JOURNAL OF BACTERIOLOGY, June 1973, p. 1346-1350
Vol. 114, No. 3

Printed in U.S.A.

Internal Membrane Control in *Azotobacter vinelandii*

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Received for publication 22 February 1973

Azotobacter vinelandii was grown on N_2, NH_4^+, or NO_3^-, and an internal membrane network was observed by electron microscopy of thin sections of cells. Cells obtained in early exponential growth contained less internal membrane than did cells from cultures in late exponential growth. It seems likely that O_2 has a role in regulating the amount of internal membrane structure.

When *Azotobacter vinelandii* is fixing N_2, its growth is inhibited by high concentrations of O_2, but this O_2 sensitivity is not seen when the cells are growing in the presence of sufficient NH_4^+ to repress nitrogenase synthesis (2). Inhibition of growth by O_2 has been attributed to the extreme O_2 lability of the protein components of nitrogenase (2). One hypothesis for nitrogenase protection in vivo is that nitrogenase is conformationally protected from O_2 inactivation (2). When cells are broken with a French pressure cell, nitrogenase is quite O_2 insensitive until the components are separated on a (diethylaminoethyl cellulose) column. It has been suggested that the conformation protection was possibly mediated by reduced form of nicotinamide adenine dinucleotide dehydrogenase, which protects nitrogenase from O_2 inactivation in vitro (15). Another protective mechanism was hypothesized to be respiratory protection (2) by which O_2 is reduced rapidly (presumably via the cytochromes) and thereby is unable to inactivate nitrogenase. In support of this hypothesis are the reports of Oppenheim and Marcus (9; J. Oppenheim and L. Marcus, Bacteriol. Proc., p. 63, 1970) and Hill et al. (5) who claim that an extensive internal membranous network is seen in electron micrographs of sections of *A. vinelandii* that have been grown on N_2 and that only slight quantities of internal membrane, concentrated around the periphery of the cell, are seen when cells are grown with excess NH_4^+. This finding is not supported, however, by the work of Phillips and Johnson (10) who showed that the great O_2 demand of *A. vinelandii* is independent of nitrogen source. This report contradicts previous work (5, 9; J. Oppenheim and L. Marcus, Bacteriol. Proc., p. 63, 1970) and shows that the internal membrane network is found in both N_2- and NH_4^+-grown cells.

MATERIALS AND METHODS

The strain used was *A. vinelandii* OP. Chemicals and methods of culture and derepression have been described previously (13, 14).

Cells were fixed with osmium tetroxide in Veronal-acetate buffer by the method of Kellenberger et al. (6). Fixed cells were dehydrated in a graded series of ethyl alcohol and propylene oxide and embedded in Epon 812 by the procedure of Luft (7). Thin sections were cut by using an ultramicrotome (Porter-Blum MT-2) and a diamond knife (E. I. DuPont de Nemours & Co. Inc.). Sections were collected on Parlodion and carbon-coated grids and stained for 10 min with 2% aqueous uranyl acetate and with lead citrate for 10 min thereafter (11). Micrographs were made with an electron microscope (Hitachi HU11E) at 75 kV with a 50-μm objective aperture. All figures are representative of many fields.

RESULTS

By using techniques previously described (9, 13) we were unable to duplicate the results of Oppenheim and Marcus (9) and Hill, et al. (5) who found the extensive internal membrane network absent from cells that have been growing on excess NH_4^+ or excess $NaNO_3$. The membrane network was present in thin sections of cells that had been grown on N_2, ammonium acetate, NH_4Cl, and $NaNO_3$ (Fig. 1). Both components of nitrogenase are completely repressed by the concentration of ammonium acetate or NH_4Cl used (13). It is unlikely, therefore, that these membranes have a sole function of protecting nitrogenase from O_2 inactivation.

A condition that does seem to change the amount of internal membrane material is the

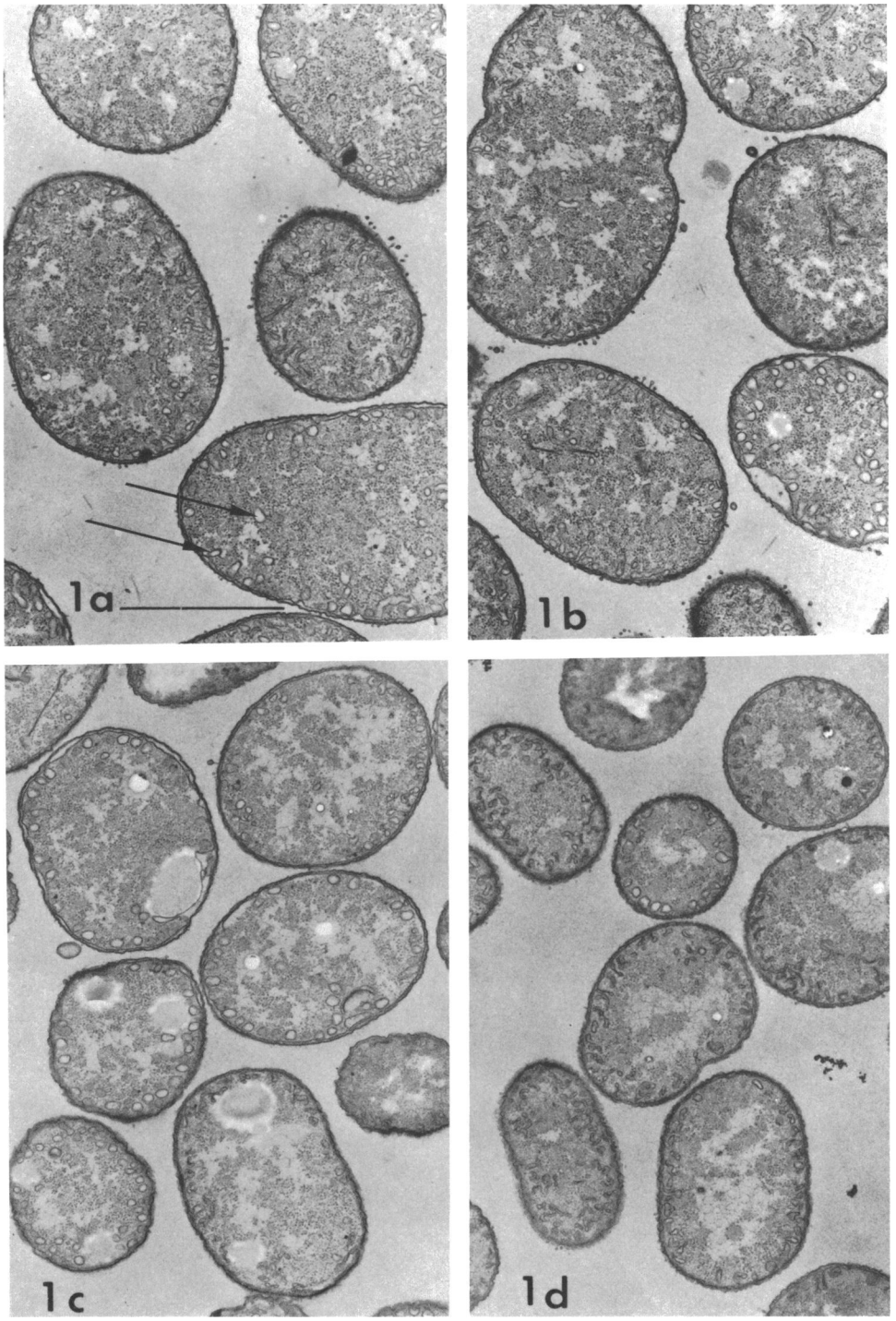

FIG. 1. *Sections of cells of A. vinelandii taken from exponentially growing cultures with different sources of nitrogen. The cultures were harvested during exponential phase at a cell titer of 2 × 10^8/ml. The sources of nitrogen were: 1a, air (N_2); 1b, 400 μg of nitrogen/ml as NH_4 acetate; 1c, 400 μg of nitrogen/ml as NH_4Cl; 1d, 400 μg of nitrogen/ml as $NaNO_3$. Arrows in 1a indicate internal membranes. The marker bar in 1a represents 1 μm. All micrographs are at the same magnification.*

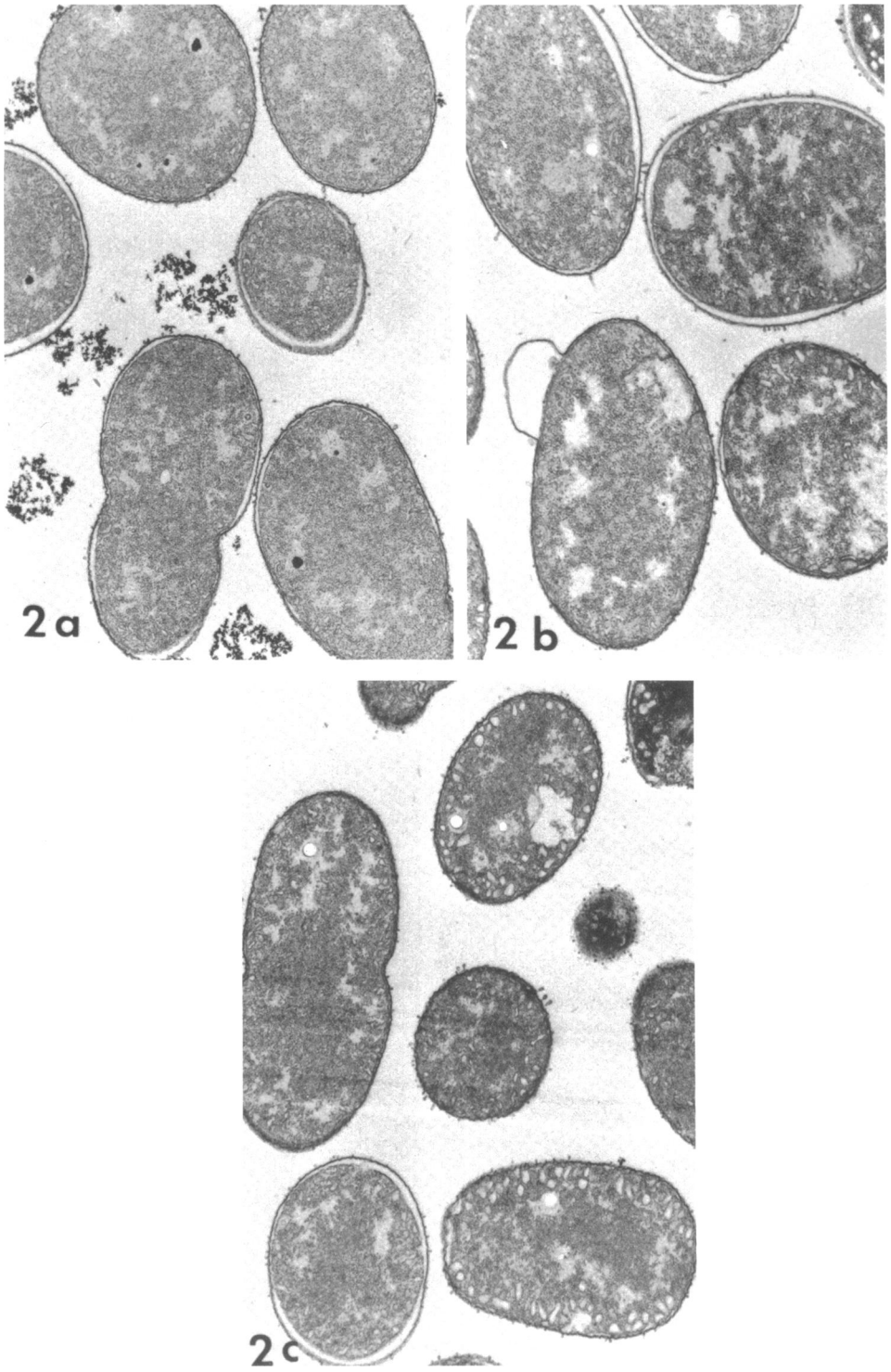

FIG. 2. *Sections of cells of A. vinelandii grown on atmospheric nitrogen. The cultures were harvested at different cell titers. Cell titers at time of harvest were* 2.6×10^7*/ml,* 6×10^7*/ml, and* 1.7×10^8*/ml for cells shown in 2a, 2b, and 2c, respectively.*

cell population and rate of agitation of the culture, irrespective of nitrogen source. N_2-grown cells (fig. 2a) were harvested at a low cell density (2.6×10^7 cells/ml; dissolved O_2 concentration was 6.5 ppm). The membrane network seemed to be only at the periphery of the cells, but at a higher density of cells (6×10^7 cells/ml; dissolved O_2 concentration was 3.2 ppm), the membranes seemed to become more predominant (Fig. 2b). When cells reached a population density of 1.7×10^8 Cells/ml, the dissolved O_2 concentration was 0.6 ppm, and the extensive network was observed (Fig. 2c). The same phenomenon of membrane quantity varying with cell density also was seen with cells that had been growing in a medium containing excess NH_4^+. Cells grew at the same rate up to cell densities greater than 2×10^8 cells/ml. At cell densities between 1×10^8 cells/ml and 2×10^8 cells/ml, presence of the membrane network depended upon whether the growth flask had baffles and upon the speed of shaking. At a given cell density, cells that were harvested from a flask without baffles had a more extensive membrane network than did cells from a flask with baffles.

DISCUSSION

An indication that growth on N_2 is not required for formation of the internal membrane network has been reported by Zey et al. (P. Zey et al., Abstr. Ann. Meet. Amer. Soc. Microbiol., p. 155, 1972) who stated that this membrane structure is found in cells grown on N_2 as well as on NH_4^+. However, they used methylamine to repress nitrogenase. Methylamine is capable of uncoupling phosphorylation (4) and does not repress nitrogenase synthesis (12). In support of the original (9) membrane results, Oppenheim and Marcus (J. Oppenheim and L. Marcus, Bacteriol. Proc., p. 148–149, 1970) and Marcus and Kaneshiro (8) showed that phospholipid content is greater in N_2-grown cells than in NH_4^+-grown cells of *A. vinelandii*. However, Drozd et al. (3) claim to have found no such differences in phospholipid content between cells of *A. chroococcum* that are repressed or derepressed for nitrogenase synthesis.

The results presented in this paper indicate that the internal membrane network might be produced in response to O_2 availability rather than to nitrogen source. The cells seem to respond to dissolved O_2 concentration by synthesizing more membrane material when O_2 is limiting. These membranes could function to increase the surface area and could be able to sequester enough O_2 to allow the bacteria to remain in the exponential-growth phase.

A freshly inoculated culture of *A. vinelandii* will have a shorter lag period if the culture is not shaken for several hours before it is placed on the shaker (2). Ackrell and Jones (1) have reported that the length of the lag period is directly related to the rate of aeration of cells of *A. vinelandii*. Our results offer a possible explanation for this phenomenon. Usually, an inoculum from a slant or turbid "overnight" culture is used to inoculate fresh medium in which O_2 is not limiting. Cells that had been growing at a high density are suddenly diluted into fresh medium. These cells, therefore, are suddenly in an environment in which the surface area of membrane in contact with the dissolved O_2 is greater than that actually needed. Perhaps excess respiration under these conditions has a detrimental effect on growth. Resumption of growth might begin after these extra membranes are degraded.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, by National Science Foundation grant GB36787, and by Public Health Service Grant GM-19859-01 from the National Institute of General Medical Sciences.

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