JOURNAL OF BACTERIOLOGY, Mar. 1973, p. 1455-1461
Vol. 113, No. 3
Copyright © 1973 American Society for Microbiology
Printed in U.S.A.

Fine Structural Changes of *Acetobacter suboxydans* During Growth in a Defined Medium

BARRY L. BATZING[1] AND G. W. CLAUS

Department of Microbiology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received for publication 8 December 1972

Cytological differences were observed between stationary- and exponential-phase cells of *Acetobacter suboxydans* grown in a defined medium. Unstained cells observed with the light microscope just after entering the stationary phase differed from exponentially growing cells in that the former exhibited localized increases in density, particularly in the polar regions. Electron microscopy of thin sections revealed that early stationary-phase cells possessed predominantly polar complexes of intracytoplasmic membranes accompanied by polar increases in ribosomal material. When cultures were allowed to continue far into the stationary phase, cells contained extensive aggregations of membrane-like material as the predominant fine-structural feature. In contrast, thin sections of exponentially growing cells exhibited only occasional indications of intracytoplasmic membranes. Intracytoplasmic membranes heretofore have been observed only rarely in the heterotrophic *Pseudomonadales*.

The fine structure of both photoautotrophic (4, 6, 10, 11, 18) and chemoautotrophic (9, 16, 17, 19, 22) members of the order *Pseudomonadales* has been studied extensively, but little information exists concerning the fine structure of the heterotrophic members. Several recent reports show that extensive intracytoplasmic membrane systems are a constant and characteristic feature in some heterotrophic, methane-oxidizing bacteria (7, 20). Studies of *Pseudomonas aeruginosa* (2), *Caulobacter crescentus* (5), and a marine pseudomonad (24) indicate that other heterotrophic *Pseudomonadales* also may possess membranous organelles under certain environmental conditions.

Preliminary evidence for the presence of intracytoplasmic membranes in *Acetobacter suboxydans* was previously noted by Claus and Roth (3). The present investigation was undertaken to determine the extensiveness of these membranes in *A. suboxydans* and to ascertain whether they represent a constant or variable feature of this cell's structure.

MATERIALS AND METHODS

Growth conditions. Standard inocula of *A. suboxydans* ATCC 621 were prepared by incubating cells in 50 ml of a complex glycerol-yeast extract medium (14) at 28 C with shaking until an optical density of 1.0 at 620 nm was obtained (approximately 1.9×10^9 cells per ml). A 10-ml sample of this culture was centrifuged, and the cells were washed four times with 10 ml of 0.9% NaCl in 0.028 M *cis,cis,cis,cis*-1,2,3,4-cyclopentanetetracarboxylic acid buffer (CPTCA) (15), and suspended in 10 ml of this buffered saline. A 0.2-ml amount of this cell suspension was used as the standard inoculum.

Cells used in fine structure studies were grown on a chemically defined medium containing 0.0004% (wt/vol) L-glutamic acid; 0.002% (wt/vol) each of calcium pantothenate, nicotinic acid, *para*-amino-benzoic acid, $FeSO_4$, $MnSO_4$, and K_2HPO_4; 0.02% (wt/vol) each of NaCl, $(NH_4)_2SO_4$, and KH_2PO_4; 5.0% (wt/vol) glycerol; and 0.055 M CPTCA buffer. The medium was adjusted to a pH of 6.0 with KOH and heat sterilized. Growth was initiated with standard inocula, and cells were incubated with shaking (200 strokes per min; 1.5-inch stroke amplitude) at 28 C. Figure 1 represents a typical growth curve and illustrates the periods where the culture was sampled for microscopic observation.

Viability was determined as colony-forming units by spreading the sample on plates containing the glycerol-yeast extract medium (14) and 1.5% agar.

Light microscopy. Cells were immobilized by trapping between a Noble agar-coated microscope slide and a glass coverslip. A Zeiss model WL microscope was adjusted for Köhler illumination,

[1] Present address: Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830.

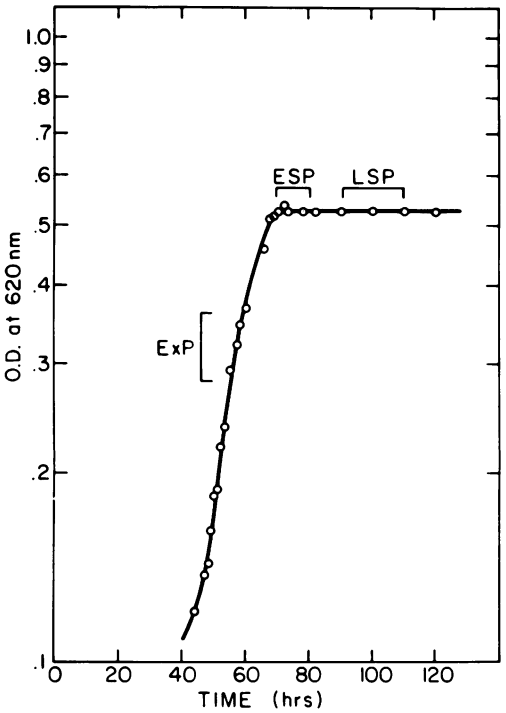


FIG. 1. *Growth of A. suboxydans on the chemically defined medium. Periods during which cell samples were taken for microscopic observation are indicated with brackets, and the cells sampled within these periods are referred to as exponential-phase (ExP), early stationary-phase (ESP), and late stationary-phase (LSP) cells.*

and all photographic records were made by using a 350 to 600 nm band-pass filter, a 1.4 numerical aperture (NA) achromatic condenser with the iris adjusted to a standard setting for all observations, a 40× planapochromatic 1.0 NA oil immersion objective, and a 2.0× Optovar setting. Kodak 35-mm high-contrast copy film was used to record all images, and this was developed in Kodak D-76.

Electron microscopy. Osmium tetroxide was added directly to the culture medium so that its final concentration was 0.1%. The cells were immediately removed by centrifugation and then fixed according to the procedure of Kellenberger et al. (12). Fixed cells were suspended in a small volume of warm 2% agar. This suspension was extruded from a Pasteur pipette into chilled uranyl acetate. After postfixation and dehydration by passage through a graded acetone series, the cells were embedded in vinylcyclohexane dioxide as described by Spurr (21). This medium was found to be superior to the ethyl and *n*-butyl methacrylate mixture previously used to embed *A. suboxydans* (3). Other reagents, such as Epon, Araldite, or combinations of these two, were tried but failed to provide adequate cell infiltration. Thin sections were cut on an LKB Ultrotome and stained with commercial lead citrate (23). Thin sections were examined with either a Hitachi HU-11E or a Philips EM-300 electron microscope with accelerating voltages of 60 to 80 kV.

RESULTS

Light microscopy. Unstained cells from exponentially growing populations appeared essentially homogenous when observed with the bright-field microscope (Fig. 2a). About half of these cells showed a tendency for one pole to be slightly more dense than the remainder of the cell. An occasional cell appeared to contain a single dense polar region.

As these populations entered the maximum stationary phase of growth, all cells exhibited regions of extreme density at both poles as well as other regions of less density (Fig. 2b). This striking change in cellular appearance as exponential growth is terminated prompted us to examine thin sections of cells from both growth phases for possible differences in cell fine structure.

Electron microscopy. Cells in the exponential growth phase appeared to have no extraordinary fine-structural features (Fig. 3). The cell wall had a prominant triple-layered structure typical of gram-negative bacteria, and its trilaminar portion was separated from the plasma membrane by the typically diffuse electron opaque area (3). The plasma membrane was ca. 7.5 nm in diameter and usually could be followed around the cell periphery when oriented perpendicular to the plane of section. Although an occasional fragment of intracytoplasmic membrane was observed in close proximity to the plasma membrane, no extensive elaboration of intracytoplasmic membranes was observed within cells sampled from exponentially growing populations. The chromatin, although slightly aggregated, was basically fibrillar in appearance. Ribosomes appeared to be most predominant along the cell periphery.

Cell viability reached a maximum upon entering the stationary phase, and it dropped no more than 0.1 log during the early stationary-phase period of observation (Fig. 1). Nevertheless, a marked alteration in cell fine structure in all longitudinally sectioned cells was observed as soon as the population entered the stationary growth phase. These sections revealed that virtually all cells contained a polar system of whorled intracytoplasmic membranes (Fig. 4). The poles of these cells also contained a greater concentration of ribosomal material than that observed in exponentially growing cells, and these ribosomal clusters frequently appeared to obscure much of the

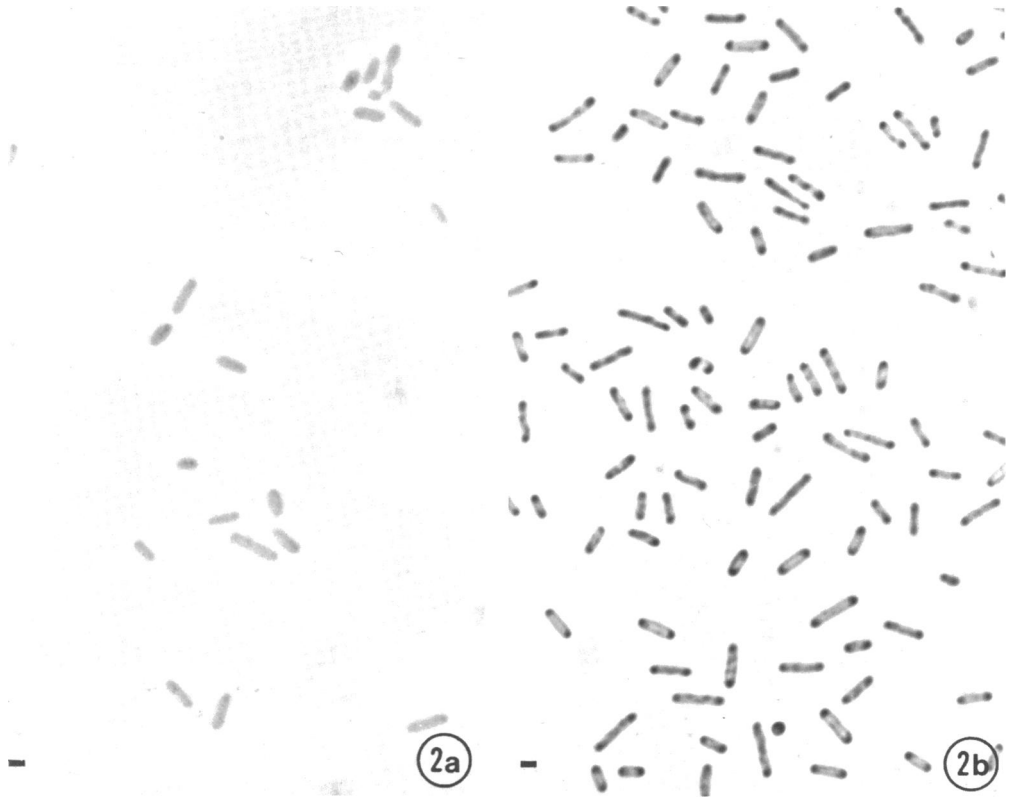


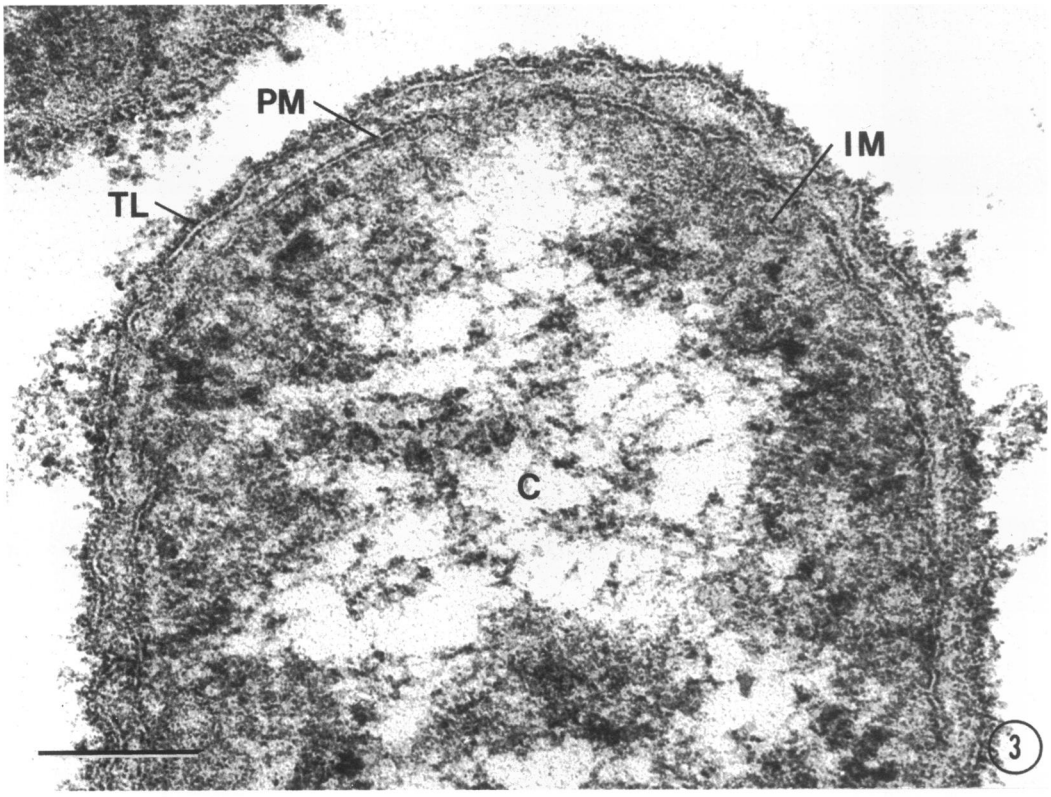
FIG. 2. *Bright-field microscopic appearance of unstained A. suboxydans during growth on the chemically defined medium. All microscopic adjustments and photographic procedures were identical for both observations. Figure 2a is representative of a culture sampled during exponential growth (0.32 optical density). The same culture was sampled 1 h after obtaining its maximum optical density, and its appearance is shown in Fig. 2b. Bar marker represents 1.0 μm.*

intracytoplasmic membrane. The frequency and location of these regions in longitudinal sections appeared to be identical to the pattern observed with the light microscope (Fig. 2b). Transverse sections through polar regions frequently revealed loops of membrane around the cell's periphery.

Cell viability was assayed at the beginning of the late stationary phase (Fig. 1) and found to be only 0.4 log less than at the beginning of the early stationary phase. The characteristic structural feature of cells sampled during this period was complex regions of membrane or membrane-like material found predominantly at the cell poles and with lesser frequency than in other regions of the cell. The internal appearance of these complex regions was markedly different from the whorled membranes observed in early stationary phase, and their appearance varied with the plane of section. When they were next to an obliquely sectioned cell wall, they appeared to lack a definite fine structure and appeared amorphous (Fig. 5). Two or three of these regions were frequently observed within one cell. Occasionally, the amorphous regions were bounded by a trilaminar membrane (Fig. 6). On the other hand, sections containing these complexes adjacent to a more perpendicularly sectioned cell wall demonstrated an apparent array of tightly stacked membrane (Fig. 7).

DISCUSSION

This investigation has shown that both the light microscope appearance and the fine structure of early stationary-phase cells is very different from that of exponential-phase cells. Unstained exponential-phase cells appear relatively undifferentiated when observed with the light microscope, and this lack of differentiation is also observed when sections are examined with the electron microscope. On the



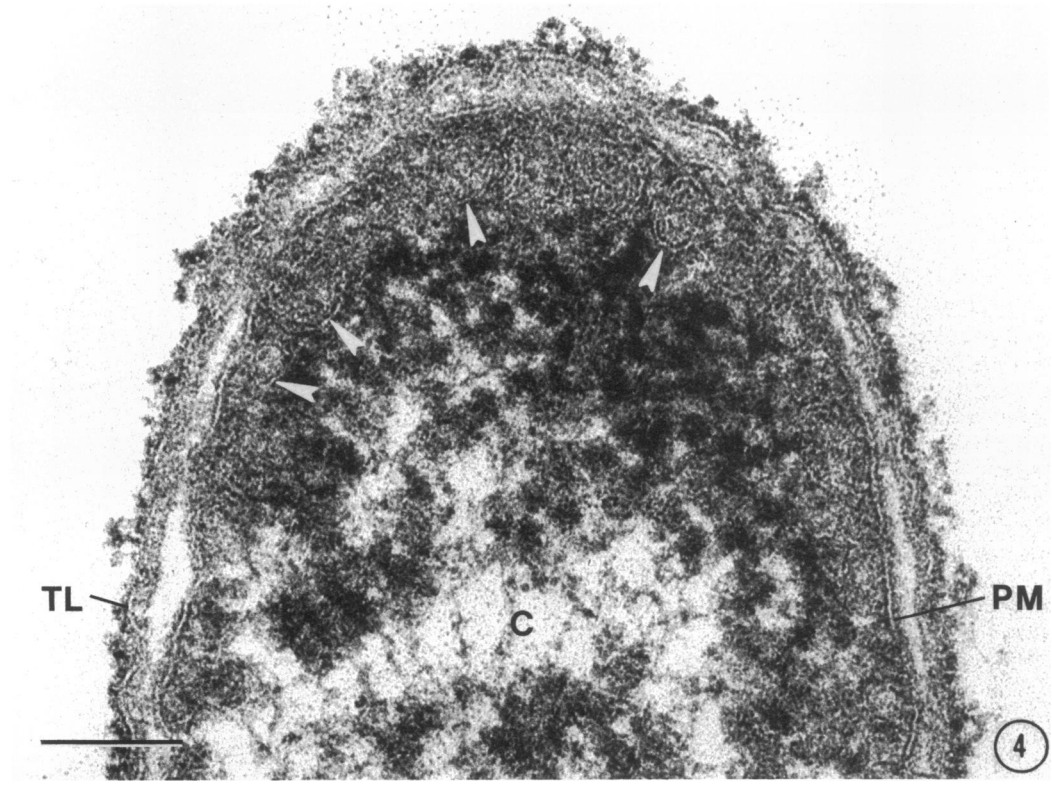
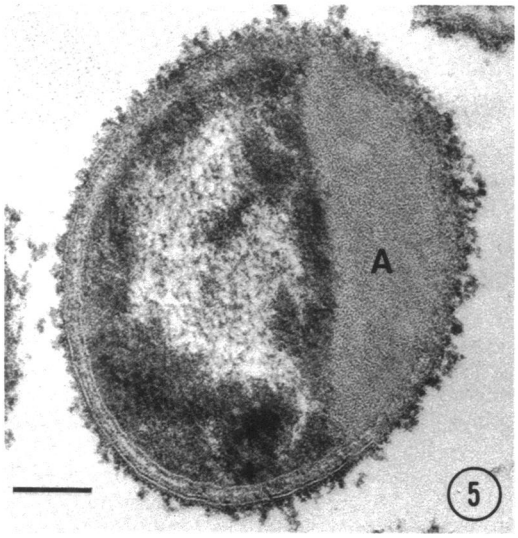
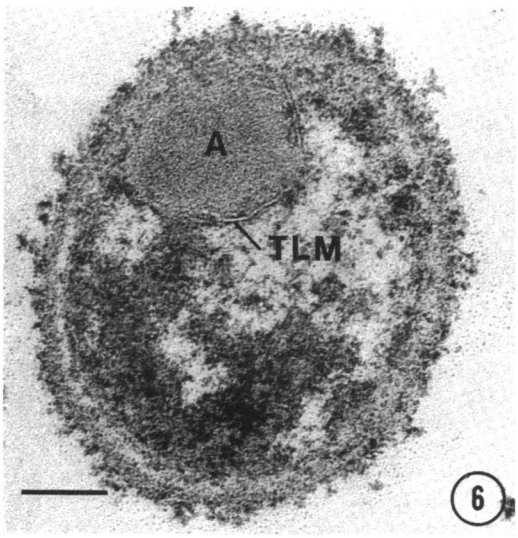


FIG. 3. *Longitudinal section demonstrating the polar region of a representative exponential-phase cell. Fibrillar chromatin material (C) is slightly aggregated. The plasma membrane (PM) is continuously parallel with the trilaminar (TL) portion of the cell wall. Occasionally, small segments of intracytoplasmic membrane (IM) are observed. The bar marker in this and all following figures represents 0.1 μm.*

FIG. 4. *Longitudinal section demonstrating the polar region of a representative early stationary-phase cell. Whorls of intracytoplasmic membranes (arrows) are situated close to the cell periphery. Polar clustering of ribosomal material is also demonstrated.*





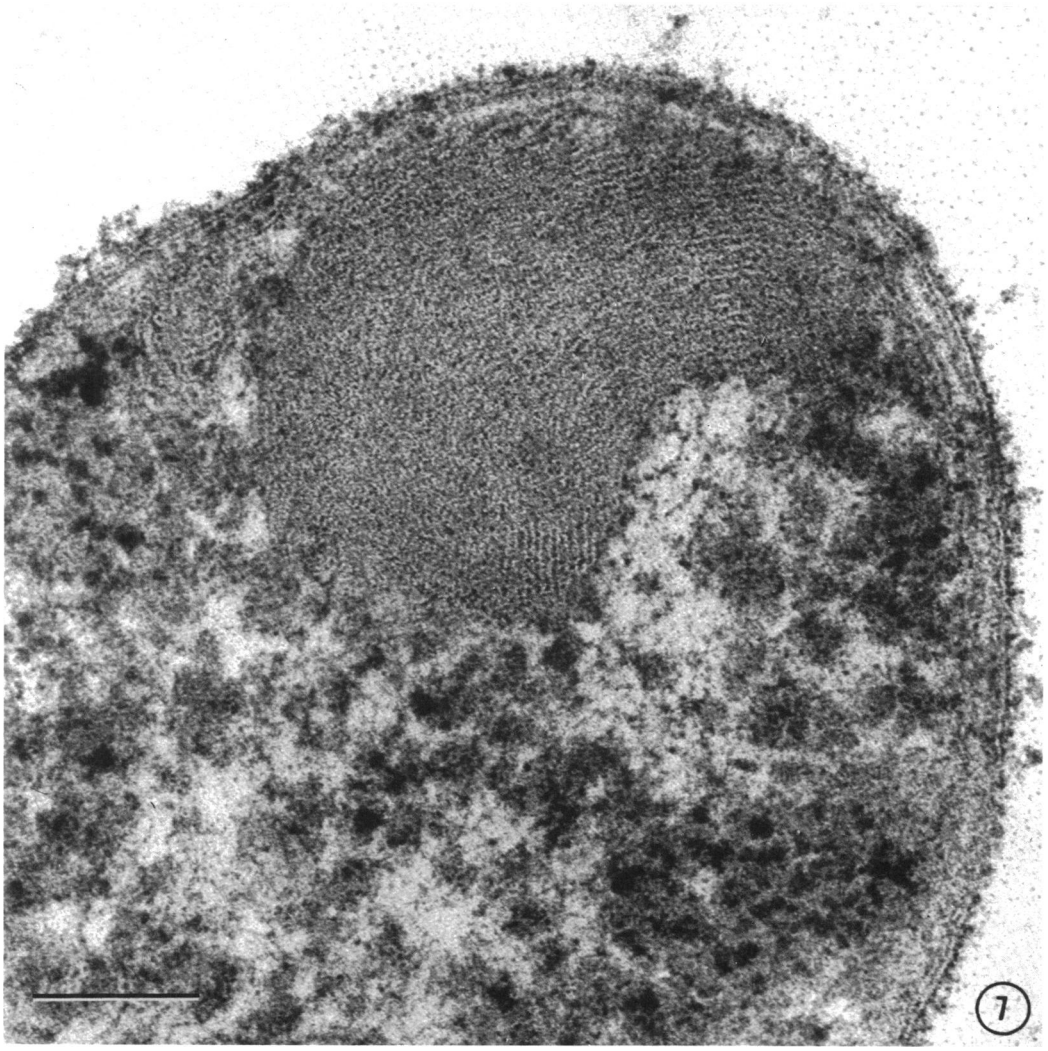


FIG. 5–7. *Thin sections of late stationary-phase cells demonstrating complex regions of membrane or membrane-like material. Usually these appear as amorphous regions (A) as demonstrated in Fig. 5. Occasionally an amorphous region is seen limited by a trilaminar membrane (TLM) as shown in Fig. 6. In sections where these inclusions are adjacent to a perpendicularly sectioned wall, a lamellar type of organization is revealed (Fig. 7).*

other hand, viable early stationary-phase cells show regions of intense density at the poles as well as areas of lesser density throughout the cell when observed with the light microscope. In thin sections of these early stationary-phase cells, localized intracytoplasmic membranes and ribosome accumulations in longitudinal sections closely match both cellular location and observed frequency of the dense regions seen with the light microscope. Others, using phase-contrast instead of bright-field microscopy, have also shown that dark inclusions observed in unstained cells closely match the frequency and arrangement of intracytoplasmic membrane masses in a photosynthetic bacterium (18) and a marine nitrifier (16).

The organization and extent of the intracytoplasmic membrane system in early stationary-phase cells of *A. suboxydans* do not closely resemble those of other systems previously described for heterotrophic or autotrophic species in the *Pseudomonadales*. Intracytoplasmic membranes of some heterotrophic methane-oxidizing bacteria are organized into bundles of vesicular discs (7) which resemble the stacked arrangement observed in *Ectothiorhodospira mobilis* by Remsen et al. (18). Other methane oxidizers have multilayered arrays of intracytoplasmic membrane (1, 7) that are similar to the cytomembranes of some nitrifying bacteria (16, 17, 22) and the thylakoids of certain photosynthetic bacteria (4, 11, 18). The organization of intracytoplasmic membranes in *A. suboxydans* does resemble that of certain nitrifying (9, 22) and photosynthetic bacteria (4) in that they are predominantly present in the polar regions of the cell. However, their loosely coiled or whorled arrangement and the high concentration of ribosomal material associated with these polar regions have not been commonly observed in the bacteria.

The complex regions of membrane-like material noted in a few early stationary-phase and many late stationary-phase cells are interesting from a purely structural viewpoint. Although a lamellar organization is observed in certain sections, these regions generally appear as amorphous masses occasionally contained within a trilaminar membrane. The commonly observed amorphous appearance resembles that noted in the obliquely sectioned stacked membranes of *E. mobilis* (18) and certain methane-oxidizing bacteria (7). It is possible that *A. suboxydans* accomplishes a transformation from convoluted polar membrane to the amorphous or lamellar masses and that the latter may be functionally significant in viable cells. On the other hand, the appearance of these complex regions is accompanied by a decrease in apparent culture viability, and these regions may only be significant in reflecting cellular deterioration.

The most significant result arising from this study is that fully viable cells develop a polar system of intracytoplasmic membranes upon cessation of active population growth. We have preliminary evidence demonstrating that chemical and physiological changes accompany this membrane formation, but, at present, the full physiological significance of this membrane development is unclear. Energy for growth of *A. suboxydans* is reportedly derived from single-step oxidation of polyhydroxy substances (13) catalyzed by particulate dehydrogenases thought to be bound to the plasma membrane (8). This dependance of *A. suboxydans* on membrane-associated enzymes is strikingly similar to the situation existing in many autotrophic bacteria and the methane-oxidizing species of the order *Pseudomonadales*. It is attractive to speculate that the potential to elaborate a system of intracytoplasmic membranes under certain environmental conditions may play a functional role in both autotrophic and heterotrophic members of this order.

ACKNOWLEDGMENTS

We greatfully acknowledge the use of the Hitachi HU-11E electron microscope generously provided by Stuart Patton and the Department of Dairy Science, Pennsylvania State University.

This investigation was supported in part by grant GB-17493 from the National Science Foundation.

LITERATURE CITED

1. Boer, W. E. de, and W. Hazeu. 1972. Observations on the fine structure of a methane-oxidizing bacterium. Antonie van Leeuwenhoek J. Microbiol. Serol. **38**:33-47.
2. Carrick, L., Jr., and R. S. Berk. 1971. Membranous inclusions of *Pseudomonas aeruginosa*. J. Bacteriol. **106**:250-256.
3. Claus, G. W., and L. E. Roth. 1964. Fine structure of the gram-negative bacterium *Acetobacter suboxydans*. J. Cell Biol. **20**:217-233.
4. Cohen-Bazire, G. 1971. The photosynthetic apparatus of procaryotic organisms, p. 65-90. *In* Colloquium, biological ultrastructure: the origin of cell organelles. Oregon State University Press, Corvallis.
5. Cohen-Bazire, G., R. Kunisawa, and J. S. Poindexter. 1966. The internal membranes of *Caulobacter crescentus*. J. Gen. Microbiol. **42**:301-308.
6. Cohen-Bazire, G., and W. R. Sistrom. 1966. The procaryotic photosynthetic apparatus, p. 313-341. *In* L. P. Vernon and G. R. Seely (ed.), The chlorophylls. Academic Press Inc., New York.
7. Davis, S. L., and R. Whittenbury. 1970. Fine structure of methane and other hydrocarbon-utilizing bacteria. J.

Gen. Microbiol. **61**:227-232.
8. DeLey, J. 1960. "Oxidosomes" in bacterial anatomy. Nature (London) **188**:331.
9. Gool, A. P. van, R. Lambert, and H. Laudelout. 1969. The fine structure of frozen etched *Nitrobacter* cells. Arch. Mikrobiol. **69**:281-293.
10. Holt, S. C., and A. G. Marr. 1965. Location of chlorophyll in *Rhodospirillum rubrum*. J. Bacteriol. **89**:1402-1412.
11. Holt, S. C., H. G. Truper, and B. J. Takacs. 1968. Fine structure of *Ectothiorhodospira mobilis* strain 8113 thylakoids: chemical fixation and freeze-etching studies. Arch. Mikrobiol. **62**:111-128.
12. Kellenberger, E., A. Ryter, and K. Sechaud. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Cell Biol. **4**:671-678.
13. King, T. E., and V. H. Cheldelin. 1953. Sources of energy and the dinitrophenol effect in the growth of *Acetobacter suboxydans*. J. Bacteriol. **66**:581-584.
14. Kitos, P. E., C. H. Wang, B. A. Mohler, T. E. King, and V. H. Cheldelin. 1958. Glucose and gluconate dissimilation in *Acetobacter suboxydans*. J. Biol. Chem. **233**:1295-1298.
15. Mallette, M. F. 1967. A *p*H 7 buffer devoid of nitrogen, sulfur, and phosphorus for use in bacteriological systems. J. Bacteriol. **94**:283-290.
16. Murray, R. G. E., and S. W. Watson. 1965. Structure of *Nitrocystis oceanus* and comparison with *Nitrosomonas* and *Nitrobacter*. J. Bacteriol. **89**:1594-1609.
17. Remsen, C. C., F. W. Valois, and S. W. Watson. 1967. Fine structure of the cytomembranes of *Nitrocystis oceanus*. J. Bacteriol. **94**:422-433.
18. Remsen, C. C., S. W. Watson, J. B. Waterbury, and H. G. Truper. 1968. Fine structure of *Ectothiorhodospira mobilis* Pelsh. J. Bacteriol. **95**:2374-2392.
19. Shively, J. M., G. L. Decker, and J. W. Greenawalt. 1970. Comparative ultrastructure of the thiobacilli. J. Bacteriol. **101**:618-627.
20. Smith, U., and D. W. Ribbons. 1970. Fine structure of *Methanomonas methanooxidans*. Arch. Mikrobiol. **74**:116-122.
21. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31-43.
22. Tsien, H. C., R. Lambert, and H. Laudelout. 1968. Fine structure and the localization of the nitrite oxidizing system in *Nitrobacter winogradski*. Antonie van Leeuwenhoek J. Microbiol. Serol. **34**:483-494.
23. Venable, J. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. **25**:407-408.
24. Wiebe, W. J., and G. B. Chapman. 1968. Variation in the fine structure of a marine achromobacter and a marine pseudomonad grown under selected nutritional and temperature regimes. J. Bacteriol. **95**:1874-1886.