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Ultrastructure of *Nitrobacter agilis* Grown Under Autotrophic and Heterotrophic Conditions

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Nitrobacter agilis, grown through seven transfers heterotrophically in the absence of nitrite, was examined in the electron microscope. The ultrastructure of such cells closely resembled that of autotrophically grown *N. agilis*. It was thus further established that the organisms growing heterotrophically were indeed *N. agilis* and, therefore, that *N. agilis* is a facultative autotroph. Acetate incorporation into poly-β-hydroxybutyrate was confirmed cytologically.

The nitrifying bacterium *Nitrobacter agilis* has long been regarded as an obligate chemoautotroph, since repeated attempts to grow this organism on organic media in the absence of nitrite have been unsuccessful. Recent work in several laboratories has clearly established that growing cultures and cell suspensions of *N. agilis* can assimilate organic carbon into cell material (2, 4, 11). Furthermore, Smith and Hoare (11) were able to grow *N. agilis* on an organic medium in the absence of nitrite. However, the growth rate was exceedingly slow. These authors proposed that the strain of *N. agilis* used in their investigations was a facultative chemoautotroph. Physiological and biochemical studies on *N. agilis* grown under different conditions also supported this proposition. *N. agilis* has a distinctive morphology and ultrastructure (8). Therefore, we examined the effect of growth conditions on its morphology and ultrastructure. Particular attention was directed to the ultrastructure of organisms grown heterotrophically in order to determine whether heterotrophically grown cells display the characteristic ultrastructural features of *N. agilis* (8); thus, we could further establish that the heterotrophically growing cells were indeed *N. agilis*. Cytological evidence was also sought to confirm the massive accumulation of poly-β-hydroxybutyrate (PHB) reported by Smith and Hoare (11) in cultures grown in the presence of acetate and limiting concentrations of nitrite. PHB has been isolated and characterized from the closely related chemoautotroph *N. winogradskyii* (16).

MATERIALS AND METHODS

N. agilis was obtained from C. C. Delwiche (University of California, Davis). The organism was grown in a mineral medium with nitrite, with or without the further addition of sodium acetate (final concentration, 5 mM), as described by Smith and Hoare (11). *N. agilis* was grown under heterotrophic conditions in a nitrite-free mineral medium supplemented with sodium acetate (5 mM) and Difco casein hydrolysate (0.075%, w/v). This medium was inoculated with autotrophically grown organisms, and the culture was incubated at 25 C on a rotary shaker. After 2 to 3 weeks, the organism was transfered to fresh medium of the same composition; a 5% (v/v) inoculum was routinely used. This procedure was repeated seven times before a sample of organisms was removed for examination in the electron microscope. All cultures were routinely screened for contamination as described previously (11).

Cells were washed twice and resuspended in deionized water. Specimens for thin sectioning were fixed for 30 min in unbuffered potassium permanganate (2%, w/v; 7) and were washed five times in deionized water. The cells were dehydrated in a graded ethyl alcohol series and two changes of acetone. The specimens were embedded in a plastic mixture consisting of 70% dodecenyl succinic anhydride, 20% Araldite 6005, and 10% Epon 812 (Shell Chemical Co.) with one drop of DMP-30 [2,4,6,Tri(dimethylaminomethyl)phenol (Rohm & Haas Co., Philadelphia, Pa.)] per ml of plastic and were polymerized at 60 C. Sections were cut on a Sorvall Porter Blum MT-2 microtome with a diamond knife. All sections were stained with Reynold's lead citrate for 1 to 2 min (10). Specimens were viewed with a Hitachi HS-7S electron microscope. Micrographs were taken on Kodak, contrast grade, projector slides.

RESULTS AND DISCUSSION

N. agilis was grown under strictly autotrophic conditions in a mineral medium with nitrite as the energy source and CO_2 as the sole carbon source. The ultrastructure of organisms from autotrophic cultures in the exponential phase of growth (Fig. 1) was essentially the same as that described by Murray and Watson (8). This organism was characteristically pear-shaped and

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possessed a unique lamellar array of membranes located peripherally at the swollen end of the cell. Each cell had a granular cytoplasm and several electron dense bodies. Amorphous inclusions of moderate density were also observed in sections of *N. agilis* by Murray and Watson (8). Some of the structures shown in Fig. 1 appear to be hexagonal in shape and in this respect resemble the "polyhedral bodies" seen in ultrathin sections of blue-green algae (6) and in other chemoautotrophs (3, 5, 15; J. B. Waterbury, C. C. Remsen, and S. W. Watson, Bacteriol. Proc., p. 45, 1968, and *personal communication*).

N. agilis was also grown in a nitrite mineral medium containing sodium acetate. Cells from cultures in the logarithmic phase of growth were very similar to autotrophically grown organisms in morphology and ultrastructure (Fig. 1 to 4). The pear-shaped cells contained the characteristic peripheral lamellar membranes and electron dense bodies, but also contained a few electron transparent inclusions. During the exponential phase of growth in nitrite mineral medium supplemented with acetate, *N. agilis* readily assimilated organic carbon into most of the major constituents of the cell including PHB (11). It is therefore likely that the electron transparent areas seen in thin sections of cells grown under these conditions are deposits of PHB. Gross changes in morphology and fine structure took place when cultures were incubated for prolonged periods in the medium containing substrate concentrations of acetate and low concentrations (60 mM) of nitrite. These changes were evident after the nitrite had been completely oxidized. Most of the cells were then irregular in shape and larger than cells grown under strictly autotrophic conditions (Fig. 5). The cells contained many discrete deposits of electron-transparent material; these deposits occupied much of the cell and largely obscured the ultrastructural features that characterize this organism. Nevertheless, peripheral lamellar intrusions of the plasma membrane were apparent in some sections (Fig. 3). Cells in the exponential phase of growth (Fig. 2) contained a relatively small amount of reserve material when compared with those which had been incubated for several days in nitrite-depleted media (Fig. 3); the latter were packed with reserve material. These findings are consistent with the protein to dry weight ratios of organisms grown under similar conditions (*see* reference 11, Table 3). Thus, an organic compound (such as acetate) in the growth medium has a readily detectable effect on the ultrastructure of *N. agilis*.

N. agilis was grown through many generations in a nitrite-free organic medium as described by Smith and Hoare (11), before samples were removed for examination in the electron microscope. The cells in such cultures were pear-shaped, and some sections revealed a lamellar array of membranes disposed towards the periphery of the cell (Fig. 4). Electron-dense bodies and several deposits of electron-transparent reserve material, presumed to be PHB, were also evident. Thus, the cells from heterotrophic cultures were very similar in morphology and ultrastructure to *N. agilis* grown in a nitrite mineral medium supplemented with acetate. However, as a result of surveying large numbers of sections of heterotrophically grown cells, it was evident that the lamellar membrane system was less prominent than in sections of autotrophic cells. In this and other respects, the physiological properties and the ultrastructural features of *N. agilis* grown under autotrophic and heterotrophic conditions merit comparison. It has been established that *N. agilis* grown heterotrophically has physiological properties similar to those displayed by autotrophic cultures (11). Heterotrophic cultures retain the ability to oxidize nitrite and grow autotrophically even after many cell divisions in an organic medium in the absence of nitrite. However, cell suspensions of heterotrophically grown cultures oxidize nitrite at a slower rate (11). Nitrite oxidation is associated with the particulate fraction of cell-free extracts (1), which contain fragments of the cytoplasmic membranes. The lower nitrite oxidizing activity of heterotrophic cultures may therefore be attributed to the smaller amounts of lamellar membranes in such cultures. The results of our investigations of the ultrastructure of *N. agilis* grown under different conditions support earlier physiological findings that the strain of *N. agilis* used in these investigations is a facultative chemoautotroph.

The characteristic pear shape of *N. agilis* (Fig. 1 to 4) suggests that this organism may reproduce by asymmetric fission or "budding." The precise nature of the normal mode of division of *N. agilis* is uncertain, although there are several reports that this organism reproduces by budding (12, 17, 18, 19). Zavarzin and co-workers propose that *N. winogradskyii* reproduces by the formation of a bud on the narrow end of the characteristically pear-shaped cell. The similarities in cell shape and gross ultrastructure of *N. agilis* and the purple nonsulfur photosynthetic bacterium *Rhodopseudomonas palustris* (9, 14) are noteworthy, particularly since a study of the growth of individual cells of *R. palustris* has shown that this organism reproduces by budding (17). A similar approach might resolve the problem of whether *N. agilis* normally reproduces by budding.

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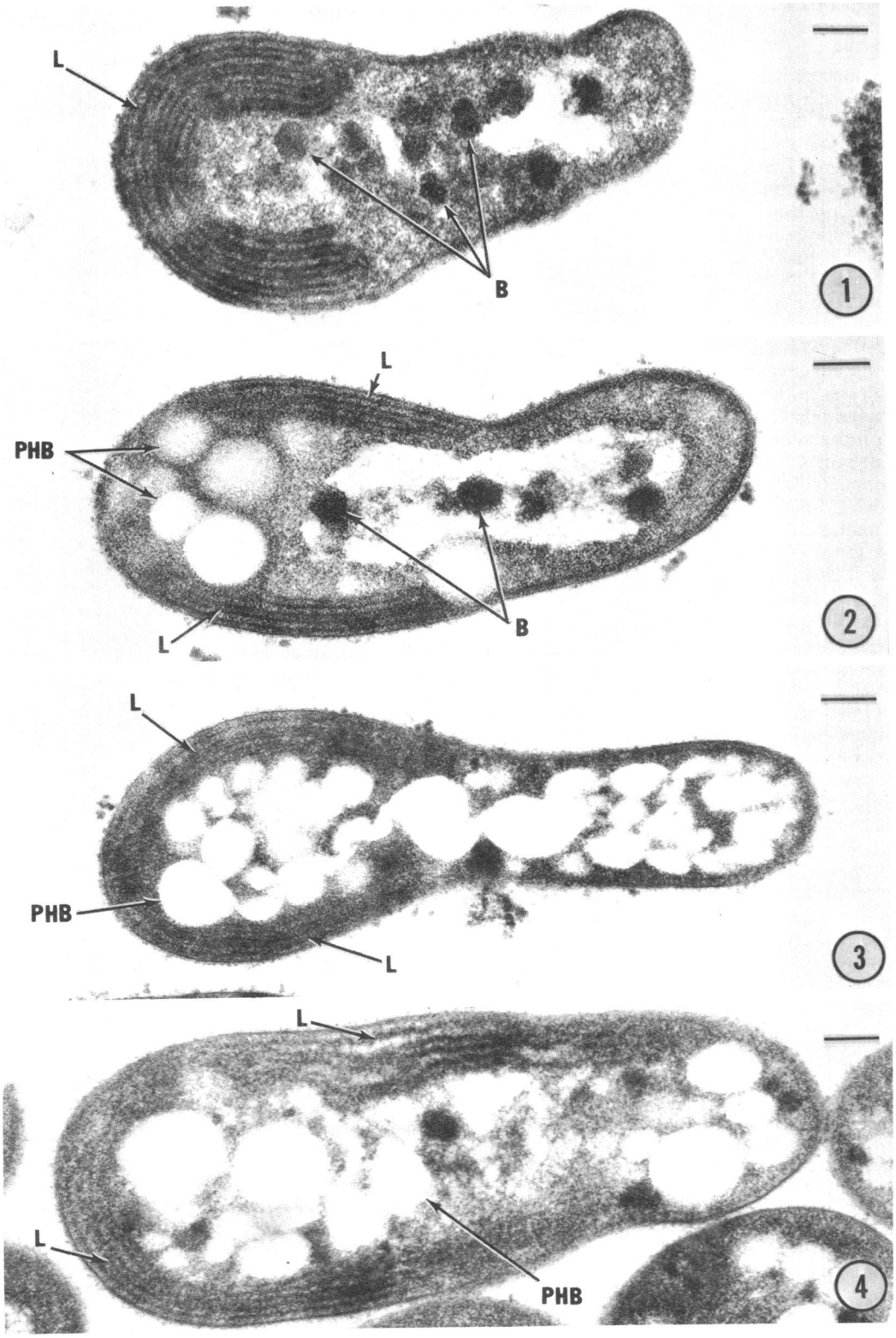

FIGS. 1–4

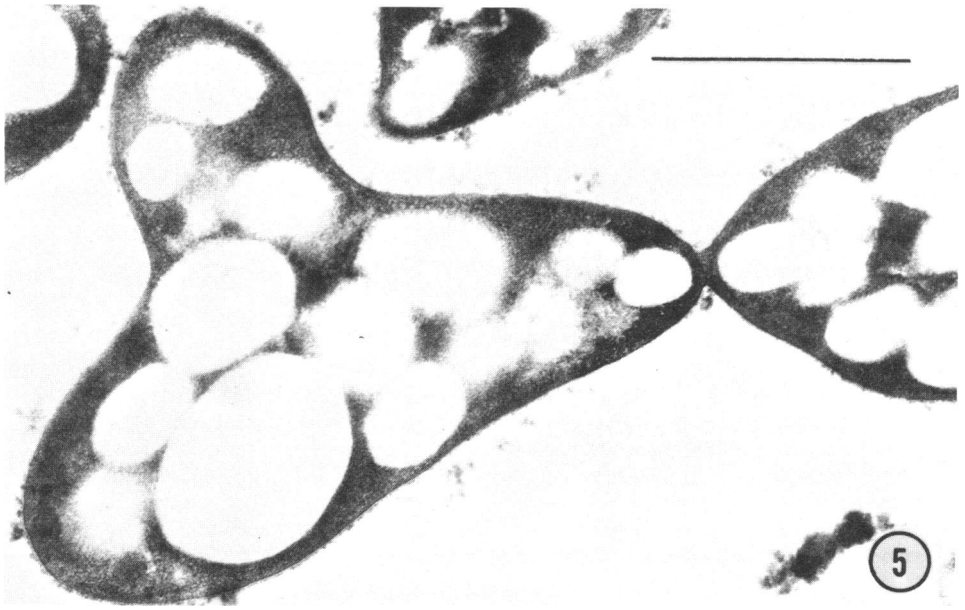

FIG. 5. *Section of N. agilis grown on nitrite mineral salts medium supplemented with 5 mM sodium acetate harvested after all added nitrite had been oxidized. Section shows cells distorted and stuffed with reserve material.*

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FIG. 1. *Section of N. agilis, grown chemoautotrophically and harvested during the exponential phase of growth, showing lamellar membrane system (L) at the swollen end of the cell and electron dense polyhedral bodies (B).*

FIG. 2. *Section of N. agilis grown on nitrite mineral salts medium supplemented with 5 mM sodium acetate, harvested during the exponential phase of growth. Section shows lamellae (L), polyhedral bodies (B), and electron-transparent bodies believed to be poly-β-hydroxybutyrate (PHB) reserve material.*

FIG. 3. *Section of N. agilis grown on nitrite mineral salts medium supplemented with 5 mM sodium acetate, harvested during the "postexponential" phase of growth when all added nitrite had been oxidized. Section still shows lamellae (L), but cell is packed with electron-transparent reserve material (PHB).*

FIG. 4. *Section of N. agilis grown heterotrophically in the absence of nitrite. Section shows lamellae (L), electron transparent reserve material (PHB), and electron dense bodies (B).*