JOURNAL OF BACTERIOLOGY, July 2000, p. 3854–3857 Vol. 182, No. 13
0021-9193/00/$04.00+0

Role of the *Azotobacter vinelandii* Nitrogenase-Protective Shethna Protein in Preventing Oxygen-Mediated Cell Death

R. J. MAIER* AND F. MOSHIRI†

Department of Microbiology, University of Georgia, Athens, Georgia 30602-2605

Received 21 January 2000/Accepted 7 April 2000

Azotobacter vinelandii **strains lacking the nitrogenase-protective Shethna protein lost viability upon carbon-substrate deprivation in the presence of oxygen. This viability loss was dependent upon the N_2-fixing status of cultures (N_2-fixing cells lost viability, while non-N_2-fixing cells did not) and on the ambient O_2 level. Supra-atmosheric O_2 tensions (40% partial pressure) decreased the viable cell number of the mutant further, and the mutant had a slightly higher spontaneous mutation frequency than the wild type in the high-O_2 conditions. Iron starvation conditions, which resulted in fourfold-reduced superoxide dismutase levels, were also highly detrimental to the viability of the protective protein mutants, but these conditions did not affect the viability of the wild-type strain. Nitrogenase or other powerful reductants associated with N_2 fixation may be sources of damaging partially reduced oxygen species, and the production of such species are perhaps minimized by the Shethna protein.**

Whereas O_2 can cause irreversible damage to nitrogenase both in vitro and in vivo, for most nitrogen fixing organisms controlled levels of O_2 have beneficial effects on whole-cell N_2 fixation. As the terminal acceptor for aerobic respiration, O_2 stimulates oxidative phosphorylation, thus providing the ATP supply to fuel both nitrogenase activity and its synthesis (see reference 3). The optimum oxygen concentration for maximal N_2 fixation rates varies widely among N_2-fixing organisms, with some showing considerably more O_2 tolerance than others (see reference 4). In vivo, nitrogenase is not only a potential target for inactivation by O_2 but may also be a source of O_2-derived radicals that can further the cellular O_2-related damage (3). For example, under certain oxygen conditions, the iron-protein of nitrogenase can reduce O_2 in a reaction that produces either H_2O_2 or O_2^- as an intermediate (16). Many powerful reductants associated with nitrogen fixation, such as flavodoxin, ferredoxin, and nitrogenase, or (in root nodules) leghemoglobin-related products, have been proposed to be likely generators of superoxide, hydrogen peroxide, or hydroxy radicals; all produced from reactions due to the partial reduction of oxygen (3). Other common iron-sulfur enzymes may be major sources of superoxide production by bacterial cells in vivo (5, 14).

Azotobacter spp. have a very high respiratory rate, and their ability to fix N_2 in O_2 tensions at and above air saturation levels has intrigued researchers for many years (3, 12). One mechanism *Azotobacter* species use to protect nitrogenase from O_2 damage is termed conformational protection (12) and involves the association of an FeSII protein with nitrogenase during periods of oxygen stress (9, 11, 13). In this state, the nitrogenase component proteins are in an inactive but protected state capable of reconversion to the catalytically active state when the redox environment becomes favorable for nitrogenase function (11). The spectral and physical properties of FeSII (also known as the Shethna protein) of *Azotobacter vinelandii* has been well characterized, and the protein has been crystallized (8). It contains two [2Fe-2S] clusters that undergo oxidation-reduction, and it is presumed that this redox-active nature of the protein mediates its interactions with nitrogenase (8, 13). Specific amino acid residues important in the function, including in presumably recognizing the nitrogenase component proteins, have recently been proposed based on analysis of site-directed mutations within the FeSII protein (6). Initially, the phenotype of the mutant strains was indistinguishable from that of the wild type, but this was attributed to the high respiratory rate (and consequent O_2 removal during growth on an excellent carbon [sucrose] substrate [9]). The nitrogenase component proteins in *A. vinelandii* strains containing a deletion at the *feSII* locus exhibited a hypersensitivity to O_2-mediated inactivation during conditions of carbon substrate starvation; upon nutrient deprivation, both the MoFe and Fe proteins of nitrogenase were degraded much more rapidly in an FeSII deletion strain than in the wild-type strain (9). Such carbon starvation conditions negate respiratory protection so that only conformational protection operates. In the course of studying the FeSII mutants, we noticed a loss of viability in the *feSII* deletion mutants, as nitrogen-fixing cultures entered stationary phase at the time of carbon starvation. We have now characterized this behavior and show that this cytotoxic effect is related to the presence of the nitrogenase components, oxygen, and the FeSII protein.

The growth behavior of *A. vinelandii* cultures was monitored by measuring both the culture optical density at 600 nm (OD_{600}) and viable cell numbers based on plate counts. Strains CA (wild type) and Δ*fesII*:KIXXI were described previously (9). All the results shown here that indicate use of an FeSII$^-$ strain are with Δ*fesII*:KIXX, but some experiments done with Δ*fesII*:KIXX4 (9) gave similar results. During growth in N-free Burks medium with 10 mM sucrose, the exponential growth rate of the wild type and the FeSII deletion mutant were essentially indistinguishable (9). In this low-carbon medium, the viable cell count for both strains began to level off at 1.6×10^8 to 1.9×10^8 cells per ml. By closely monitoring cell growth as reflected by the OD at 30-min intervals (data not shown), the transition point for cessation of exponential growth to the start of stationary phase could be reproducibly identified, and that point is referred to as the $t = 0$ point. At that same point,

* Corresponding author. Mailing address: University of Georgia, Department of Microbiology, 815 Biological Sciences Bldg., Athens, GA 30602-2605. Phone: (706) 542-2323. Fax: (706) 542-2674. E-mail: rmaier@arches.uga.edu.

† Present address: The Monsanto Company, St. Louis, MO 63198.

TABLE 1. Viable cell number (% initial) upon depletion of carbon substrate (at $t = 0$) in wild type and in an FeSII$^-$ mutant strain of *A. vinelandii*[a]

Medium and strain (% O_2)	Mean viable cell no. (±SD) at:		
	1.5 h	3 h	4.5 h
N-free medium[b]			
WT (20)	108 ± 13	129 ± 15	138 ± 5
FeSII$^-$ (20)	74 ± 13	66 ± 10	72 ± 8
WT (40)	82 ± 8	80 ± 14	117 ± 23
FeSII$^-$ (40)	64 ± 4	44 ± 11	39 ± 7
NH_4^+ medium			
WT (20)	116 + 6	113 ± 9	143 ± 4
FeSII$^-$ (20)	136 ± 5	152 ± 12	142 ± 8
WT (40)	128 ± 10	146 ± 11	152 ± 10
FeSII$^-$ (40)	126 ± 11	141 ± 6	149 ± 7

[a] The $t = 0$ (100%) viable cell numbers (at an OD_{585} of approximately 0.60 to 0.64) was about the same for the wild type (WT) (2.02×10^6/ml) and the mutant (1.88×10^6/ml). Cultures were grown in 300-ml baffled sidearm flasks with 25 ml of culture media (Burk plus 10 mM sucrose) and were shaken (210 rpm) at 30°C. Ammonium media contained 25 mM ammonium acetate. The plating medium was Burk plus ammonium. Results are the mean ± the standard deviation for five replicate plates.

[b] Statistical analysis (Student t test) of the results for the N-free medium showed that the FeSII$^-$ strain values were significantly less ($\alpha' = 0.01$, confidence level of 99%) than the wild-type values at all points for the 20% O_2 values and for the 3-h and 4.5-h 40% O_2 values. The FeSII$^-$ strain is significantly less than the wild type at $\alpha' = 0.05$ for the 1.5-h 40% O_2 values (N-free medium).

viable cell numbers were determined by plate counts for the cultures under the various conditions. The $t = 0$ point is deemed the 100% viability level for ease of data interpretation. As can be seen for the experiment shown in Table 1, the viable cell counts of a nitrogen-fixing culture of the wild type increased slowly after the $t = 0$ point, but the viable cell counts for the FeSII mutant in 20% O_2 had decreased, to 72% of the $t = 0$ level after 4.5 h. Due to the number of replicates performed, the value for the mutant even at the 1.5 h point is statistically significantly less than that for the wild type (see Table 1 footnote). This loss of cell viability after the $t = 0$ point is related to the nitrogen-fixing status of the cells; non-nitrogen-fixing cells of either the wild-type or the mutant strain (grown in ammonium-supplemented medium) showed no loss of viability (Table 1) upon cessation of exponential growth.

The loss of cell viability seen in the FeSII deletion strain was also dependent on the level of oxygen. When the O_2 concentration was increased to 40% at $t = 0$ and the flasks were stoppered, the viability of both the wild type and the FeSII mutant were adversely affected; however, the FeSII strain was more sensitive to oxygen-mediated killing (Table 1); at 4.5 h it had only 39% of the viable cell number compared to the $t = 0$ point. We have consistently observed that upon carbon substrate depletion, where respiratory protection is inoperable (9), the FeSII strains lose viability. This viability loss is clearly related to nitrogen fixation as ammonium-grown cells (Table 1) are not adversely affected by oxygen, even by the supra-atmospheric O_2 treatment. Neither the wild type nor the mutant were affected by the exposure to the 40% O_2 level when cells were grown in ammonium medium.

In another experiment, the effect of anaerobic incubation at $t = 0$ was studied to see if the viability loss of the FeSII strain was indeed due to oxygen exposure. At the $t = 0$ point the cultures of the wild type and an FeSII strain were stoppered and flushed with 100% argon and then the flasks were returned to the shaker (see Table 2 footnote). To exclude oxygen as much as possible, cell sampling at the 1.5-h time intervals and dilution for plating was also done under conditions to exclude oxygen. This required the use of serum stopper-sealed tubes, argon-sparged dilution media, and syringes for transfer of cells. It may be important that the dilution media contained sucrose in addition to ammonium (see Table 2 footnote) so that respiratory protection could begin to operate soon after samples were removed from the flasks. Indeed, we found that respiratory rates (as assayed by O_2 electrode) resumed normal levels within 2 min upon placing the Ar-incubated C-starved cells into the carbon-containing dilution medium. The cell viability results for the wild type and the mutant strain were then compared to the normal aerobic condition, like the conditions that had been used for the Table 1 results. The viable cell counts for argon-incubated cultures of both the wild type or the deletion mutant were less than for the same time points corresponding to aerobically incubated wild type but, more importantly, the argon-treated mutant was similar to the wild type in its viability in argon. Considering the adverse affect of anaerobiosis alone on the wild type, the results indicate the O_2-dependent killing affect on the FeSII mutant upon carbon starvation is indeed due to oxygen and not to some other factor. In addition to the viability results shown (Tables 1 and 2), we have determined that the spontaneous mutation rate (to rifampin resistance) is two- to threefold greater in the FeSII mutant strain than the wild type when cells are shifted to 40% partial pressure O_2 at the $t = 0$ time point (and plated on rifampin-containing media [25 μg/ml] at the 3-h time point). In a typical experiment the number of Rif^r mutants per 10^8 cells (mean ± the standard deviation for five replicate samples) was 2.6 ± 0.3 for the wild type and 6.4 ± 0.3 for the mutant strain.

The FeSII protein does not have superoxide dismutase or catalase activities (F. Moshiri and R. J. Maier, unpublished), so that direct detoxification of partially reduced oxygen species was ruled out as a mechanism for O_2 detoxification by the FeSII protein. Nevertheless, we considered the possibility that the viability loss in the FeSII strain could be related to superoxide levels in the cell (perhaps generated by exposure of nitrogenase to oxygen [see above]). If this were true the viability loss associated with the FeSII deletion mutation would be more pronounced if the *A. vinelandii* superoxide dismutase (SOD) levels could be reduced. SOD levels in *Escherichia coli*

TABLE 2. Effect of anaerobic incubation of cells at the time of carbon depletion ($t = 0$) in the wild type and in an FeSII$^-$ mutant strain[a]

Strain (gas condition)	Mean (% of initial) viable cell no. (± SD) at:		
	1.5 h	3 h	4.5 h
WT (20% O_2)	101 ± 17	131 ± 10	136 ± 8
FeSII$^-$ (20% O_2)	78 ± 12	69 ± 11	74 ± 5
WT (argon)	86 ± 7	85 ± 5	83 ± 8
FeSII$^-$ (argon)	92 ± 4	103 ± 12	97 ± 7

[a] Sidearm flasks with media as described in Table 1 footnote were removed when cells reached an OD_{585} of 0.60 ($t = 0$), and one set of flasks was tightly stoppered and flushed with argon for 5 min by use of inflow and outflow needles through the stopper. The four flasks were returned to the shaker, and samples taken for determination of viable cell number at 1.5, 3, and 4.5 h after $t = 0$. Dilutions were done to minimize O_2 exposure (use of argon-flushed dilution media and Ar-sparged stoppered tubes), and 5 mM sucrose was included in the Burk (plus N) dilution medium. According to the Student's t distribution test, data for the argon-incubated samples of both strains were significantly less than for the O_2-incubated samples of the wild type at $\alpha' = 0.01$ (99% confidence level) for the 3- and 4.5-h samples. In comparing the wild type (WT) and the mutant in 20% O_2, the mutant results are statistically significantly less than those for the wild type at $\alpha' = 0.01$ for the 3- and 4.5-h time points.

TABLE 3. Effect of iron deficiency on SOD activity and viable cell number upon depletion of carbon substrate (at $t = 0$) in the wild type and in a mutant strain of *A. vinelandii*[a]

Strain and iron supplementation (mg/liter) in Burk (no N) medium	SOD activity at $t = 0$ (U/mg of protein)	% Viable cell no. at:		
		0 h	1.5 h	4.5 h
FeSII$^-$				
0.015	1.6 ± 0.5	100	61 ± 9	32 ± 6
0.05	1.8 ± 0.3	100	59 ± 7	34 ± 4
0.5	6.9 ± 2.2	100	85 ± 12	70 ± 12
1.0	7.1 ± 1.3	100	76 ± 13	73 ± 9
3.0	7.6 ± 2.0	100	84 ± 10	68 ± 15
Wild type				
0.015	1.0 ± 0.4	100	93 ± 4	92 ± 8
0.05	1.1 ± 0.6	100	115 ± 8	96 ± 11
0.5	5.9 ± 0.9	100	96 ± 12	120 ± 15
1.0	6.3 ± 2.0	100	109 ± 13	118 ± 15
3.0	5.9 ± 2.1	100	114 ± 8	132 ± 6

[a] The medium was Burk containing 10 mM sucrose (without iron) and was supplemented with $FeCl_3$ to the iron level indicated. Dilutions were done in Burk plus N (see text). For SOD activities, cells were harvested and then washed in ice-cold 50 mM HEPES buffer (pH 7.4), and cell extracts were prepared by French pressure cell lysis as described previously (8). Samples (25 μl) containing between 5 to 6.5 mg of protein per ml were added to cuvettes containing 1.5 ml of 0.05 M potassium phosphate (pH 7.8) with all of the other ingredients as indicated previously (7), and 6 μl of xanthine oxidase (Sigma grade IV) was added to start the assay. The viable cell numbers for the two lowest iron supplements are significantly lower than the values for the other iron levels at $\alpha' = 0.01$ (Student's t test analysis) for the FeSII$^-$ strain. Results are mean ± the standard deviation for five replicate plates (viable cell number data) or three replicate assays (SOD activities).

are subject to regulatory mechanisms that are in turn regulated by iron levels supplied to the cells (10, 15, 17). We therefore attempted to produce variable SOD levels in *A. vinelandii* by varying the iron supply, to perhaps correlate the viability loss with SOD levels. SOD levels were determined essentially as described by McCord and Fridovitch (7), by monitoring cytochrome *c* reduction by determining the absorbance at 550 nm over a 3- to 4-min period on a Perkin-Elmer model 557 dual-beam spectrophotometer. One unit of activity is the amount required to inhibit the rate of reduction of cytochrome *c* by 50%, in our case to a rate of 0.013 units per min; all conditions were as described previously (7) except that 0.02 mM cytochrome *c* was used. Also, the sample size was 1.5 ml in a quartz semimicro quartz cuvette (10-mm pathlength). Xanthine (grade sigmaultra) and cytochrome *c* (type VI, horse heart) were from Sigma Chemical Co.

As the iron level was decreased in the N-free medium, the SOD activity of cell extracts also decreased for both strains (Table 3). There was a correlation in loss of subsequent viable cell number with lowered SOD levels for the FeSII$^-$ strain; in the two iron levels at which the SOD levels were <20% of the other iron-supplemented levels, the viable cell number (at $t =$ 4.5 h) was about one-half of that in the other iron supplement regimes. Still, the conditions consisting of the highest iron supplement, i.e., the 3.0-mg/ml level (and the highest SOD level) did not confer complete protection from killing; 68% of the $t = 0$ cell number was obtained at 4.5 h. At the highest iron supplementation the mutant still had only about one-half of the viable cell number achieved by the wild type at $t = 4.5$ h. The wild-type viability was essentially unaffected by iron starvation and therefore by the low SOD activities. The results are interesting in that they correlate the loss of viability with loss of SOD activity for the FeSII$^-$ strain, but further studies are needed to firmly conclude that the killing effect by O_2, when cells lack the Shethna protein, is due to nitrogen fixation-generated toxic oxygen species. Also, the iron deprivation affect may be especially complex, as the many regulators of antioxidant activities (see reference 14) may themselves by subject to iron level-dependent regulation. The results shown here are strictly correlative.

Considering that nitrogenase can account for ca. 10% of the total *Azotobacter* cell protein (1) and therefore represent a significant source of iron-sulfur clusters for O_2-dependent oxidation, the damaging O_2-derived partially reduced species may accumulate to toxic levels. A by-product of iron-sulfur cluster damage is that large amounts of iron may be released, furthering the cell damage by causing (in conjunction with H_2O_2) oxidation of DNA (see reference 14). A number of redox titration experiments performed using redox mediators and various O_2 levels have demonstrated that both the MoFe and Fe proteins of nitrogenase can exist in a number of reduced, oxidized, or intermediate redox states (18). It is likely that in the initial formation of the oxygen stable complex, the FeSII protein by virtue of its intermediate redox potential (8), can act as a redox mediator between the MoFe and Fe protein and O_2, maintaining the components in a stable oxidized state. In the absence of the FeSII protein, however, oxidation of the nitrogenase components rapidly proceeds to an irreversibly oxidized, inactive state, and toxic O_2-derived products may be generated as a consequence. In its role of preventing O_2 from inactivating nitrogenase, another role of the Shethna protein may be to prevent the generation of oxygen-derived radicals which would have more severe cytotoxic affects than the cessation of N_2 fixation. Perhaps *Azotobacter* sp. has other mechanisms in place in order to adapt to the extra stress that is a result of possessing the oxygen sensitive N_2 fixation machinery. For example, it is interesting to note that SOD levels were reported to be twofold greater in *Azotobacter* cells while fixing N_2 compared to levels determined for NH_4^+-grown cells (2).

This work was supported by grant NRICGP 93-37305-6583 to R.J.M. from the U.S. Department of Agriculture.

REFERENCES

1. **Dingler, C., J. Kuhla, H. Wassink, and J. Oelze.** 1988. Levels and activities of nitrogenase proteins in *Azotobacter vinelandii* grown at different dissolved oxygen concentrations. J. Bacteriol. **170:**2148–2152.
2. **Dingler, C., and J. Oelze.** 1987. Superoxide dismutase and catalase in *Azotobacter vinelandii* grown in continuous culture at different dissolved oxygen concentrations. Arch. Microbiol. **147:**291–294.
3. **Gallon, J. R.** 1992. Tansley review no. 44: reconciling the incompatible: N_2 fixation and O_2. New Phytol. **122:**571–609.
4. **Hill, S.** 1988. How is nitrogenase regulated by oxygen? FEMS Microbiol. Rev. **34:**11–130.
5. **Imlay, J.** 1995. A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*. J. Biol. Chem. **270:**19767–19777.
6. **Lou, J., F. Moshiri, M. K. Johnson, M. E. Lafferty, D. L. Sorkin, A.-F. Miller, and R. J. Maier.** 1999. Mutagenesis studies of the FeSII protein of *Azotobacter vinelandii*: roles of histidine and lysine residues in the protection of nitrogenase from oxygen damage. Biochemistry **38:**5563–5571.
7. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. **244:**6049–6055.
8. **Moshiri, F., B. R. Crouse, M. K. Johnson, and R. J. Maier.** 1995. The "Nitrogenase-protective" FeSII protein of *Azotobacter vinelandii*: overexpression, characterization, and crystallization. Biochemistry **34:**12973–12982.
9. **Moshiri, F., J. W. Kim, C. Fu, and R. J. Maier.** 1994. The FeSII protein of *Azotobacter vinelandii* is not essential for aerobic nitrogen fixation, but confers significant protection to oxygen-mediated inactivation of nitrogenase. Mol. Microbiol. **14:**101–114.
10. **Niederhoffer, E. C., C. M. Naranjo, K. L. Bradley, and J. A. Fee.** 1990. Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric iron uptake regulation (*fur*) locus. J. Bacteriol. **172:**1930–1938.
11. **Robson, R. L.** 1979. Characterization of an oxygen-stable nitrogenase complex isolated from *Azotobacter chroococcum*. Biochem. J. **181:**569–575.
12. **Robson, R. L., and J. R. Postgate.** 1980. Oxygen and hydrogen in biological

nitrogen fixation. Annu. Rev. Microbiol. **34:**183–207.
13. **Scherings, G., H. Haaker, H. Wassink, and C. Veeger.** 1983. On the formation of an oxygen tolerant three component nitrogenase complex from *Azotobacter vinelandii*. Eur. J. Biochem. **135:**591–599.
14. **Storz, G., and J. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:** 188–194.
15. **Tardat, B., and D. Touati.** 1991. Two global regulators repress the anaerobic expression of MnSOD in *E. coli*: Fur (ferric uptake regulation) and Arc (aerobic respiration control). Mol. Microbiol. **5:**455–465.
16. **Thorneley, R. N. F., and G. A. Ashby.** 1989. Oxidation of nitrogenase iron protein by dioxygen without inactivation could contribute to high respiration rates of *Azotobacter* species and facilitate nitrogen fixation in other aerobic environments. Biochem. J. **261:**181–187.
17. **Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despied.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. **177:** 2305–2314.
18. **Wang, Z.-C., A. Burns, and G. D. Watt.** 1985. Complex formation and O_2 sensitivity of *Azotobacter vinelandii* nitrogenase and its component proteins. Biochemistry **24:**214–221.