Evidence for protection of nitrogenase from O$_2$
by colony structure in the aerobic diazotroph
Gluconacetobacter diazotrophicus

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Gluconacetobacter diazotrophicus is an endophytic diazotroph of sugarcane which exhibits nitrogenase activity when growing in colonies on solid media. Nitrogenase activity of G. diazotrophicus colonies can adapt to changes in atmospheric partial pressure of oxygen (pO$_2$). This paper investigates whether colony structure and the position of G. diazotrophicus cells in the colonies are components of the bacterium’s ability to maintain nitrogenase activity at a variety of atmospheric pO$_2$ values. Colonies of G. diazotrophicus were grown on solid medium at atmospheric pO$_2$ of 2 and 20 kPa. Imaging of live, intact colonies by confocal laser scanning microscopy and of fixed, sectioned colonies by light microscopy revealed that at 2 kPa O$_2$ the uppermost bacteria in the colony were very near the upper surface of the colony, while the uppermost bacteria of colonies cultured at 20 kPa O$_2$ were positioned deeper in the mucilaginous matrix of the colony. Disruption of colony structure by physical manipulation or due to ‘slumping’ associated with colony development resulted in significant declines in nitrogenase activity. These results support the hypothesis that G. diazotrophicus utilizes the path-length of colony mucilage between the atmosphere and the bacteria to achieve a flux of O$_2$ that maintains aerobic respiration while not inhibiting nitrogenase activity.

**Keywords:** Acetobacter diazotrophicus, diffusion resistance, N$_2$ fixation, nitrogen, oxygen

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**INTRODUCTION**

Gluconacetobacter diazotrophicus (Yamada et al., 1997), formerly Acetobacter diazotrophicus (Gillis et al., 1989), is an N$_2$-fixing bacterium that inhabits intercellular spaces of sugarcane (Dong et al., 1994). An unusual feature of this bacterium is the ability to fix N$_2$ *in vitro* on semi-solid (Cavalcante & Döbereiner, 1988) and solid media (Dong et al., 1995; Pan & Vessey, 2001) in the presence of relatively high (approx. 20 kPa) partial pressures of O$_2$ (pO$_2$) in the atmosphere. The nitrogenase enzyme is oxygen labile; however in aerobic diazotrophs nitrogenase activity requires substantial amounts of ATP and reductant derived from aerobic respiration (Hunt & Layzell, 1993). Diazotrophs therefore need to protect nitrogenase from O$_2$ inactivation by regulating intracellular concentrations of free O$_2$. The mechanisms by which diazotrophs reduce free O$_2$ concentrations while permitting aerobic respiration has been the subject of much research (Robson & Postgate, 1980; van Cauwenberghe *et al.*, 1993; Oresnik & Layzell, 1994).

Sugarcane does not form any specialized structure to host G. diazotrophicus (Dong *et al.*, 1994) that may aid in the regulation of O$_2$ flux as root nodules do in legume plants (Hunt & Layzell, 1993). Nitrogenase activity by G. diazotrophicus in liquid medium was optimized when the dissolved oxygen content of the medium was equilibrated with 0.2 kPa O$_2$ in the gas phase (Reis & Döbereiner, 1998). However, G. diazotrophicus is able to use N$_2$ as its sole nitrogen source under 21 kPa O$_2$.
on a semi-solid medium (Cavalcante & Döbereiner, 1988). Under these conditions, distinct colonies are not formed; rather, the bacteria grow just below the surface of the media. This behaviour may help to optimize the O₂ flux to the bacterium as seen in other aerotactic diazotrophs (Zhulin et al., 1996). On solid medium, distinct, superficial colonies of G. diazotrophicus form thick, mucilaginous matrices and are able to grow on N₂ as the sole nitrogen source at 20 kPa O₂ (Dong et al., 1994). Pan & Vessey (2001) showed that bacterial respiration and nitrogenase activity by G. diazotrophicus in colonies adapted over long-term exposures (i.e. several days) to different atmospheric pO₂ (10, 20 and 30 kPa). Optimal nitrogenase activity by G. diazotrophicus colonies occurred at or slightly above (i.e. +10 kPa) the O₂ concentrations at which they were grown (Pan & Vessey, 2001).

Since nitrogenase is active in G. diazotrophicus colonies grown on solid media, the bacterium must have means of ensuring an appropriate concentration and flux of O₂ to balance aerobic respiration and nitrogenase activity. In this study, we test the hypothesis that G. diazotrophicus positions itself within the mucilaginous matrix of its colony to achieve an appropriate O₂ environment for nitrogenase activity, and that an intact colony structure is required to maintain this nitrogenase activity. This hypothesis was tested by comparing G. diazotrophicus colony structure when grown on solid medium under 2 and 20 kPa pO₂, correlating nitrogenase activity to colony development, and observing nitrogenase activity in response to disruption of colony structure.

**METHODS**

**Assessment of colony structure.** G. diazotrophicus strain JO-2 was originally isolated from a Cuban line of sugarcane (Dong et al., 1995) and strain PAL 5 (ATCC 49037) was originally isolated from sugarcane in Brazil (Gillis et al., 1989). Colonies were cultured at 30 °C in Petri dishes on a modified version of LGI-P medium the same as that described in Pan & Vessey (2001) except that sugarcane extract was not added to the medium. This medium is free of mineral nitrogen. Colonies were grown for 4–5 days at either 2 or 20 kPa O₂, and N₂ was used to bring the gas blends to 100 kPa (Pan & Vessey, 2001). At this stage of development, colonies were of similar size in both pO₂ treatments.

Colony structure was examined in both live, intact colonies and fixed, sectioned colonies. Cells of G. diazotrophicus accumulated the pH indicator bromothymol blue from the LGI-P medium. Fluorescence of the pH indicator permitted the visualization of cells within live colonies using confocal laser scanning microscopy (CLSM). A minimum of six randomly selected 4-day-old colonies of G. diazotrophicus PAL 5 from each of the two pO₂ treatments were examined using a Bio-Rad MRC600 CLSM equipped with a 514 Argon laser utilizing a GHS filter block (514 nm DF excitation/550 nm LP emission). An inverted stage and 32 × open air objective were used to view the colonies, which were removed with a thin layer of submerging agar from the plates on which they were grown. Optical sections (Z-series) were initiated at the top of the colony mucilage and collected at 5 μm intervals down through each colony toward the agar substrate, to the maximum penetration depth of the laser (120 μm). A montage of the Z-series was produced using Bio-Rad’s Confocal Assistant v. 4.02.

For light microscopy, a freeze-substitution method was used because standard fixatives flooded over the agar surface caused the colonies to rupture. Five-day-old colonies of G. diazotrophicus JO-2, supported by small pieces of the submerging LGI-P agar, were plunged into the freezing mixture (isopentane/methyl cyclohexane, 1:1, at the melting point). Freeze substitution in dry acetone resulted in the formation of sucrose crystals (from the medium), which damaged the structure of the colonies. The colonies were therefore freeze-substituted in methanol/acrolein (10:1) at −80 °C for 7 days. Under these conditions, sucrose crystals formed at the bottom of the vial or on the surface of the agar, from which they were easily removed.

Freeze-substituted colonies were gradually warmed (−20 °C overnight, then +5 °C for 24 h) in methanol on ice, post-fixed with 1 % OsO₄ in methanol for 1 h on ice, then rinsed again with three changes of methanol. The methanol was replaced by the transition solvent acetone in a graded series (5, 10, 20, 50, 70, 90 and 100 % acetone; two 10 min changes per step). Colonies were gradually infiltrated in Spurr’s resin monomer mixture (Spurr, 1969), with the concentration of the resin in acetone reaching 5 % at 90 min, 10 % at 150 min, 25 % at 210 min and 75 % at 330 min. The vials were then covered with perforated foil to allow evaporation of the remaining acetone overnight. The next day, resin in the vials was replaced with 100 % resin and polymerized at 70 °C overnight. Mid-colony transverse sections were cut with glass knives, stained with toluidine blue O (0.05 % in benzoate/borate buffer at pH 4.4), mounted in immersion oil and viewed with an Olympus Vanox microscope using phase-contrast and brightfield optics. Transmission electron microscopy was performed to confirm that stained bodies seen in the light microscopy corresponded to bacteria (data not shown).

**Observation of nitrogenase activity.** Nitrogenase activity was assayed for developing G. diazotrophicus colonies and for mature colonies before and after physical disruption. Nitrogenase activity was measured by H₂ evolution in the presence of Ar/O₂ (Hunt & Layzell, 1993) in a flow-through gas-exchange system (Pan & Vessey, 2001). To assess the effect of physical disruption of colony structure on nitrogenase activity, G. diazotrophicus PAL 5 was grown on solid, modified LGI-P medium (Pan & Vessey, 2001). At 6 days after inoculation (DAI), 20 Petri dishes containing 100–150 colonies per dish were placed in the gas-exchange system. A gas mixture of Ar/O₂ (80:20) was passed through the chamber at the rate of 500 ml min⁻¹. Hydrogen evolution of the colonies was recorded after 1 h, then the plates were removed from the chamber. The colonies on each plate were gently disrupted by using a glass rod to smear the colonies on the surface of the agar to approximately twice their original surface area. The plates were returned to the chamber along with the bent glass rod, and once again exposed to the Ar/O₂ mixture. Hydrogen evolution by the disrupted colonies was recorded after 1 h and reported as nmol H₂ h⁻¹ per colony ± SEM.

To assess the effect of colony development and morphology on nitrogenase activity, G. diazotrophicus PAL 5 was inoculated onto Petri dishes containing solid, modified LGI-P medium (Pan & Vessey, 2001) and incubated at 30 °C. Discrete colonies were visible at 2 DAI. Nitrogenase activity of the intact colonies was measured daily from 3 to 8 DAI by H₂
evolution in the flow-through gas-exchange system. Nitrogenase measurements were performed daily on four replicates of 20 Petri dishes each (100–150 colonies per dish) as described above. Because of increasing colony size over time, bacterial titre per colony was quantified and nitrogenase activity is reported as $\text{H}_2$ evolution rate per cell number (i.e. $\mu$mol $\text{H}_2$ per $10^{10}$ cells h$^{-1}$). Concentrations of bacteria per colony were assessed by plate counting as detailed by Pan & Vessey (2001). Colonies were visually assessed daily from 3 to 8 DAI for breakdown of colony structure. Breakdown of colony structure was indicated by a ‘slumping’ of the upper layer of mucilage to one side of the colony (easily visible due to the brightly yellow stained bacteria) and a flattening of the colony profile.

RESULTS AND DISCUSSION

Dong et al. (1995) demonstrated that $G$. $\text{diazotrophicus}$ colonies on solid media have nitrogenase activity at 2 and 20 kPa $\text{O}_2$. Further to this, Pan & Vessey (2001) recently showed that nitrogenase activity by $G$. $\text{diazotrophicus}$ in colonies adapts to long-term changes in atmospheric $\text{pO}_2$. The current study supports the hypothesis that colony structure is important in the ability of nitrogenase activity by $G$. $\text{diazotrophicus}$ to adapt to long-term changes in $\text{pO}_2$.

**Influence of $\text{pO}_2$ on colony structure**

Colonies grown for 4 days under 2 and 20 kPa $\text{pO}_2$ were lens-shaped in transverse section. In both treatments, two bacterial populations were seen embedded in the matrix of each colony, one adjacent to the agar medium, the other in a layer closer to the upper surface of the colony, with a low density of cells in between the two (Figs 1 and 2). The toluidine-blue-stained colonies (Fig. 1) clearly demonstrate that the position of the upper population varied with $\text{pO}_2$ treatment. At 20 kPa $\text{O}_2$, the uppermost population of bacteria was midway between the surface and the base of the colony (Fig. 1a). In contrast, the upper population of bacterial cells in colonies grown at 2 kPa $\text{O}_2$ was positioned just below the surface of the colony (Fig. 1b). The direction of the knife blade sectioning these colonies was from the top of the colony to the bottom. The vertical striations in these sections (particularly Fig. 1a) were due to fine crystals of sucrose remaining in the colony after fixation.

The darkfield images of $G$. $\text{diazotrophicus}$ colonies (Fig. 2), at slightly lower magnification than those in Fig. 1, confirm that $\text{pO}_2$ affects the relative position of the upper population of bacteria in the colonies. Bromothymol blue (displaying bright yellow in colour) is accumulated by the bacterial cells, but not the mucilaginous matrix, and clearly indicates that the uppermost population of bacteria in colonies grown at 20 kPa $\text{O}_2$ were located deeper in the colony matrix (Fig. 2).
2a) than the uppermost population of the colonies grown at 2 kPa O₂ (Fig. 2b). These images also more clearly show the lower population of bacteria at the base of the colonies in both treatments. It is unknown if these lower populations of bacteria represent dead or live cells. Some smearing of stain and vertical striations in the images (especially Fig. 2b) are scratches in the embedding resin due to fine sucrose crystals, as seen in the images (especially Fig. 2b). These images also more clearly show the lower population of bacteria at the base of the colonies in both treatments. It is unknown if these lower populations of bacteria represent dead or live cells. Some smearing of stain and vertical striations in the images (especially Fig. 2b) are scratches in the embedding resin due to fine sucrose crystals, as seen in the images (especially Fig. 2b).

Because fixation processes have the potential of disrupting the structure of what is being observed, live colonies grown at 2 and 20 kPa O₂ were also observed by CLSM. The difference in location of the upper population of bacteria between the pO₂ treatments was also evident in intact colonies of G. diazotrophicus (Fig. 3). Each panel in these images represents a 5 µm optical section through a living colony, starting at the top of the dome of the colony (upper left panel) and moving down towards the base of the colony (lower left panel). The whitish fluorescence indicates the presence of bacteria due to laser-induced excitation of bromothymol blue bound to bacterial capsular material. For colonies grown at 20 kPa O₂, the highest density of the upper population was typically located at a depth of 85–100 µm below the highest point of the colony surface (Fig. 3a). At 2 kPa O₂, the majority of cells of the upper population was typically located only 45–60 µm below the top surface of the mucilage (Fig. 3b). The haloing effect seen in the sections from 60 to 95 µm (Fig. 3b) shows that this upper population of bacteria is following the contour of the dome-shaped colony. Because the penetration of the CLSM system was limited to just beyond the upper 100 µm of the colony, the lower population of cells near the base of the colonies (Fig. 2) could not be imaged.

The microscopic imaging of G. diazotrophicus colonies (Figs 1, 2 and 3) clearly indicates that the uppermost population of bacteria of colonies grown at 20 kPa O₂ is located deeper in the colony matrix than that of colonies grown at 2 kPa O₂. Since nitrogenase activity by G. diazotrophicus colonies is known to adapt in the long term to changes in atmospheric pO₂ (Pan & Vessey, 2001), the microscopic evidence presented here suggests that the bacteria use the path-length of mucilage between the surface of the colony and the site of nitrogenase activity to affect the rate of O₂ diffusion and achieve a proper flux of O₂ for aerobic respiration without inhibiting nitrogenase activity. Bacterial mucilage is known to decrease the rate of O₂ diffusion to cells (Brown, 1970). The presence of extracellular polysaccharide surrounding Beijerinckia derxii cells is necessary to maintain nitrogenase in this organism (Barbosa & Alterthum, 1992). Derxia gummosa forms small non-fixing colonies if grown at 20 kPa O₂; however if grown at 5 kPa O₂, the bacterium forms large, highly mucilaginous colonies which fix N₂ (Hill, 1971; Hill et al., 1972). The motile diazotroph Azospirillum brasilense (Zhulin et al., 1996) displays aerotaxis within suspensions to achieve the appropriate O₂ environment for N₂ fixation. G. diazotrophicus is also motile (Gillis et al., 1989); however it is unknown at this time whether, if

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<th align="left">Serial optical sections (Z series) at low magnification down through intact, live colonies of G. diazotrophicus grown at an atmospheric pO₂ of 20 kPa (a) and 2 kPa (b) for 4 days. Reading from left to right and top to bottom, optical sections start at the colony surface (section 1) and each image of the montage is 5 µm deeper into the colony to a depth of 100 µm (section 20). Cells were stained with bromothymol blue absorbed from the culture medium and viewed by CLSM. Because of the fluorescence of the bromothymol blue, the bacterial cells fluoresce white. The relative intensity of the whitish fluorescence indicates the relative density of cells. The diameter of each colony was approximately 0.8 mm. Note that the highest density of cells is located 85–100 µm from the surface (sections 17–20) of the colony grown under 20 kPa O₂ (a) and only 45–60 µm from the surface (sections 9–12) of the colony grown under 2 kPa O₂ (b).</th>
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colonies were switched between 20 and 2 kPa $O_2$, the upper population of bacterial cells in colonies would migrate upwards or a new population of bacteria would grow nearer the surface of the colony. Continuous or near-continuous imaging of intact, living colonies over many hours after switching between the two $O_2$ conditions would be necessary to conclusively determine if migration or differential growth of bacteria within the colony were responsible for such a change in location of the upper bacterial population.

**Nitrogenase activity as influenced by physical disruption of colonies and by colony development**

Nitrogenase activity of intact *G. diazotrophicus* colonies grown at 20 kPa $O_2$ at 6 DAI was $1.14 \pm 0.07$ nmol H$_2$ h$^{-1}$ per colony. After physical disruption of colony structure by smearing colonies across the agar surface with a glass rod, nitrogenase activity was decreased by 96.7% to $0.038 \pm 0.006$ nmol H$_2$ h$^{-1}$ per colony. Likewise, breakdown in colony structure due to development/ageing (Figs 4 and 5) also resulted in declines in nitrogenase activity.

Colonies of *G. diazotrophicus* change as colonies develop (Fig. 4). Starting at 3 DAI, the accumulation of the pH indicator bromothymol blue by the cells from the medium resulted in a disc-shaped, stained (yellow) area inside the translucent, hemispherical colony mucilage (Fig. 4, colony images from 4 to 6 DAI). However, as colonies continued to develop, their structure began to break down, taking on a ‘slumped’ morphology (Fig. 4, colony image from 7 DAI). The first slumped colonies were recorded at 5 DAI, and by 7 DAI the percentage of collapsed colonies had increased significantly (Fig. 5a). At 8 DAI, about 75% of the colonies on each plate had slumped (Fig. 5a). Breakdown of bacterial colony structure with age is not uncommon and may be caused by enzymic depolymerization and loss of viscosity of the exopolysaccharide matrix of the colony (Sutherland, 1999).

Nitrogenase activity of the colonies grown at 20 kPa $O_2$ (Fig. 5b) was detectable at 3 DAI, although at a very low rate of $<0.2$ µmol H$_2$ per $10^{10}$ cells h$^{-1}$. Nitrogenase activity increased daily to a maximum value of $0.697$ µmol H$_2$ per $10^{10}$ cells h$^{-1}$ at 6 DAI. After this time, nitrogenase activity decreased, declining to only 76% of the maximum at 8 DAI. Hence, the increase in the breakdown in colony structure due to ageing (Fig. 5a) was coincident with the decline in nitrogenase activity per cell within the colonies (Fig. 5b).

Disruption of colony structure due to either manipulation or ageing would compromise all spatial relationships between the bacterial cells and path-length of mucilage to the open atmosphere. Decline in the path-length between bacteria and the atmosphere would result in a dramatic increase in $O_2$ flux to the bacteria and in the concentration of free $O_2$ at the sites of nitrogenase activity. Pan & Vessey (2001) demonstrated that *G. diazotrophicus* displays a rapid switch-off protection phenomenon when $O_2$ flux rapidly increases to *G. diazotrophicus* within colonies. Alternatively, a
rapid increase in O$_2$ flux to *G. diazotrophicus* with disruption of colony structural integrity could result in a reversible inhibition of nitrogenase activity (Burris, 1991).

Physical disturbance of the colonies by manipulation with a glass rod resulted in a much greater decline in nitrogenase activity (96.7%) than that caused by the ‘slumping’ of colonies due to ageing between 6 and 8 DAI (Fig. 5b; a 24% decline in nitrogenase activity). However, this is not unexpected as the smearing of the colony with the glass rod is a much more severe physical disturbance than that induced by colony slumping.

**Conclusion**

The results of both the imaging of colonies grown at different pO$_2$ values and the correlation of nitrogenase activity with colony intactness are consistent with the hypothesis that the mucilaginous matrix of *G. diazotrophicus* colonies is important in the protection of nitrogenase activity by the bacterium from excessive O$_2$ flux. Likewise, the position of *G. diazotrophicus* within the colony appears to be a component of the bacterium’s long-term adaptation to changes in pO$_2$ in the surrounding atmosphere. However, the actual concentration of free and dissolved O$_2$ within the colonies at the sites of nitrogenase activity is as yet unknown. Reis & Dobereiner (1998) found that nitrogenase activity of *G. diazotrophicus* in liquid cultures was maximal when the culture was at equilibrium with 0.2 kPa O$_2$ in the gas phase. However, the actual concentration of dissolved O$_2$ at the site of nitrogenase activity in any medium is dependent upon the concentration of O$_2$ in the gas phase, the diffusion rate through the medium, and the rate of O$_2$ consumption by bacterial respiration (Hunt & Layzell, 1993). The current study supports the hypothesis that *G. diazotrophicus* utilizes the path-length of colony mucilage between the atmosphere and the bacteria to achieve this optimal flux and concentration of O$_2$ for respiration and nitrogenase activity.

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