represent a highly ordered organization of the catalase, alcohol oxidase, and other oxidases present (23). Sahm and co-workers (17) noted the absence of the crystalloid structures in a mutant of *Candida boidinii* lacking alcohol oxidase.

The microbodies observed in the two methane-grown yeasts differ from those in methanol yeasts in size, shape, organelle structure, and location within the cell. These organelles were similar in appearance to the microbodies of *R. glutinis* grown on hexadecane and of other yeasts capable of growth on hydrocarbons (5, 11, 12). Crystalline structures were absent in *R. glutinis* grown on hexadecane and in other hydrocarbon-grown yeasts (10, 25). The absence of a crystalloid structure in the microbodies of the methane-utilizing yeasts coincides with an inability to detect significant alcohol oxidase activity in cell-free extracts of these methanotrophic yeasts (unpublished data). The number of microbodies present in cells of the organisms grown on methane did not change with culture age, a phenomenon that was observed in this study with hexadecane-grown cells of CY and reported to occur in *Candida tropicalis* pk 233 (11).

Catalase was determined to be one of the enzymes present in the microbodies of methane-utilizing yeasts. Thus, these organelles can be called peroxisomes. The detection of catalase in the microbodies of *R. glutinis* grown on hexadecane was consistent with the observations of others studying hydrocarbon-utilizing yeasts (11, 12), although the role of this enzyme in alkane metabolism is unknown (18). The more intense staining of the microbodies relative to the mitochondria in methane-grown cells compared to hydrocarbon-grown cells suggests a more important role for catalase in eucaryotic methane metabolism. Preliminary studies indicated the specific activity of catalase is 3.5 times higher in methane-grown cells of *S. roseus* than in glucose- or acetate-grown cells (unpublished data).

The multilayered membranous structure associated with hydrocarbon growth of *R. glutinis* is the first report of this type of structure in yeasts grown on hydrocarbons. Similar structures have been reported in several plants and fungi and given the name lomasomes or plasmalemmasomes, depending on whether they originate from cytoplasmic membrane systems or from the plasma membrane, respectively (6, 9). It is not possible from this study to predict the function of the plasmalemmasomes observed. They may be involved in hydrocarbon

metabolism since they were observed in cells only when hydrocarbons served as the substrates for growth. The plasmalemmasomes were most abundant during the period of active cell growth. They may be produced when plasma membrane production is not balanced by cell expansion, as suggested by Heath and Greenwood (6). They might also represent a degenerative membrane structure.

Other structural changes observed in methane-grown cells of *S. roseus* and in methane- or hexadecane-grown cells of *R. glutinis* may be related to the slow growth rates of these organisms on these substrates. The lower distribution of ribosomes and mitochondria in cells grown under these conditions is consistent with this explanation. The invagination of the plasma membrane may be due to a lower solute concentration in the cytosol caused by slower growth. Alternatively, the invaginations may represent a modification to increase the surface area of the plasma membrane available for hydrocarbon accumulation, as suggested by Ludvík et al. (8) for *Candida lipolytica* grown on hydrocarbons. The invaginated plasma membrane observed in *R. glutinis* grown on hexadecane coincided with observations made by other scientists for yeasts grown on hydrocarbons (8, 10).

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