Response of the Endophytic Diazotroph *Gluconacetobacter diazotrophicus* on Solid Media to Changes in Atmospheric Partial O₂ Pressure

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*Gluconacetobacter diazotrophicus* is an N₂-fixing endophyte isolated from sugarcane. *G. diazotrophicus* was grown on solid medium at atmospheric partial O₂ pressures (pO₂) of 10, 20, and 30 kPa for 5 to 6 days. Using a flowthrough gas exchange system, nitrogenase activity and respiration rate were then measured at a range of atmospheric pO₂ (5 to 60 kPa). Nitrogenase activity was measured by H₂ evolution in N₂-O₂ and in Ar-O₂, and respiration rate was measured by CO₂ evolution in N₂-O₂. To validate the use of H₂ production as an assay for nitrogenase activity, a non-N₂-fixing (Nif⁻) mutant of *G. diazotrophicus* was tested and found to have a low rate of uptake hydrogenase (Hup⁺) activity (0.016 ± 0.009 μmol of H₂ 10¹⁰ cells⁻¹ h⁻¹) when incubated in an atmosphere enriched in H₂. However, Hup⁺ activity was not detectable under the normal assay conditions used in our experiments. *G. diazotrophicus* fixed nitrogen at all atmospheric pO₂ tested. However, when the assay atmospheric pO₂ was below the level at which the colonies had been grown, nitrogenase activity was decreased. Optimal atmospheric pO₂ for nitrogenase activity was 0 to 20 kPa above the pO₂ at which the bacteria had been grown. As atmospheric pO₂ was increased in 10-kPa steps to the highest levels (40 to 60 kPa), nitrogenase activity decreased in a stepwise manner. Despite the decrease in nitrogenase activity as atmospheric pO₂ was increased, respiration rate increased marginally. A large single-step increase in atmospheric pO₂ from 20 to 60 kPa caused a rapid 84% decrease in nitrogenase activity. However, upon returning to 20 kPa of O₂, 80% of nitrogenase activity was recovered within 10 min, indicating a “switch-off/switch-on” O₂ protection mechanism of nitrogenase activity. Our study demonstrates that colonies of *G. diazotrophicus* can fix N₂ at a wide range of atmospheric pO₂ and can adapt to maintain nitrogenase activity in response to both long-term and short-term changes in atmospheric pO₂.

*Gluconacetobacter diazotrophicus* (47) (previously known as *Acetobacter diazotrophicus* [15]) is a strict aerobe and an N₂-fixing endophyte originally isolated from sugarcane roots and stems (6). It has been estimated that *G. diazotrophicus* can fix up to 150 kg of N ha⁻¹ year⁻¹ in sugarcane (2). Such high levels of N₂ fixation have not been reported in any other system outside legume-rhizobium symbioses. The bacterium has subsequently been isolated from sweet potato (38), coffee (23), pineapple (44), sorghum (22), finger millet (31), and several other tropical grass species (24).

Aerobic endophytic diazotrophs require a high flux of O₂ to their respiratory systems to enable an adequate supply of reductant and ATP to support N₂ fixation (e.g., see reference 13), yet paradoxically, an excessive flux of O₂ to the bacterium can result in an inhibition of nitrogenase activity (14, 21, 26). The inhibition of nitrogenase activity by O₂ in aerobic diazotrophs can be reversible or irreversible, depending on the organism and the nature (i.e., duration and severity) of the increase in O₂ flux (33, 37, 39). Reversible inhibition of nitrogenase activity (i.e., a temporary “switch-off” of the nitrogenase activity while O₂ flux is excessive) can be due to a conformational change in nitrogenase, as seen in *Azotobacter* (11, 32), to an ADP-ribosylation of dinitrogen reductase, as seen in the purple nonsulfur bacteria (46) and *Azospirillum* (49), or to a diversion of electrons from nitrogenase to other reduction pathways, as proposed for *Azotobacter* (16, 29).

*G. diazotrophicus* has the ability to fix N₂ at ambient atmospheric partial O₂ pressures (pO₂) (i.e., approximately 20 kPa of O₂) in semisolid medium (6) and as colonies on solid medium (10). The ability to fix N₂ in colonies on solid medium is especially interesting, as there is evidence that *G. diazotrophicus* exists in situ in the intercellular spaces of sugarcane vascular tissue as mucoid microcolonies (9). Dong (8) also reported that colony morphology on solid medium and the relative distribution of the bacteria within these highly mucilaginous colony changed with changes in the partial pressure of O₂ surrounding the colonies.

Reis and Döbereiner (40) measured nitrogenase activity in liquid cultures of *G. diazotrophicus* by acetylene reduction in closed batch assays and found that activity was maximal when the culture was at equilibrium with 0.2 kPa of O₂ in the gas phase. However, nitrogenase activity of *G. diazotrophicus* grown in colonies on solid medium in response to changes in atmospheric pO₂ has not yet been well characterized. Given that *G. diazotrophicus* exists in situ as microcolonies adhering to plant cell walls (9), characterization of the response of the bacterium on solid medium to changes in atmospheric pO₂ is particularly relevant.

The objective of our study was to characterize the effect of atmospheric pO₂ on nitrogenase activity of *G. diazotrophicus* grown on solid medium using flowthrough gas exchange mea-
measurements. Treatments included long-term growth of the bacterium on a range of atmospheric \( \text{pO}_2 \) (10 to 30 kPa) and subsequent rapid changes in atmospheric \( \text{pO}_2 \) in small (5- to 10-kPa) and large (40-kPa) steps. We found that nitrogenase activity by \( G. \text{diazotrophicus} \) is adaptive to both short-term and long-term changes in atmospheric \( \text{pO}_2 \) and that the bacterium has a switch-off/switch-on mechanism for protection of nitrogenase from rapid changes in atmospheric \( \text{pO}_2 \).

**MATERIALS AND METHODS**

**Organism and culture.** \( G. \text{diazotrophicus} \) PAL-5 (ATCC 49037; obtained from the American Type Culture Collection, Manassas, Va.) was cultured for 2 days at 30°C, shaken at 150 rpm in LGI-P liquid medium (M. McCully [Carleton University], personal communication), a modified version of LGI medium (6). LGI-P medium differs from the original LGI medium in containing 0.02 g of Na\(_2\)MoO\(_4\), 2H\(_2\)O liter\(^{-1}\), 0.1 mg of biotin liter\(^{-1}\), 0.2 mg of pyridoxal HCl liter\(^{-1}\), and 5 ml of sucargene juice (pressed from fresh sugarcane stem) liter\(^{-1}\), and the final pH was adjusted to 5.5 using 1% acetic acid. Diluted cells were spread on solid LGI-P agar medium (15 g of agar liter\(^{-1}\) plus 50 mg of yeast extract liter\(^{-1}\) before serial dilution.) A 5-mm diameter colony was vortexted with glass beads to prevent clumping of the colonies and to obtain an even distribution of individual separate colonies on the petri plates.

\( G. \text{diazotrophicus} \) was grown on solid LGI-P medium in petri plates for 5 or 6 days at 30°C prior to gas exchange measurements. Cultures were grown under ambient atmospheric \( \text{pO}_2 \) (approximately 20 kPa) or in a gas exchange chamber (see below) with 10 or 30 kPa of \( \text{O}_2 \) and delivery, an infrared gas \( \text{CO}_2 \) analyzer (ADC-225MKS; Analytical Development Co. Ltd., Hoddesdon, United Kingdom) for measurement of respiration rate, and an \( \text{H}_2 \) analyzer (27) for measurement of nitrogenase activity. A chamber with inner dimensions of 50 by 20 by 5 cm with four shelves for holding up to 40 petri plates was constructed in a small (5- or 10-kPa) and large (40-kPa), single-step increases and decreases in atmospheric \( \text{pO}_2 \). Gas exchange measurements were initiated at the atmospheric \( \text{pO}_2 \) under which the bacteria had grown (approximately 20 kPa), and then atmospheric \( \text{pO}_2 \) was increased in a single step to 60 kPa, where it remained for approximately 15 min before being returned to 20 kPa in a single step. All gas exchange measurements were made at room temperature (22 ± 1°C). Preliminary experiments showed that once steady-state rates of \( \text{H}_2 \) and \( \text{CO}_2 \) evolution were reached, they remained steady for many hours (i.e., up to 12 h).

Production of \( \text{H}_2 \) is an obligate reaction of the nitrogenase enzyme complex during the fixation of \( \text{N}_2 \) (4). The rate of \( \text{H}_2 \) evolution in \( \text{N}_2\text{-O}_2 \) is a measure of partial or “apparent” nitrogenase activity (i.e., proton reduction to \( \text{H}_2 \) by nitrogenase in the presence of \( \text{N}_2 \) fixation) (21, 26). The rate of \( \text{H}_2 \) evolution in Ar-\( \text{O}_2 \) is a measure of total nitrogenase activity (i.e., in the absence of \( \text{N}_2 \) as a substrate, total electron flow through nitrogenase is reduced to \( \text{H}_2 \) (20, 21, 26). In \( \text{N}_2\text{-O}_2 \), the proportion of total electron flow through nitrogenase being directed to \( \text{N}_2 \) fixation is known as the electron allocation coefficient (EAC) (12) and is calculated as 1 – (\( \text{H}_2 \) evolution in \( \text{N}_2\text{-O}_2 \)/\( \text{H}_2 \) evolution in Ar-\( \text{O}_2 \)). EAC can be viewed as a measure of an aspect of nitrogenase “efficiency” (i.e., the higher the EAC, the greater the proportion of electrons going to the “wasteful” process of proton reduction). The accuracy of measuring nitrogenase activity by \( \text{H}_2 \) evolution is dependent upon a lack of hydrogenase activity leading to \( \text{H}_2 \) production or consumption by the test organism under the assay conditions. Experiments with a non-\( \text{N}_2 \)-fixing (Nifmutant of \( G. \text{diazotrophicus} \) (strain MAD3A) (42) were performed to determine if \( \text{H}_2 \) evolution from \( G. \text{diazotrophicus} \) was associated only with nitrogenase activity and if the bacterium had hydrogenase uptake (Hup) activity. The Nifmutant was designed by insertional mutagenesis of the nifD gene of wild-type \( G. \text{diazotrophicus} \). The resulting mutant strain (MAD3A) was generously donated by C. Kennedy, University of Arizona. \( G. \text{diazotrophicus} \) MAD3A was grown and handled as described above for wild-type \( G. \text{diazotrophicus} \) PAL5 except that 200 mg of kanamycin ml\(^{-1}\) was added to the medium. The growth rate of the Nifmutant was not significantly different from that of wild-type \( G. \text{diazotrophicus} \) for the first 5 days of culture.

Three replicates of 40 plates each of \( G. \text{diazotrophicus} \) MAD3A were tested for \( \text{H}_2 \) production in air and Ar-\( \text{O}_2 \) (80:20) in preliminary experiments in our gas exchange system. MAD3A did not produce \( \text{H}_2 \) production under any conditions (data not shown).

Hydrogenase uptake activity by \( G. \text{diazotrophicus} \) was assessed in flowthrough and closed-assay systems. For the flowthrough assay, MAD3A was grown for 4 days on \( \text{H}_2 \)-containing \( \text{pO}_2 \) (described above). Three replicates of 20 petri plate cultures were then placed in the gas exchange chamber and flushed continuously with air containing 2 ppm (vol/vol) of \( \text{H}_2 \) at a flow rate of 20 ml min\(^{-1}\) for approximately 24 h. This concentration of \( \text{H}_2 \) was used because it is the typical level of \( \text{H}_2 \) evolution from wild-type \( G. \text{diazotrophicus} \) in air under our normal assay conditions. After exposure to 2 ppm of \( \text{H}_2 \) for 24 h, the gas flow rate was increased to 500 ml min\(^{-1}\) (the normal flow rate for our assays) and the concentration of \( \text{H}_2 \) exiting the chamber was measured. For the closed assay, wild-type and Nifmutant strains of \( G. \text{diazotrophicus} \) were grown on solid medium for 4 days. On the fifth day, 10 petri plate cultures were placed in the gas exchange chamber and the chamber was flushed with 50 ppm of \( \text{H}_2 \) in air at flow rate of 20 ml min\(^{-1}\). After 24 h at this flow rate, the chamber was sealed, and evolution (wild-type strain) and consumption (Nif strain) were monitored immediately after sealing and then every 30 to 60 min for the next 6 to 8 h. Gas samples (1 ml) were taken from the chamber and injected into an air stream entering the \( \text{H}_2 \) analyzer at a flow rate of 300 ml min\(^{-1}\) for analysis as described by Layzell et al. (27). Hydrogen consumption and evolution rates were calculated by linear regression. The tests were replicated four times each for the wild-type and Nif strains of \( G. \text{diazotrophicus} \).

The aerobic, facultative chemolithoautotroph \( Ralstonia eutrophus \) (ATCC 17699) was used as a positive control in the assessment of \( \text{H}_2 \) uptake. Early-log-phase cells grown in Difco 0003 liquid medium (Becton Dickinson, Franklin Lakes, N.J.) were plated onto Difco 0001 solid medium and grown for 4 days before being assayed. \( \text{H}_2 \) consumption by these colonies was assay as described above for \( G. \text{diazotrophicus} \) MAD3A (i.e., closed assays at 50 ppm of \( \text{H}_2 \) for 8 h).
Four experiments were conducted to test the response of *G. diazotrophicus* to changes in atmospheric pO$_2$. The experiments consisted of (i) testing responses of *G. diazotrophicus* grown at 20 kPa of O$_2$ to small (5- or 10-kPa) stepped changes in atmospheric pO$_2$; (ii) testing responses of *G. diazotrophicus* grown at 20 kPa of O$_2$ to a large (40-kPa) stepped change in atmospheric pO$_2$; (iii) testing responses of *G. diazotrophicus* grown at 10 kPa of O$_2$ to small (5- or 10-kPa) stepped changes in atmospheric pO$_2$; and (iv) testing responses of *G. diazotrophicus* grown at 30 kPa of O$_2$ to small (10-kPa) stepped changes in atmospheric pO$_2$. Gas exchange measurements in a single chamber containing 20 to 40 petri plates of *G. diazotrophicus* cultures was considered a single replicate. For each experiment, gas exchange measurements were replicated four times. All data were normalized by calculating gas evolution per cell (cell number was determined for each replication of each experiment; see enumeration method above). Data were analyzed using the general linear model of the SAS statistical package (SAS Institute, Cary, N.C.), assuming a completely randomized design, and mean separation was tested using the least-significant-difference procedure ($P$ value of $\leq 0.05$).

**RESULTS**

**Effects of small stepped changes in atmospheric pO$_2$ on *G. diazotrophicus* grown at 20 kPa of O$_2$.** For *G. diazotrophicus* grown at 20 kPa of O$_2$, 10-kPa stepped increases in atmospheric pO$_2$ above 30 kPa of O$_2$ resulted in a decrease in total nitrogenase activity (H$_2$ evolution in Ar-O$_2$) (Fig. 1). However, nitrogenase was still active even at atmospheric pO$_2$ of 60 kPa (29% of the rate at 20 kPa of O$_2$). Stepped decreases in atmospheric pO$_2$ from 20 to 10 to 5 kPa also resulted in decreases in total nitrogenase activity. The optimal atmospheric pO$_2$ for *G. diazotrophicus* grown at 20 kPa of O$_2$ were 20 and 30 kPa of O$_2$.

Stepped increases of 10 kPa of O$_2$ above 20 kPa had no significant effect on the EAC of nitrogenase activity (Fig. 2). However, as atmospheric pO$_2$ was lowered from 20 kPa to 10 and 5 kPa of O$_2$, the EAC decreased.

As atmospheric pO$_2$ was increased from 20 to 60 kPa of O$_2$ in 10-kPa steps, the respiration rate of *G. diazotrophicus* cells increased marginally (Fig. 3). For example, the threefold increase in atmospheric pO$_2$ from 20 to 60 kPa resulted in an 11% increase in CO$_2$ evolution per cell. In contrast, decreasing atmospheric pO$_2$ from 20 to 10 kPa and then 5 kPa resulted in severe decreases in respiration rates of 39 and 51%, respectively.

**Effects of a large (40-kPa) stepped change in atmospheric pO$_2$ on *G. diazotrophicus* grown at 20 kPa of O$_2$.** When *G. diazotrophicus* colonies grown at atmospheric pO$_2$ of 20 kPa were exposed to a 40-kPa single-step increase in atmospheric pO$_2$, nitrogenase activity decreased rapidly and severely (Fig. 4). After this decrease, nitrogenase activity at 60 kPa of O$_2$ was steady at approximately 26% of the activity at 20 kPa of O$_2$. After 15 min at 60 kPa of O$_2$, oxygen concentration was then switched back to 20 kPa, and nitrogenase activity increased almost immediately. Within 10 min of returning to 20 kPa of O$_2$, nitrogenase activity by *G. diazotrophicus* had recovered to approximately 80% of the original activity. Changes in nitrogenase activity (Fig. 4) and respiration rate (data not shown) in

![FIG. 1. Effect of atmospheric pO$_2$ on total nitrogenase activity (H$_2$ evolution in Ar-O$_2$) of *G. diazotrophicus* colonies grown at 20 kPa of O$_2$. Data are means plus standard errors ($n = 4$). Results with different letters are significantly different at a $P$ value of $\leq 0.05$.](http://aem.asm.org/)

![FIG. 2. Effect of atmospheric pO$_2$ on EACs of nitrogenase of *G. diazotrophicus* grown at 20 kPa of O$_2$. Data are means plus standard errors ($n = 4$). Data with different letters are significantly different at a $P$ value of $\leq 0.05$.](http://aem.asm.org/)

![FIG. 3. Effect of atmospheric pO$_2$ on respiration rate (CO$_2$ evolution in N$_2$-O$_2$) of *G. diazotrophicus* colonies grown at 20 kPa of O$_2$. Data are means plus standard errors ($n = 4$). Data with different letters are significantly different at a $P$ value of $\leq 0.05$.](http://aem.asm.org/)
response to the single-step change from 20 to 60 kPa of O₂ were similar in magnitude (i.e., a 74% decrease in nitrogenase activity and an approximate 10% increase for respiration) to those observed when the increase in from 20 and 60 kPa of O₂ took place in several 10-kPa steps (Fig. 1 and 3).

Effects of small stepped changes in atmospheric pO₂ on G. diazotrophicus grown at 10 or 30 kPa of O₂. G. diazotrophicus colonies were grown under 10 or 30 kPa of atmospheric O₂ for 5 to 6 days and then assayed for total nitrogenase activity at a range of atmospheric pO₂ (5 to 60 kPa) (Fig. 5 and 6). In both cases, maximal nitrogenase activity occurred at 10 to 20 kPa of O₂ above the atmospheric pO₂ at which the colonies had been grown. For colonies grown under 10 kPa of O₂, nitrogenase activity was maximized at 20 and 30 kPa of O₂ (Fig. 5). For colonies grown under 30 kPa of O₂, nitrogenase activity was maximized at 40 kPa of O₂ (Fig. 6).

Hydrogenase uptake activity of G. diazotrophicus. There was no detectable H₂ consumption by the Nif⁻/H₁₁₀₀₂ mutant of G. diazotrophicus (MAD3A) under typical conditions experienced by the wild-type strain when nitrogenase activity was assayed (2 ppm of H₂ in air in a flowthrough system at ambient atmospheric pO₂). However, when the strain was supplied with 50 ppm of H₂ in air in a closed-assay system, we detected a H₂ consumption rate of 0.016 ± 0.009 μmol of H₂ 10¹⁰ cells⁻¹ h⁻¹. This rate is low, as the H₂ evolution rate by wild-type G. diazotrophicus assayed under the same conditions was 0.362 ± 0.027 μmol of H₂ 10¹⁰ cells⁻¹ h⁻¹ and H₂ consumption rate by the Hup⁺ aerobe R. eutropha was 0.080 ± 0.003 μmol of H₂ 10¹⁰ cells⁻¹ h⁻¹.

DISCUSSION

The concentration of O₂ at the sites of nitrogenase activity in aerobic and microaerophilic diazotrophs is the result of the interplay among (i) the concentration of O₂ in the surrounding atmosphere, (ii) the diffusion rate of O₂ from the surrounding atmosphere to the sites of nitrogenase activity, (iii) the consumption rate of O₂ in the vicinity of nitrogenase (predominantly via oxidative phosphorylation), and (iv) the role of carriers of O₂ which facilitate diffusion in some systems (such as leghemoglobin in legume nodules) (21). The present study investigated responses in nitrogenase activity to changes in atmospheric pO₂ around colonies in a flowthrough system; previous studies (5, 40) injected enough pure O₂ into a previously anaerobic, closed liquid system to achieve target pO₂. All these studies enable observation of changes in nitrogenase activity in response to relative changes in O₂ flux to the bac-
teria; however, neither the actual flux of O₂ to the diazotrophs nor the actual concentration of O₂ at the sites of nitrogenase activity was determined.

In our study, nitrogenase activity by *G. diazotrophicus* was tolerant of atmospheric pO₂ as high as 60 kPa (Fig. 1). These findings are not in conflict with previous studies (5, 40) that found that nitrogenase activity by *G. diazotrophicus* in liquid culture was totally inhibited when the culture was at equilibrium with 6 kPa of O₂ in the gas phase. These findings simply reflect that in our study, atmospheric pO₂ surrounding the colonies was changed, and in the previous studies, the partial pressure of dissolved oxygen in liquid cultures was changed. However, comparison of these studies indicates that *G. diazotrophicus* can use the milieu of a colony as an effective resistance to O₂ diffusion, resulting in an O₂ concentration and O₂ flux within the colony which enable the bacteria to fix N₂ in a broad range of atmospheric pO₂ surrounding the colony.

Using H₂ production as a measure of nitrogenase activity in closed-assay systems, Dong et al. (8, 10) showed that *G. diazotrophicus* could fix N₂ in colonies with 2 and 20 kPa of O₂ in the surrounding atmosphere and suggested that colony structure and location of bacteria within the colony played a role in the protection of nitrogenase from excessive O₂ flux. Bacterial mucilage is known to decrease the rate of oxygen diffusion to cells (3). The presence of extracellular polysaccharide surrounding *Beijerinckia derxii* cells is necessary to maintain nitrogenase in this organism (1). *Derxia gummosa* forms small nonfixing colonies if grown at 20 kPa of O₂; however, if grown at 5 kPa of O₂, the bacterium forms large, highly mucilaginous colonies which fix N₂ (17, 18). The motile diazotroph *Azospirillum brasilense* (50) is known to display aerotaxis within suspensions to achieve the appropriate O₂ environment for N₂ fixation.

For colonies grown at 20 kPa of O₂ and assayed at the same atmospheric pO₂, total nitrogenase activity was approximately 0.5 μmol of H₂ 10¹⁰ cells⁻¹ h⁻¹ (Fig. 1). Is this a relatively low or high rate of nitrogenase activity? We have compared the level of nitrogenase activity in *G. diazotrophicus* to that of *Bradyrhizobium japonicum* in a typical soybean (*Glycine max* [L.] Merr.) nodule. Based on measurements of nodules on 5-week-old soybean plants, Lin et al. (28) found that nodules contained approximately 10⁸ B. japonicum bacteroids each and that total nitrogenase activity was in the range of 2.0 to 4.0 μmol of H₂ 10¹⁰ cells⁻¹ h⁻¹. Nitrogenase activity for *G. diazotrophicus* colonies at ambient atmospheric pO₂ in our study was approximately 12 to 25% of the rates calculated for *B. japonicum* in soybean nodules. We consider such a level of nitrogenase activity by *G. diazotrophicus* in colonies to be remarkably high considering that a soybean nodule is a highly sophisticated organ designed to provide a highly conducive milieu (in terms of O₂ flux, carbon supply, assimilation of fixed N, etc.) for bacteroids to fix N₂.

Our study is the first measure of EACs for *G. diazotrophicus*. We found that the EAC of *G. diazotrophicus* at 20 kPa of O₂ was approximately 0.6 (Fig. 2), meaning that in air, approximately 60% of electron flow through nitrogenase would be allocated to reduction of dinitrogen and 40% would be allocated to proton reduction. Again, the EAC of *G. diazotrophicus* can be put into context by comparing it to that of rhizobia in legume nodules. The theoretical maximum for EAC is 0.75 (i.e., at least one H₂ produced for every N₂ fixed by nitrogenase) (43). The EACs of legume symbioses are commonly between 0.59 and 0.70 (21). The reason for the variability in the EAC is not clearly understood (19, 26). Our measurements of the EAC for nitrogenase in *G. diazotrophicus* grown on solid medium at ambient atmospheric pO₂ is in the same range as EACs commonly observed in legume nodules.

The accuracy of measurements of nitrogenase activity by H₂ evolution is dependent upon the lack of hydrogenase uptake activity (21, 26). Although the Nif⁺ mutant of *G. diazotrophicus* was seen to have a low level of Hup⁺ activity when supplied with relatively high levels of H₂ (50 ppm) in a closed-assay system, under the standard conditions in our flowthrough assay system (i.e., 2 ppm of H₂), Hup⁺ activity was not detectable.

Small (5- to 10-kPa) decreases in atmospheric pO₂ resulted in declines in nitrogenase activity and respiration rate in *G. diazotrophicus* grown at 20 kPa of O₂ (Fig. 1). This is clearly representative of an O₂ limitation of cellular metabolism and has been seen in other aerobically functional N₂-fixing systems, such as *Azotobacter* (48) and soybean nodules (25). However, small stepwise increases in atmospheric pO₂ above 20 kPa also resulted in declines in nitrogenase activity (Fig. 1). The declines in nitrogenase activity with small (10-kPa) increases in atmospheric pO₂ could occur for one of two reasons: (i) an irreversible O₂-induced denaturation of the nitrogenase enzyme or (ii) a reversible controlled down-regulation of nitrogenase activity (14). The time course of nitrogenase activity in response to single-step, 40-kPa changes in atmospheric pO₂ (Fig. 4) indicates that the latter and not the former mechanism is at work in *G. diazotrophicus*. The rapid decrease in nitrogenase activity when atmospheric pO₂ was switched from 20 to 60 kPa, and the subsequent rapid recovery when the bacteria returned to 20 kPa, indicate that *G. diazotrophicus* has a switch-off/switch-on protection mechanism in response to changes in atmospheric pO₂.

Reversible inhibition of nitrogenase activity has been seen in a number of diazotrophs in response to increases in pO₂, and to the addition of ammonium. Three underlying physiological mechanisms have been associated with switch-off/switch-on kinetics of nitrogenase in diazotrophs. The switch-off/switch-on mechanism can be the result of an O₂-induced conformational change in nitrogenase as seen in the Mo-dependent nitrogenase system of *Azotobacter* (11, 32, 35, 36, 41). Switch-off/switch-on mechanisms can also be facilitated by an ADP-ribosylation of dinitrogen reductase which halts nitrogenase activity. This mechanism is coded for by the *draT* and *draG* genes and has been observed in a number of diazotrophs, including *Rhodobacter capsulata* (46), *Rhodospirillum rubrum*, *Azospirillum brasilense*, and *Azospirillum lipoflorum* (34, 49). Finally, the nitrogenase switch-off/switch-on mechanism in a number of diazotrophs may involve diversion of electrons from nitrogenase to other (unidentified) electron acceptors (16) and/or an ATP limitation of nitrogenase activity, possibly due to a switch to uncoupled respiratory chain as proposed for *Azotobacter vinelandii* (29).

Which if any of the above identified switch-off/switch-on mechanisms are at work in *G. diazotrophicus* was not investigated in our study. However, it is highly unlikely that the mechanism involves the ADP-ribosylation of dinitrogen reductase. Although Burris et al. (5) found that *G. diazotrophicus*
had “a rather sluggish” response to ammonium addition and required 10 μM NH₄⁺ to switch off nitrogenase, they found no evidence of ADP-ribosylation of dinitrogen reductase or of the draT-drgG gene complex in G. diazotrophicus. Recently, S. Norlund (personal communication) also found evidence of a switch-off/switch-on phenomenon in G. diazotrophicus in response to changes in pO₂, possibly involving a conformational change in nitrogenase medium by a Shetha-like protein (32).

In this study, long-term adaptation of G. diazotrophicus to different atmospheric pO₂ was tested by growing the bacterium for 5 or 6 days at 10, 20, or 30 kPa of O₂ before nitrogenase activity was measured. Although culture conditions were not exactly the same for all the cultures (see Material and Methods), some trends are consistent in all three treatments. During assays of nitrogenase activity, when atmospheric pO₂ was decreased below the concentration at which the bacteria were cultured, nitrogenase activity was always lower (Fig. 1, 5, and 6). This appears to be due to a generalized O₂ limitation of cellular metabolism (Fig. 3) (25, 48). G. diazotrophicus cultures which were grown under different atmospheric pO₂ also showed different optimal atmospheric pO₂ for nitrogenase activity. The optimal atmospheric pO₂ for cultures grown at 10 and 20 kPa of O₂ was 20 to 30 kPa of O₂ (Fig. 1 and 5); the optimal atmospheric pO₂ for nitrogenase activity for cultures grown at 30 kPa of O₂ was 40 kPa of O₂ (Fig. 6) The fact that the cultures grown at the highest atmospheric pO₂ showed a higher optimal pO₂ for nitrogenase activity indicates a long-term adaptation of G. diazotrophicus colonies to different pO₂. Other aerobically functional N₂-fixing systems such as A. vinelandii (30) and the B. japonicum-soybean symbiosis (7) are known to make long-term adaptations of nitrogenase activity to nonambient pO₂. Dong (8) noted differences in colony morphology of G. diazotrophicus between cultures grown long-term on 2 and 20 kPa of atmospheric pO₂. We are currently investigating whether morphologic and structural characteristics of the colonies contribute to these long-term adaptations.

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REFERENCES


