Mo-Independent Nitrogenase 3 Is Advantageous for Diazotrophic Growth of *Azotobacter vinelandii* on Solid Medium Containing Molybdenum

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Competition experiments between wild-type *Azotobacter vinelandii* and a mutant lacking Mo-independent nitrogenase 3 indicate that nitrogenase 3 provides an advantage during diazotrophic growth on agar media containing 100 to 500 nM Na₂MoO₄ but not in liquid media under the same conditions. Expression of nitrogenase 3 in wild-type cells growing on agar surfaces was verified with an *anfH-lacZ* fusion and by detection of nitrogenase 3 subunits. These results show that nitrogenase 3 is important for diazotrophic growth on agar medium at molybdenum concentrations that are not limiting for Mo-dependent diazotrophic growth in liquid medium.

Azotobacter vinelandii is an aerobic nitrogen-fixing soil bacterium. This diazotroph is able to reduce N₂ to NH₄⁺ by using any of three distinct nitrogenases. These enzymes are encoded by three separate sets of structural genes. *nifHDK* encodes the subunits of the molybdenum (Mo)-containing nitrogenase (nitrogenase 1), *nifHDGK* encodes the subunits of the vanadium (V)-containing nitrogenase (nitrogenase 2), and *anfH-DGK* encodes the subunits of the iron (Fe)-only nitrogenase (nitrogenase 3) (1, 4, 5). Nitrogenase 1 is expressed in medium containing Mo, whereas nitrogenases 2 and 3 are repressed by Mo. Nitrogenase 2 is expressed in Mo-deficient medium containing V, while nitrogenase 3 is expressed in medium lacking both Mo and V (5). All three nitrogenases are composed of two components: dinitrogenase reductase and dinitrogenase (5).

Recently, two other diazotrophs, *Rhodobacter capsulatus* (16, 20) and *Rhodospirillum rubrum* (12, 13), have been found to have alternative nitrogenases that are similar to nitrogenase 3 from *A. vinelandii*. Since Mo-independent nitrogenases are likely to be found in a wide range of diazotrophs, the existence of these enzymes poses interesting questions concerning the role(s) that they might play under natural conditions. These nitrogenases are expressed only in the absence of Mo; therefore, it is reasonable to surmise that they might function under conditions of Mo limitation such as in acid soils with high iron oxide concentrations or where the natural abundance of Mo is low. Another possibility is the formation of Mo-depleted microzones through Mo accumulation by organisms in ecosystems such as microbial mats, plant rhizospheres, and the canopies of tropical rain forests and by some of those in symbiotic relationships (1). Limitation of Mo-dependent nitrogen fixation due to high concentrations of sulfate in oxic marine environments has been hypothesized to account for the chronic nitrogen depletion observed in these environments (8). Accordingly, we thought that Mo-independent nitrogenases might provide an advantage to organisms growing diazotrophically in environments with such high sulfate concentrations. We found, however, that diazotrophic growth of *A. vinelandii* was Mo dependent even in media containing extremely high

ratios of sulfate/molybdate (17). In addition to the availability of Mo, temperature may affect the requirement for alternative nitrogenases. Recent findings suggest that the V-containing nitrogenase 2 may function more efficiently at low temperatures than does Mo-containing nitrogenase 1; thus, this nitrogenase may be involved in low-temperature diazotrophy (15, 23).

There has been an increasing awareness that gene expression in response to nutrients during growth on substrate surfaces such as agar medium can be quite different from gene expression during growth in liquid media. Much of the information concerning regulation of gene expression, however, has been derived from studies with liquid culture (22), where all free-swimming cells are exposed to the same concentration of micronutrients in the medium. In contrast, microorganisms growing on a substrate surface have the potential to create microzones of nutrient deficiency through the process of nutrient removal (19).

Since *A. vinelandii* is known to have a powerful Mo accumulation system (18), we hypothesized that diazotrophic growth of *A. vinelandii* cells on a solid medium surface might create Mo-depleted microzones, whereas in liquid shake cultures this should not occur. Thus, nitrogenase 3 should provide an advantage to cells growing diazotrophically on a solid substrate surface. To test this hypothesis, competition experiments between wild-type strain CA (6) and strain CA70 (10), a strain lacking the structural genes encoding nitrogenase 3 (*ΔanfHD70::kan*), were performed. Strains CA and CA70 were mixed to give a ratio of approximately 1:1. N-free liquid Burk medium (21) containing 100 nM Na₂MoO₄ (30 ml in a 300-ml sidearm flask) was inoculated with 1 × 10⁸ to 5 × 10⁸ cells (in a 100-μl volume) of the mixed inoculum. Agar plates (30 ml of agar medium per plate) of N-free Burk medium containing 100, 250, 500, or 1,000 nM Na₂MoO₄ were inoculated by spreading the mixed inoculum (1 × 10⁸ to 5 × 10⁸ cells in a 100-μl volume) on the agar surface with a glass rod. The mixed cultures were transferred and assayed for each strain at 48-h intervals. Agar plate cultures were suspended in 2 ml of Burk buffer (7 mM potassium phosphate, pH 7.2) (21), and approximately 10⁸ cells were transferred to fresh Burk agar plates containing appropriate concentrations of Na₂MoO₄. Approxi-

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FIG. 1. Competition experiments between strains CA and CA70. (A) Comparison of CA/CA70 ratios from agar (●) and liquid (Δ) medium cultures containing 100 nM Na₂MoO₄. (B) Effect of different Na₂MoO₄ concentrations on CA/CA70 ratios. □, 1,000 nM Na₂MoO₄; +, 500 nM Na₂MoO₄; Δ, 250 nM Na₂MoO₄; ●, 100 nM Na₂MoO₄.

mately 10^8 cells were also used for liquid culture transfers. The proportion of CA and CA70 cells in the mixed cell populations was determined by differential plate counting. Burk agar medium containing 28 mM ammonium acetate (Burk+N) was used to determine the total number of CFU, and Burk+N containing kanamycin (10 μ g/ml) was used to determine the CFU for CA70. The difference between the total number of CFU and the number of CFU determined for CA70 represents the number of CFU for strain CA. From these numbers, CA/CA70 ratios were determined at each transfer. Each ratio was based on a count of approximately 1,000 CFU for the total population. Results of these experiments (Fig. 1A) show that the log ratio of CA/CA70 did not change during 10 transfers in liquid medium containing 100 nM Na₂MoO₄, whereas a 3.5-log-unit increase was observed after 10 transfers on agar medium (and after 19 transfers, a 6-log-unit increase was observed [results not shown]). Increasing Na₂MoO₄ concentrations led to diminished increases in the CA/CA70 ratios, and at 1,000 nM Na₂MoO₄, strain CA70 seemed to have a slight advantage over CA. Thus, increases in the ratios appear to be inversely correlated with the Na₂MoO₄ concentrations; i.e., the higher the Na₂MoO₄ concentration, the smaller the ratio increase (Fig. 1B). This would be expected if increased Na₂MoO₄ concentrations reduced the requirement for nitrogenase 3. The ratio did not change during transfer on Burk medium containing 28 mM ammonium acetate, indicating that the observed ratio increases can be attributed to differences in the nitrogen fixation capabilities of the two strains.

Results from the competition experiments strongly suggest

that nitrogenase 3 plays a role in diazotrophic growth on agar surfaces even in the presence of Na₂MoO₄ concentrations that completely repress nitrogenase 3 in liquid medium. To test for expression from the *anfH* promoter, agar and liquid Burk media were inoculated with a strain (CA73) which contains an *anfH-lacZ* transcriptional fusion (11). Agar and liquid media contained 100, 250, 500, or 1,000 nM Na₂MoO₄ and were supplemented with 10 mM urea (a nonrepressing source of nitrogen) or with 28 mM ammonium acetate. Cells were harvested after 24 h of incubation for both agar and liquid cultures. Each value is the average obtained from two separate cultures. Five- and 50- μ l aliquots of each culture were used for

TABLE 1. Expression of an *anfH-lacZ* fusion in the presence of Na₂MoO₄

Addition to medium	% β -Galactosidase activity ^a with:	
	Agar	Liquid
None	100	100
100 nM Mo	50	56
250 nM Mo	36	2.0
500 nM Mo	30	0.25
1,000 nM Mo	6.0	0.0
10,000 nM Mo	0.14	0.0
100 nM Mo plus NH ₄ ⁺	3.0	0.0

^a Percentages were based on β -galactosidase activity determined for cells cultured in the absence of Mo and V. (Activity of 100% is 13,290 Miller units on agar and 19,510 Miller units in liquid.)

FIG. 2. Two-dimensional protein gel electrophoresis of cell extracts of *A. vinelandii* CA cultured in the presence of 250 nM Na_2MoO_4 . Gels from an agar plate culture (A) and from a liquid culture (B) are shown. Numbered spots are as follows: D1, dinitrogenase 1 subunits; DR1, dinitrogenase reductase 1; DR2, dinitrogenase reductase 2 (this nitrogenase component is required for expression of nitrogenase 3 [11]); D3, dinitrogenase 3 subunits; and DR3, dinitrogenase reductase 3. The identities of nitrogenase subunits on two-dimensional protein gels have been established previously (3, 7).

β -galactosidase assays as described by Miller (14). After an incubation period of 24 h, β -galactosidase activity was readily detected in cells grown on agar medium in the presence of 100, 250, or 500 nM Na_2MoO_4 , but in liquid medium, activity was found only in cells exposed to 100 nM Na_2MoO_4 (Table 1). As expected, the level of expression of the *anfH-lacZ* fusion was inversely correlated with the concentration of Na_2MoO_4 in the agar growth medium.

To ascertain the presence of nitrogenase 3 subunits in strain

CA growing on agar in the presence of Mo, two-dimensional protein gel electrophoresis was conducted with cell extracts prepared from cultures growing on N-free Burk agar media and in liquid N-free media in the presence of 100, 250, 500, or 1,000 nM Na_2MoO_4 . Cells from three separate cultures grown for 48 h at 30°C on or in 30 ml of N-free Burk medium were combined before cell extract preparation. Cell extract preparation and two-dimensional protein gel electrophoresis were done as described previously (3) except that for the agar cultures, cells were removed from the agar surface of each plate by suspending the cells in 3 ml of Burk buffer (7 mM K phosphate, pH 7.2). Nitrogenase 3 subunits were found in cell extracts from agar medium cultures exposed to all of the Na_2MoO_4 concentrations tested. With liquid cultures, these subunits were detected only in extracts of cells grown in the presence of 100 nM Na_2MoO_4 . Although nitrogenase 1 subunits were detected in cells grown in the presence of all Na_2MoO_4 concentrations tested, the amounts of nitrogenase 3 subunits were roughly equal to those of nitrogenase 1 in cell extracts from cultures grown in the presence of the 100 and 250 nM concentrations. Results for the 250 nM Mo concentration are shown in Fig. 2.

The results of competition experiments together with the experiments showing nitrogenase 3 expression in cells growing on agar surfaces lend strong support to the hypothesis that alternative nitrogenase 3 plays an important role in diazotrophic growth of *A. vinelandii* on solid substrate surfaces even in the presence of concentrations of Mo that completely repress nitrogenase 3 expression in liquid culture (2, 9). The findings of this study relate to the more general area of inquiry into gene expression during growth on substrate surfaces in comparison with gene expression during growth in liquid media. Thus, if altered concentrations of a micronutrient such as Mo lead to corresponding changes in gene expression, we might expect populations growing on surfaces to be heterogeneous with respect to gene expression, whereas in liquid cultures gene expression would tend to be more homogenous. This topic is of increasing interest in the field of microbiology because, in nature, many microorganisms grow on surfaces.

In summary, the results presented in this study suggest that one probable role for Mo-independent nitrogenases like nitrogenase 3 is the ability to fix nitrogen in conditions under which the microenvironment is likely to become Mo deficient, such as on solid substrate surfaces.

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