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NOTES

Influence of Oxygen on Phospholipid Production and Colony Formation in a Nitrogen-Fixing Mutant of *Azotobacter vinelandii*

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Colony dimorphism in a conditional nitrogen-fixing mutant of *Azotobacter vinelandii* was directly influenced by fixed nitrogen and oxygen partial pressure and may be related to the production of internal peripheral membrane.

Colony dimorphism in *Derxia gummosa* was reported some years ago to be dependent upon the cell density on the agar surface (9). Thin colonies and massive colonies arose, and the number of colonies that developed into the massive, N_2-fixing type was inversely proportional to the total number of colonies present. The formation of massive colonies, even in areas of high cell density, could be stimulated by the addition of fixed nitrogen or, as shown more recently by Hill (3), by lowering the partial pressure of oxygen below 0.2 atm.

The effects of varying oxygen concentration and nitrogen source on *Azotobacter vinelandii* are somewhat different from those described in *Derxia*. It has been argued that the synthesis of internal peripheral membrane responds to oxygen availability irrespective of nitrogen source (7). Others contend that the membrane network is synthesized only in the absence of fixed nitrogen and somehow serves to protect the labile nitrogenase from inactivation by molecular oxygen (6).

We have examined a mutant of *A. vinelandii* ATCC 12837 that resembles *D. gummosa* in its dimorphic pattern of growth on N-free agar. The wild-type growth pattern can be restored in inhibited cells by the addition of fixed nitrogen or by lowering the pO_2. In addition, we report an increase in the ratio of phospholipid-to-protein in inhibited mutant cells and the apparent effect of fixed nitrogen and lowered pO_2 on production of peripheral membrane.

The typical pattern of wild-type colony formation on Burk N-free agar (10) containing 0.5% sucrose is shown in Fig. 1a. Growth was most luxuriant where the inoculum was heaviest. Colony formation of the mutant, however, was inhibited in the area of high cell density (Fig. 1b). Occasionally, fully developed colonies would arise in the inhibited region, but these infrequent colonies as well as colonies from

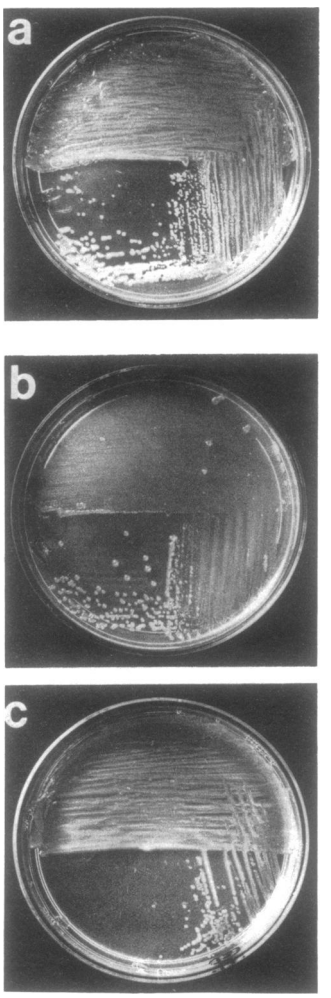

FIG. 1. *Growth pattern of the wild type on N-free agar medium in air (a); growth pattern of the mutant on N-free agar medium in air (b) and in pO_2 of 0.04 atm (c).*

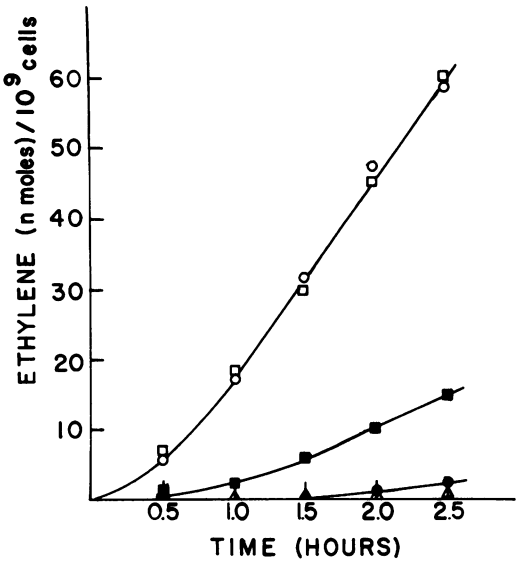

FIG. 2. *Comparative rates of acetylene reduction between mutant and wild-type cells when grown on N-free or NH_4NO_3-supplemented agar medium. A 20-ml whole-cell suspension was placed into a 70-ml serum bottle and sealed with a rubber stopper. The suspension was injected with 10 cm^3 of acetylene (0.2 atm) and incubated at 33°C with shaking. Gas samples were removed at intervals from the atmosphere above the cell suspension and injected directly into a Varian Aerograph model 90/75 gas chromatograph. Cell suspensions were standardized by viable cell count. Cell numbers were determined by dilution plating onto Burk agar medium containing 0.5% sucrose and supplemented with 0.1% Casamino Acids (Difco) and 0.1% yeast extract. Symbols: (△) mutant cells grown on NH_4NO_3-supplemented agar; (■) wild-type cells grown on NH_4NO_3-supplemented agar; (●) inhibited mutant cells grown on N-free agar; (○) well-developed mutant cells grown on N-free agar; (□) wild-type cells grown on N-free agar.*

areas of low cell density reproduced the inhibited growth pattern upon reinoculation onto N-free agar.

When nitrogen was added to the medium as NH_4NO_3, NH_4Cl, or ammonium acetate, the mutant demonstrated the wild-type growth pattern. Between 10 and 15 μg of nitrogen per ml was required to reverse inhibition, although some cells were stimulated by as little as 3 μg of nitrogen per ml.

The effect of lowered pO_2 on growth was measured by placing agar plates in desiccating jars connected to a vacuum aspirator and a manometer. The wild-type pattern of growth was restored in the mutant when the pO_2 was reduced to 0.04 atm (Fig. 1c). Growth was the same whether incubation occurred under a partial vacuum or with the evacuated atmosphere replaced with nitrogen gas.

The acetylene-reducing ability of cells from inhibited and well-developed colonies was examined, and the results are summarized in Fig. 2. The inhibited mutant cells reduced acetylene at only 7% of the wild-type rate, whereas, in cells from well-developed colonies, nitrogenase activity was comparable to that of wild-type cells. The effect of reduced oxygen concentration on acetylene reduction is shown in Fig. 3. The wild-type rate was inhibited by about 57%, which probably reflects a limiting oxygen concentration in the assay system. The critical

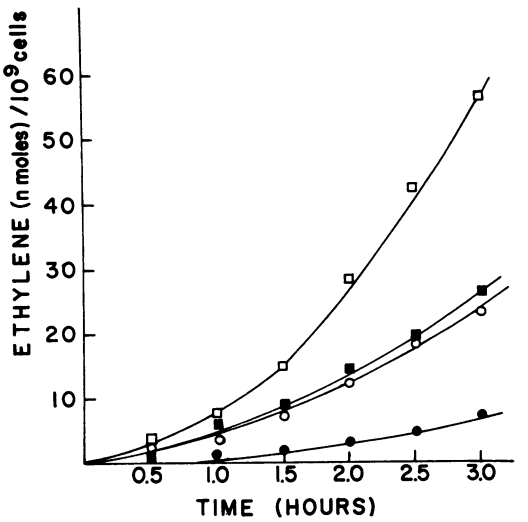

FIG. 3. *Effect of lowered oxygen concentration on the rate of acetylene reduction in mutant and wild-type cells. The atmosphere removed from desiccating jars was replaced with N_2 gas. Cultures were incubated at 33°C for 48 h. Acetylene reduction and viable cell count were determined as described in Fig. 2. Symbols: (●) mutant assayed at a pO_2 of 0.2 atm; (□) wild type assayed at a pO_2 of 0.2 atm; (○) mutant assayed at a pO_2 of 0.04 atm; (■) wild type assayed at a pO_2 of 0.04 atm.*

TABLE 1. *Oxygen uptake by mutant and wild-type cells (nanomoles per minute per 10^8 cells)*[a]

Organism	pO_2 during growth	
	0.2 atm	0.04 atm
Mutant	113.4	54.8
Wild type	96.4	56.7

[a] Initial rates of oxygen uptake were measured with a Clark polarigraph electrode. The instrument was calibrated from 0 to 96% oxygen with oxygen saturated 0.05 M KH_2PO_4 (pH 7.2) and sodium dithionite. Reaction mixtures contained in a final volume of 1.6 ml: 1.0 ml of cell suspension, 0.5 ml of KH_2PO_4 (pH 7.2), and 0.1 ml of 50 mM sucrose. Viable cell count was determined by dilution plating as described in Fig. 2.

TABLE 2. *Determination of phosphorus in mutant and wild-type cell membranes*[a]

Organism	Results (μg of phosphorus/mg of protein)[b]				
	Expt I	II	III	IV	V
Mutant (inhibited)	6.10	7.25	6.25	6.00	6.85
Mutant (developed)	5.10	5.00	5.10	–[c]	–
Mutant (pO_2-0.04 atm)	–	–	–	6.10	7.00
Wild type	5.05	5.20	5.10	5.00	5.15
Wild type (pO_2-0.04 atm)	–	–	–	5.80	6.00

[a] The membrane fraction was obtained by a modification of the method of Mavis, Bell, and Vagelos (5). Crude cell extracts were treated with pancreatic deoxyribonuclease and centrifuged at 6,000 × *g* for 10 min. The membrane fraction was collected by centrifuging the clarified extract at 50,000 × *g* for 45 min.

[b] The supernant fluid from the 6,000 × *g* centrifugation was assayed for protein content by the method of Lowry et al. (4). Phospholipids in washed membrane pellets were purified by the methanol extraction procedure of Bligh and Dyer (1). The phosphorus content of membrane fractions was determined by the method of Chen, Toribara, and Warner (2).

[c] –, Not done.

observation is that the rate of acetylene reduction in the mutant increased nearly up to the level permitted in the wild type under identical conditions.

Oxygen uptake by cells grown on N-free agar under a pO_2 of 0.2 atm and 0.04 atm was compared to determine whether the mutant was more susceptible to oxygen toxicity due to a lower maximum respiration rate (Table 1). The rate of respiration in inhibited mutant cells was comparable to the wild-type rate. When grown in a reduced oxygen environment, the respiration rates of both cell types were reduced by 40 to 50% as expected.

Pate et al. (7) suggested that the synthesis of internal membrane in *Azotobacter* increased as oxygen availability decreased. In light of this suggestion, phospholipid concentrations were measured in both the mutant and wild type. Phosphorus was used as a quantitative indicator of phospholipid content (Table 2). The ratio of phospholipid to protein in the inhibited mutant cells was 21 to 40% greater than in the wild type, whereas the ratio in fully developed mutant cells was virtually the same as in the wild type.

Preliminary electron microscopic studies by L. M. Pope in this laboratory suggest that the differences in the phospholipid-to-protein ratio may reflect changes in total membrane production rather than a simple increase in membrane phosphorus or decrease in protein synthesis. Invaginations of the cytoplasmic membrane, which appear as vesicles in cross section, occur only peripherally in wild-type (Fig. 4a) and well-developed mutant cells (Fig. 4b). However, in inhibited mutant cells this membrane network occurs in great quantity and extends well into the cell cytoplasm (Fig. 4c).

The influence of growth at a pO_2 of 0.04 atm on the phospholipid-to-protein ratio is shown in experiments IV and V. The phospholipid-to-protein ratio increased by only 1 to 2% when the mutant was grown at low pO_2, whereas the wild-type growth at reduced oxygen tension resulted in a 16.5% increase.

The data show that colony formation and N-fixing ability in the mutant are influenced by atmospheric oxygen concentration. The susceptibility of these cells is not the result of a reduced rate of respiration. Although the unusually high phospholipid-to-protein ratio found in inhibited mutant cells does not unequivocally prove excessive membrane production, the electron microscopic evidence does indicate that aberrant membrane regulation exists.

We are most grateful to Leodocia Pope, who kindly provided the electron micrographs.

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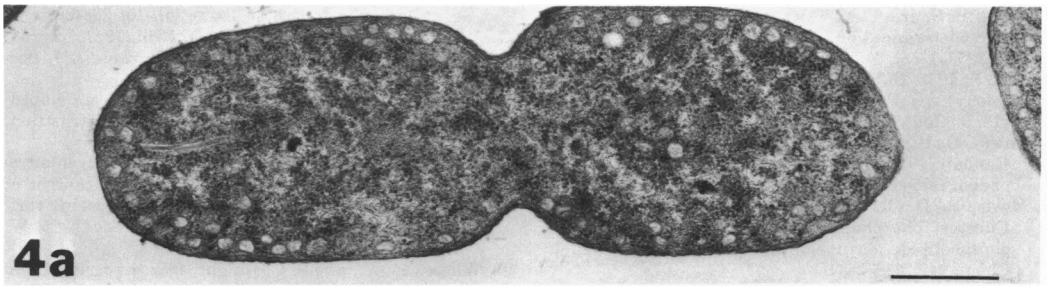
FIG. 4. *Thin sections of A. vinelandii wild type and mutant showing aberrant quantities of membrane in inhibited mutant cells. Fixation was carried out overnight in a solution of 0.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) followed by postfixation for 2 h in 1% OsO_4 in the same buffer. Samples were stained in a solution of 0.5% uranyl acetate for 18 h and dehydrated in increasing concentrations of acetone. The material was then imbedded in Spurr (8) low-viscosity medium prior to sectioning: (a) Wild type; (b) well-developed mutant; (c) inhibited mutant. All cells were grown on N-free agar and harvested after 48 h. Bars represent 0.5 μm.*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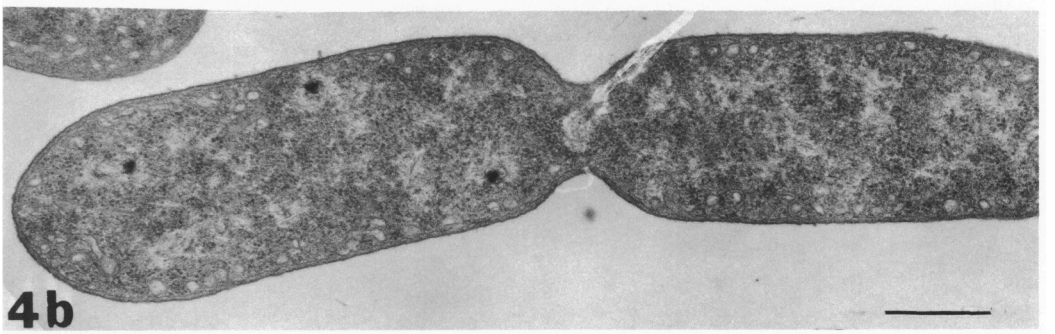
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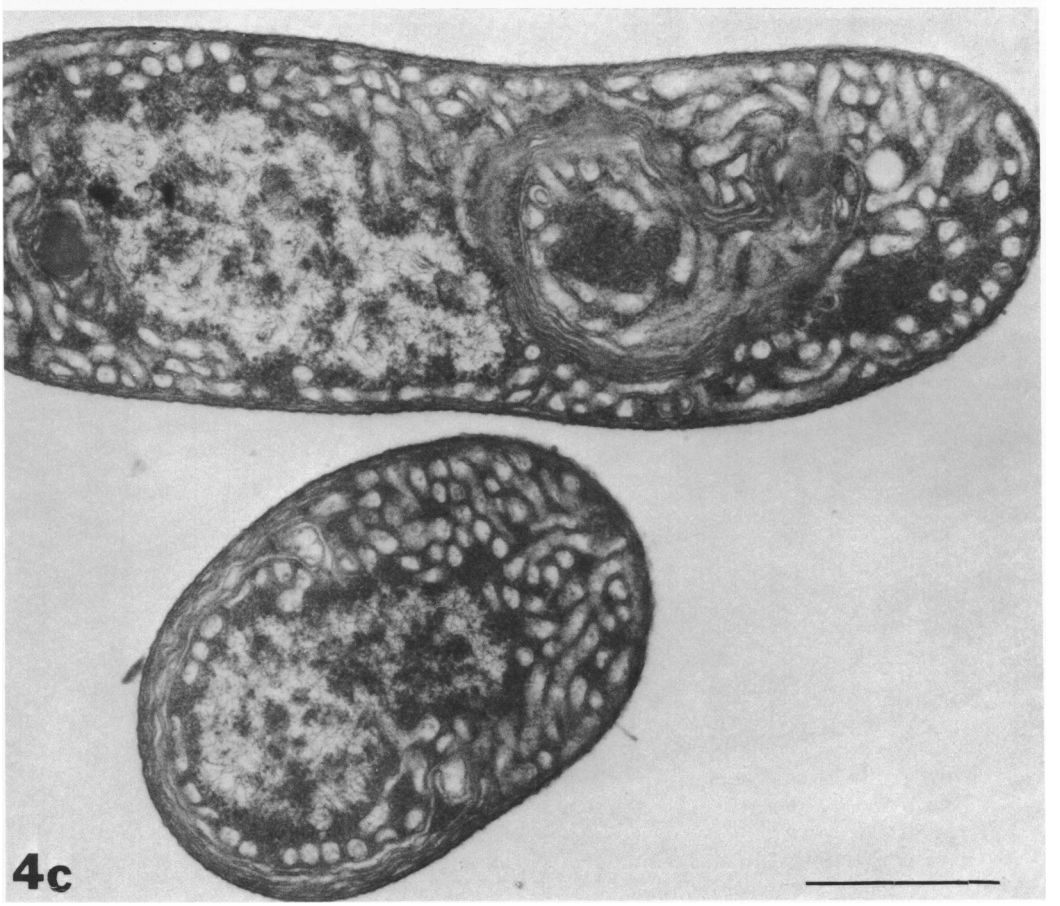
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