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Micromorphology of Gram-Negative Hydrogen Bacteria

II. Cell Envelope, Membranes, and Cytoplasmic Inclusions

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Abstract. The fine structure of the cell envelope, of membrane systems and of cytoplasmic inclusions of Gram-negative aerobic hydrogen bacteria has been studied. The results have been tabulated, and three main groups could be recognized: Group 1: *Alcaligenes eutrophus*, *A. paradoxus*, *A. ruhlandii*, *Pseudomonas facilis*, *P. flava*, *P. pseudoflava*, *P. palleronii*, and *Aquaspirillum autotrophicum*; Group 2: "*Corynebacterium*" *autotrophicum* and strains MA 2 and SA 35; Group 3: *Paracoccus denitrificans*. Special structures related to the chemoautotrophic way of life of the hydrogen bacteria were not observed.

Key words: Ultrastructure – Micromorphology – Gram-negative – Hydrogen bacteria – Cell envelope – Cytoplasmic inclusions – Membranes – Mesosomes – Glycogen – Poly-β-hydroxybutyrate – Cell wall types.

Although the cytology of hydrogen bacteria has not been systematically studied, some investigations on the cell wall fine structure of *Corynebacterium autotrophicum* strains 12/60/X (Eberhardt, 1971) and GZ 29 (Berndt et al., 1976) as well as of the unidentified Gram-positive coryneform strain 11/X (Canevascini and Eberhardt, 1975) served to describe these recently isolated hydrogen bacteria of uncertain taxonomic position. Veltri and McLear (1972) studied the cell envelope of *Alcaligenes eutrophus* by freeze-etching technique.

As stated in the preceding paper (Aragno et al., 1977) the aerobic hydrogen bacteria do not represent a taxonomic unit. Important differences in cell morphology and flagellation were observed. Moreover, within the motile species, important differences regarding the flagellar fine structure and the occurrence of pili were noted. Whether the fine structure of the cell envelope as well as the occurrence of membrane systems and of cytoplasmic inclusions will corroborate this taxonomic diversity is one of the aims of the present study.

The ability of growing autotrophically was related with special structures, as carboxysomes or elaborate membrane systems, in some thiobacilli and nitrifying bacteria (Shively et al., 1973; Purohit et al., 1976; Murray and Watson, 1965). Whether some structures may be correlated in hydrogen bacteria, with the ability of growing autotrophically will also be studied here.

Abbreviations. CM = cytoplasmic membrane; OM = outer membrane

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MATERIALS AND METHODS

Strains and Culture. The strains studied and their culture conditions are identical to those described in the previous paper (Aragno et al., 1977).

Electron Microscopy. For fixation of cells two different procedures were applied: 1. Kellenberger's method (Kellenberger et al., 1958) (Figs. 5 and 6 only); 2. double fixation with glutaraldehyde and osmium tetroxide according to Behn and Arnold (1974). After dehydration in a graded series of acetone, fixed cells were embedded either in a combined Epon-Araldite resin mixture (Behn and Arnold, 1974) or in Spurr's low viscosity medium (Spurr, 1969).

Ultrathin sectioning was performed with a LKB-Ultratome III with glass knives. Sections were collected on carbon coated formvar-copper-grids and were poststained with lead citrate (Reynolds, 1963). A block-staining with 2% uranyl acetate was included during the dehydration procedure. Electron micrographs were taken with a Philips EM 301 electron microscope. Magnifications were calibrated using a cross-lined grating replica.

Chemicals. All resin compounds and sodium barbiturate came from Serva (Heidelberg, W.-Germany), uranyl acetate from Fluka (Buchs, Switzerland). All others were purchased from Merck (Darmstadt, W.-Germany).

RESULTS

Cell Walls

The cell walls of the hydrogen bacteria studied according to their appearance in ultrathin sections were classified into three different Gram-negative types:

The first type (Fig. 1) is represented by a multilayered cell wall as described, being characteristic for Gram-negative bacteria (Costerton et al., 1974; Glauert and Thornley, 1969). Its main features were an outer membrane (OM), having almost the same dimensions and appearance as the cytoplasmic membrane (CM), and between both layers, OM and CM, a so-called dense layer. This type of arrangement was recognized in *Alcaligenes eutrophus* (Figs. 2 and 11), *A. paradoxus*, *A. ruhlandii*, *Pseudomonas facilis*, *P. flava*, *P. pallero-nii*, *P. pseudoflava*, and *Aquaspirillum autotrophicum*.

The second type (Fig. 1), concerns those cell walls, which did not show a dense layer between CM and OM. In contrast to the first type, the OM was much broader than the CM. In *C. autotrophicum* 14g, for example, the OM was 13 nm broad, the CM 7.5 nm (Fig. 12). In some strains, the innermost electron-opaque layer of the OM exhibited much more contrast than the outer one (Figs. 13 and 14). The periplasmic space varied in thickness due to different grades of plasmolysis. In some cases, bridge-like connections between the CM and the OM were visible (Figs. 13 and 14). This type was found in "*Coryne-bacterium*" *autotrophicum* (Fig. 12), in the strains MA 2 (Fig. 13) and SA 35, and in *Paracoccus denitrificans*, strain DSM 65 (Fig. 14). Almost all of these bacteria proved to be very sensitive to plasmolysis during fixation. Satisfactory results were obtained only by decreasing the buffer concentration to 0.01 M. In the case of *P. denitrificans* strain DSM 65, addition of 3 mM $CaCl_2$ was necessary.

The third type of cell wall (Fig. 1) resembles the second: No distinct dense layer was observed, and the OM was much broader compared with the CM. The latter was scarcely visible in specimens subjected to the fixation procedures described. The third differs from the second type mainly by a stronger contrast of the outermost layer of the OM and by an additional rough layer on its outside (Fig. 15), eventually a slime capsule. This type was found in *P. denitrificans* strain "Morris".

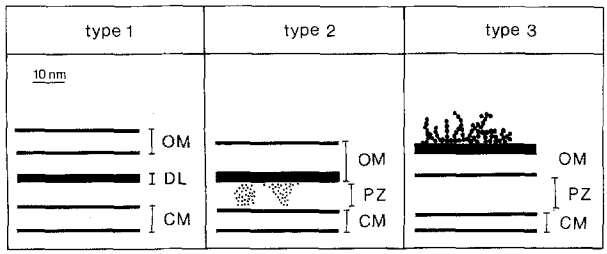


Fig. 1. Schematic diagrams of the three types of cell walls found in Gram-negative hydrogen bacteria. *OM* outer membrane; *DL* dense layer; *CM* cytoplasmic membrane; *PZ* periplasmic zone

Intracytoplasmic Membranes

Two completely different types of membrane systems were observed in the bacteria investigated. The first type was a mesosome-like structure with a spiral appearance, often located in the area of cell division or at the cell poles (Figs. 3–6). In cells fixed by Kellenberger's method they could be seen more frequently and distinctly (Figs. 5 and 6) than in cells fixed with glutaraldehyde and osmiumtetroxide (Figs. 3 and 4). These mesosome-like membranes were found in *Alcaligenes eutrophus*, *A. paradoxus*, *A. ruhlandii*, *Pseudomonas facilis*, *P. flava*, *P. palleronii*, *P. pseudo-flava*, and *Aquaspirillum autotrophicum*.

The second type of intracytoplasmic membranes consisted of more or less extensive membrane systems sometimes in regular parallel layers in the cortical region of the cell (Figs. 16 and 17). This multilayer membrane system was visible in *C. autotrophicum* and in strains MA 2 and SA 35.

Symbols used in Figures 2–18: *CM* cytoplasmic membrane; *DB* "dense bodies"; *DL* dense layer; *G Escherichia coli*-like glycogen inclusions; *N* nuclear region; *OM* outer membrane; *PHB* poly-β-hydroxybutyric acid; *PP* polyphosphate; *PZ* periplasmic zone; *R* ribosomes. Dimensions are given in μm

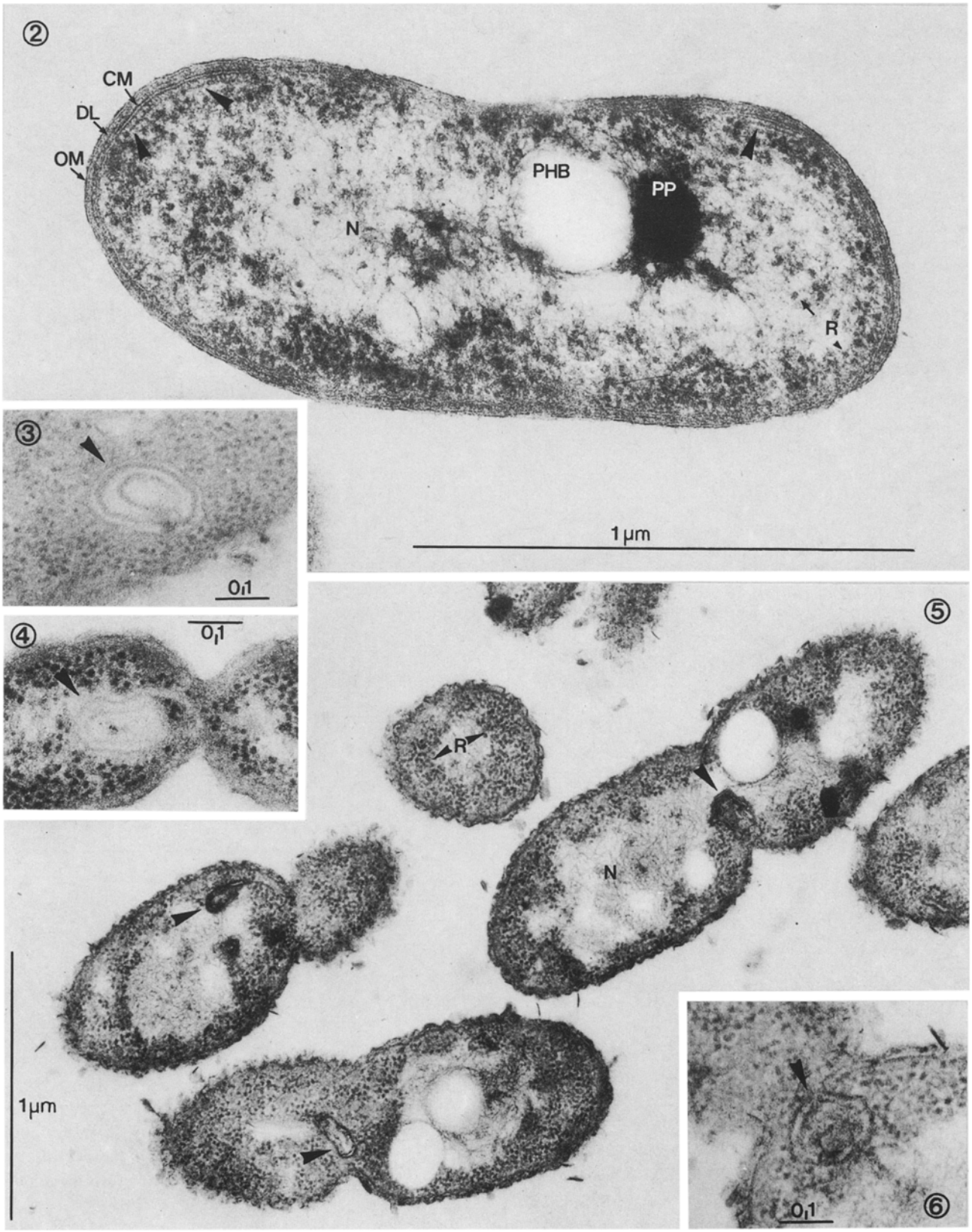
Fig. 2. Longitudinal section of *Alcaligenes eutrophus* H 16 (ATCC 17699). "Polar membranes" are visible (*arrowheads*)

Fig. 3. Mesosomal structure in *A. eutrophus*, type strain ATCC 17697 (glutaraldehyde-osmium tetroxide double fixation)

Fig. 4. Typical appearance of mesosomal structure with glutaraldehyde-osmium tetroxide double fixation (*Aquaspirillum autotrophicum*)

Fig. 5. *A. eutrophus* H 16 fixed according to Kellenberger's method. Arrowheads indicate mesosomal structures. Note the appearance and central location of the nucleoids!

Fig. 6. Typical mesosomes in the area of cell division. Same fixation as in Figure 5



Figs. 2–6

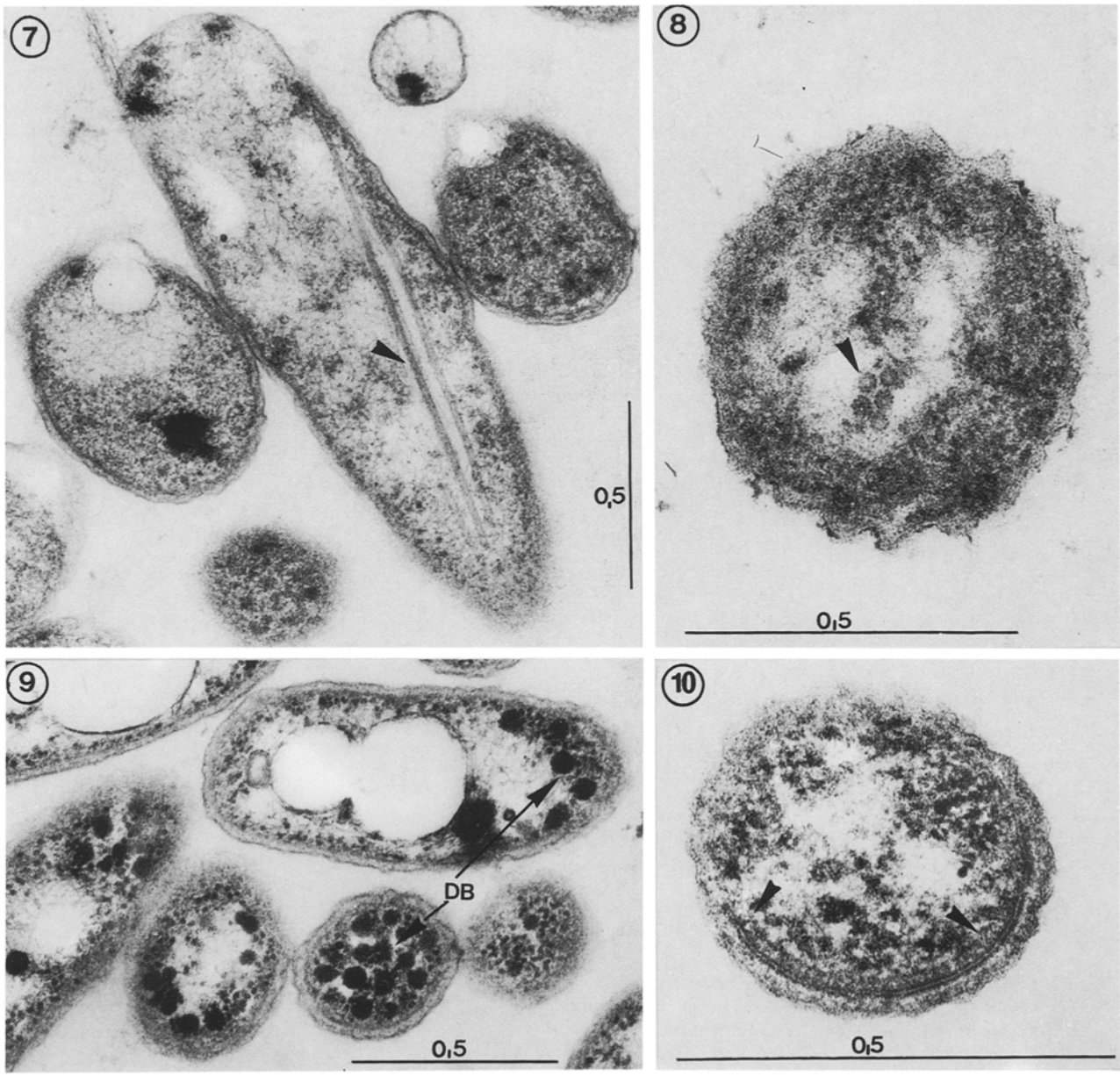


Fig. 7. Cell of *A. eutrophus* H 16 with polysheath-like structures (*arrowheads*)

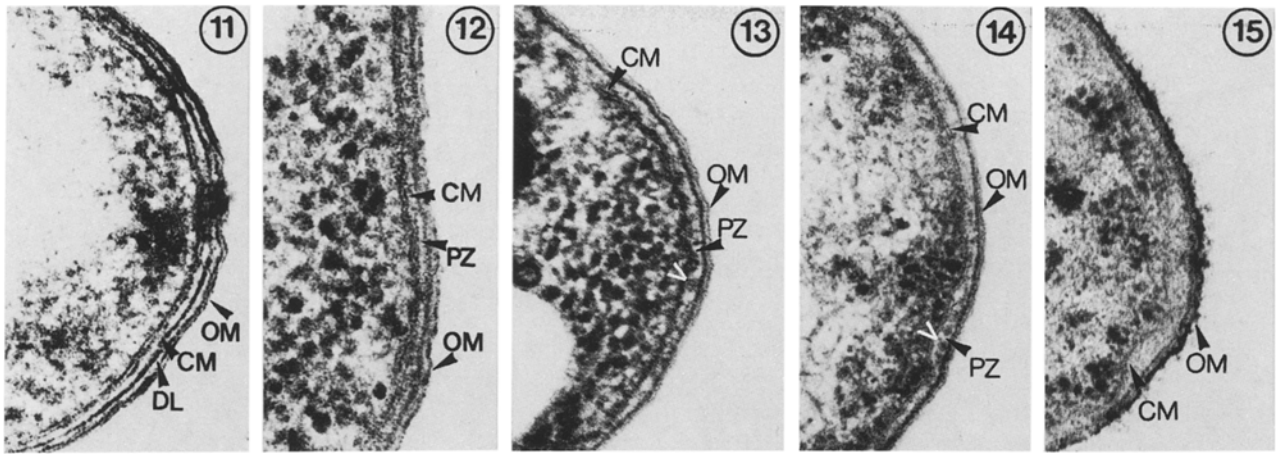
Fig. 8. Cross-sectioned bundle of five polysheaths in *A. eutrophus* H 16

Fig. 9. Section of *Pseudomonas pseudoflava* SA 27 showing "dense bodies"

Fig. 10. Section of *Aquaspirillum autotrophicum*. *Arrowheads* indicate the "polar membrane"

Cytoplasmic Inclusions

In ultrathin sections of all strains investigated here, sharply confined "vacuoles" were observed which are typical for storage of poly-β-hydroxybutyrate (Figs. 2, 5, and 9) (Kran et al., 1963; Cohen-Bazire and Kunisawa, 1963). Other typical inclusions were polyphosphates; they were found in all strains investigated and occurred in two different morphological forms. The first form appeared as an electron opaque circular inclusion, with a relatively large diameter (about 200 nm) which resembled a structure found in *Rhodospirillum rubrum* (Cohen-Bazire and Kunisawa, 1963) (Figs. 2 and 17). The number of these structures per



Figs. 11–15. Micrographs of the three different cell wall types with identical magnifications (×114000)

Fig. 11. Cell wall type 1, represented by *A. eutrophus* H 16

Figs. 12–14. Cell wall type 2, represented by "*Corynebacterium*" *autotrophicum* 14g (Fig. 12) strain MA 2 (Fig. 13), and *Paracoccus denitrificans* DSM 65 (Fig. 14). *White arrowheads* indicate bridge-like connections between *CM* and *OM* (in Figs. 13 and 14)

Fig. 15. Cell wall type 3, represented by *Paracoccus denitrificans* strain "Morris". Note the rough appearance of the layer outside the *OM*

cell – 1 to 2, seldom 3 – was rather constant and independent of the growth phase. The second form of polyphosphate inclusion appeared as small circular structures with an extremely dense periphery and a varying diameter (20–100 nm) (Fig. 16). They were abundant in *Alcaligenes eutrophus*, when the cells had been grown under conditions of polyphosphate accumulation (Schlegel and Kaltwasser, 1961) and absent in phosphate depleted cells. Their number per cell varied significantly with the growth phase. Characteristically their translucent centres turned dark on poststaining with uranyl acetate. They were not found in cells fixed by Kellenberger's method. All bacteria investigated contained both types of polyphosphate; *C. autotrophicum*, the strains MA 2 and SA 35, and *P. pseudoflava* were extraordinarily rich in the second type.

Ultrathin sections of some strains showed small circular translucent structures with a diffuse perphery (Figs. 16–18). They are supposed to be glycogen-like storage material as described for *Escherichia coli* (Cedergren and Holme, 1959; Cheng et al., 1973; Dipersio and Deal, 1974). These inclusions were found in *C. autotrophicum*, MA 2 and SA 35, *Pseudomonas facilis*, *Alcaligenes paradoxus* strain SA 29, *Pseudomonas palleronii* and *Paracoccus denitrificans* strain Morris.

In *Pseudomonas flava* and *P. pseudoflava*, especially in cells taken from the stationary growth phase, electron dense bodies were found (diameter 16–37 nm) (Fig. 9) resembling those of *Chondromyces crocatus* (MacRae and McCurdy, 1975). As it has been recently demonstrated; *P. pseudoflava* strain GA 3 stores lots of glycogen similar to that from *Arthrobacter* (Auling, personal communication); these dense bodies are thus likely to consist of glycogen. In "*Corynebacterium*" *autotrophicum* 14g, electron dense "worm-like" inclusions were visible (Fig. 17); their chemical nature has not yet been clarified.

Other Features

In *Alcaligenes eutrophus* H 16 as well as in *Aquaspirillum autotrophicum* structures were visible underneath the CM (Figs. 2 and 10) which resemble the "polar membranes" or "polar plates" (Murray and Birch-Andersen, 1962; Cohen-Bazire and London, 1967; Remsen et al., 1968). These structures are likely to be organelles of flagellar attachment. In the peritrichously flagellated *Alcaligenes eutrophus* H 16, these "polar membranes" were located at different places of the cell wall; in *Aquaspirillum autotrophicum* with bipolar tufts of flagella, they were located only at the cell poles.

In sections of *Alcaligenes eutrophus*, long tube-like structures were detected (Fig. 7). They had a diameter of 24 nm, a variable length (up to the length of the cell, i.e. about 2 μm), and they were arranged as bundles of 5–6 (maximal 24) tubes each. In cross sections (Fig. 8) a central hole with a diameter of 7 nm was recognized. Their appearance and dimensions are

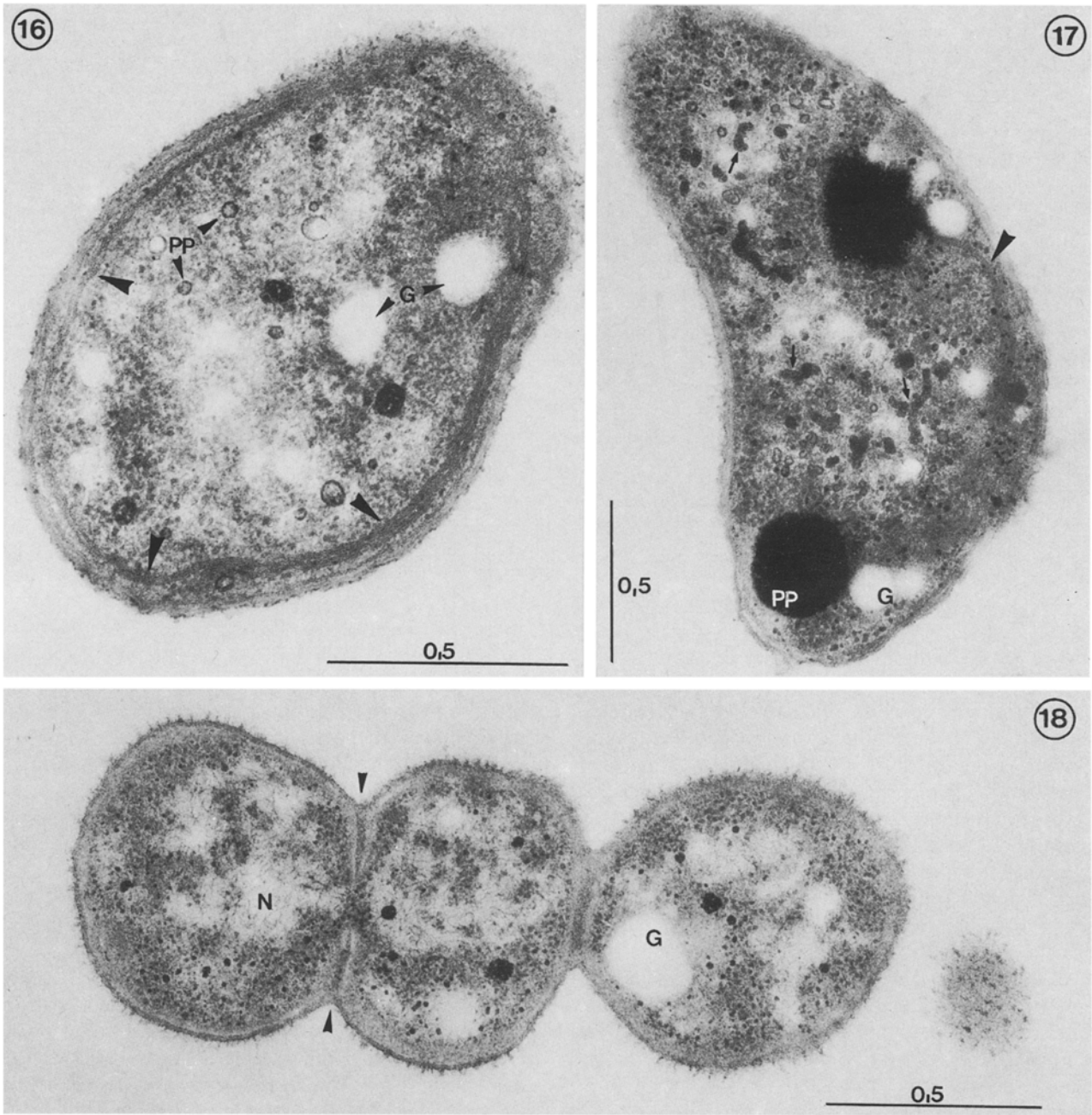


Fig. 16. "*Corynebacterium*" *autotrophicum* 14g with membrane layers parallel to the cell wall (*arrowheads*)

Fig. 17. Cell of *C. autotrophicum* 14g rich in polyphosphate inclusions of both types and of "worm-like" structures (*small arrows*). The *arrowhead* marks intracytoplasmic membranes

Fig. 18. Section of a short chain of *Paracoccus denitrificans* strain "Morris". *Arrowheads* indicate a site where cell division is nearly finished. Note the rough appearance of the cell wall!

indicative for polysheaths which are considered to be defective bacteriophages (Lotz, 1976).

In none of the strains studied, grown either autotrophically or heterotrophically, structures resembling carboxysomes were observed, although most of these bacteria have been proved to fix CO_2 through a Calvin cycle and thus to possess the enzyme ribulose-bisphosphate carboxylase (Schlegel, 1975).

DISCUSSION

When a study aims at comparing ultrastructural details, the conditions of growth, the methods of electron microscopic fixation and staining procedures should be as similar as possible for all bacterial strains. Variations in one of these parameters might influence the appearance of identical structures in different strains or change the appearance of non-identical structures in a way that they cannot be discriminated. Our investigations showed a considerable ultrastructural diversity among the Gram-negative hydrogen bacteria. No basic structural feature could be related to hydrogen lithoautotrophy.

Cell Wall

Whereas an outer membrane seems to be common for all Gram-negative bacteria (Costerton et al., 1974) including hydrogen bacteria, a great variability of the additional components could be observed. The main difference seems to be the visibility and the location of the peptidoglycane layer. For the majority of Gram-negative bacteria a clearly visible peptidoglycane layer – called "dense layer" – like that represented by the first wall-type, seems to be typical (Murray et al., 1965; Glauert and Thornley, 1969; Costerton et al., 1974). The second cell wall type, especially that found in strain MA 2, resembles that of *Escherichia coli* (Murray et al., 1965): No separate dense layer was to be seen, but the innermost layer of the OM showed more contrast than the outer one. In *Escherichia coli*, this was explained by the close neighbourhood of the peptidoglycane layer to the OM.

The cell wall of *Paracoccus denitrificans* DSM 65 appeared slightly different from that of *Micrococcus denitrificans* CCM 982 (Kocur et al., 1968). This may be caused by the different fixation procedure employed, whereas the cell wall of *Paracoccus denitrificans* "Morris" differed considerably.

Intracytoplasmic Membranes

Mesosomal structures as in *Alcaligenes eutrophus* H16, are less complex and much smaller than those described for Gram-positive bacteria (Reusch and Burger, 1973; Greenawalt and Whiteside, 1975); however, they agree well with criteria stated for mesosomes of Gram-negative bacteria (Greenawalt and Whiteside, 1975). The observation of their increased occurrence in preparations fixed according to Kellenberger's method is in accordance with recent results about mesosomes (Ghosh and Nanninga, 1976; Silva et al., 1976).

Special membrane structures were only observed in the strains of "*Corynebacterium*" *autotrophicum* and in MA 2 and SA 35, both in heterotrophically and autotrophically grown cells. Perhaps these membranes might be involved in nitrogen fixation occurring both in *C. autotrophicum* (Gogotov and Schlegel, 1974; Berndt et al., 1976) and in the strains MA 2 and SA 35 (Aragno, unpublished).

Cytoplasmic Inclusions

The different appearance of glycogen-like inclusions under the same fixation and staining conditions is striking: Either dark – as in *Pseudomonas pseudoflava* (Fig. 9) – or light – as in *C. autotrophicum* (Figs. 16 and 17). This phenomenon is obvious, too, comparing results of several authors. Robertson et al. (1975) investigated the influence of fixation and staining on the appearance of glycogen in *Nocardia corallina*. Dipersio and Deal (1974) and Cheng et al. (1973), however, showed light glycogen inclusions where they should be dark according to Robertson et al. (1975). Up to now, among the hydrogen bacteria only from *P. pseudoflava* strain GA 3 a glycogen-like polysaccharide has been isolated.

Similar structures as those assumed to be another type of polyphosphate were described for *Mycobacterium phlei* (Drews, 1960) and the mold *Rozella allomycis* (Wool and Held, 1976).

Grouping of Strains

On the basis of the morphological studies presented in the preceding paper (Aragno et al., 1977) and of the cytological investigations treated in the present paper several groups of Gram-negative hydrogen bacteria can be differentiated. The main characteristics of each species are summarized in Table 1.

Among the cytological features studied here, only the presence of poly-β-hydroxybutyrate and both types of polyphosphate inclusions were common to all Gram-negative hydrogen bacteria. With respect to the other features, three main groups can be recognized:

1. *Alcaligenes-Pseudomonas-Aquaspirillum* group: The structural features common to the strains of this group are: Cell wall of the first type (Fig. 1), the presence of mesosomes. This grouping is strikingly confirmed by the immunological relationships existing between the particulate hydrogenases of these bacteria (Schink, personal communication). The members of this group can be differentiated from each other by their cell shape, flagellation and flagellar fine structure (Aragno et al., 1977). Polar flagella show type I structure, and peritrichous ones either types II or III.

2. "*Corynebacterium*" *autotrophicum* and strains MA 2 and SA 35 group: The structural features common to this group are: Cell wall of the second type,

Table 1. Main morphological characteristics of the Gram-negative hydrogen bacteria

	Shape: rods	Shape: cocci	Shape: helical	Shape: irregular	Motility	Flagella: polar monotrichous	Flagella: bipolar polytrichous	Flagella: peritrichous	Flagellar fine structure: Type I	Flagellar fine structure: Type II	Flagellar fine structure: Type III	Flagellar diameter: < 14 nm	Flagellar diameter: 14–17 nm	Flagellar diameter: > 17 nm	Pili	Cell wall type 1	Cell wall type 2	Cell wall type 3	Mesosomes	Membrane structures of second type	Poly-β-hydroxybutyrate	Polyphosphates I	Polyphosphates II	Glycogen: Transparent inclusions	Glycogen: Opaque inclusions
Alcaligenes eutrophus	+				+			+		+		+				+			+		+	+	+		
Alcaligenes paradoxus	+				+			+		+			+		+[d]	+			+		+	+	+	+	
Alcaligenes ruhlandii	+				+			+			+			+		+			+		+	+	+	+	
Pseudomonas facilis	+				+	+			+					+	+[e]	+			+		+	+	+	+	
Pseudomonas flava	+				+	+			+			+			+[e]	+			+		+	+	+		+
Pseudomonas pseudoflava	+				+	+			?	?		+			+[e]	+			+		+	+	+		+
Pseudomonas palleronii	+				+	+			+				+		+[d]	+			+		+	+	+	+	
Aquaspirillum autotrophicum			+		+		+		+				+			+			+		+	+	+		
Corynebacterium autotrophicum	+[a]	+[a]		+[a]													+			+	+	+	+		
Strains MA 2 and SA 35	+[a]			+[a]	+			+	+				+				+			+	+	+	+		
Paracoccus denitrificans type strain		+[b]															+				+	+	+		
Paracoccus denitrificans "Morris"		+[c]																+			+	+	+	+	

[a] Depending on growth conditions
[b] Occurring in pairs
[c] Occurring in small chains
[d] Polarly located
[e] Peritrichously located

the presence of intracellular membranes, the absence of mesosomes. Other common features are the sensitivity to plasmolysis, the ability to fix nitrogen (Gogotov and Schlegel, 1974; Wiegel and Schlegel, 1976; Aragno, unpublished data), the identity of carotenoid pigments (K. Schmidt, personal communication), and the ability to grow on alcohols, including methanol (R. Opitz, personal communication; Aragno, unpublished data). Strains MA 2 and SA 35 differ from *C. autotrophicum* by the presence of flagella (Aragno et al., 1977), by a much less marked pleomorphism and by a reduced substrate spectrum (Aragno, unpublished data). It is likely that strains MA 2 and SA 35 represent a new species, related to *C. autotrophicum*.

"*Corynebacterium*" *autotrophicum*, the type strain (7C) as well as strain 14g, were originally described as Gram-positive (Tunail and Schlegel, 1974; Schneider et al., 1973; Baumgarten et al., 1974). Our results show that both strains have a Gram-negative-like cell wall, identical to that observed in *C. autotrophicum* GZ 29, which was described as Gram-negative (Berndt et al., 1976). These authors also recognized in the latter strain the citrate synthase as a NADH-sensitive enzyme. Studying several strains of *C. autotrophicum* further features characteristic for Gram-negative bacteria were found, such as the presence of ubiquinone and absence of vitamine K, the absence of corynemycolic acids and the presence of DL-diaminopimelic acid in the cell wall (J. Wiegel, unpublished data). Furthermore, the cell wall of *C. autotrophicum* differs distinctly from that of *Corynebacterium diphtheriae*, whose cell wall structure is typical for Gram-positive bacteria (Barksdale, 1970). Therefore, the original

assignment of "coryneform" hydrogen bacteria has to be revised.

3. *Paracoccus denitrifcans:* All Gram-negative, coccoid, denitrifying hydrogen bacteria are at present grouped in *P. denitrificans*. Our results, however, show some important differences between both strains studied, with respect to the cell wall structure, the occurrence in pairs, respectively in chains, and the nutritional spectrum. Further investigation is needed to establish their taxonomical relationships. The Gram-negative nature of the cell wall, previously described by Kocur et al. (1968), supports the separation of these strains from the genus *Micrococcus* (Davis et al., 1970).

The absence of special structures related to the chemoautotrophic way of life of the hydrogen bacteria may explain the widespread occurrence of this type of metabolism in taxonomically unrelated groups of bacteria.

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