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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

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 the its sole nitrogen source under 21 kPa O_2

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Abbreviations: CLSM, confocal laser scanning microscopy; DAI, days after inoculation; pO_2, partial pressure of oxygen.

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 O_2 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 N_2 as the sole nitrogen source at 20 kPa O_2 (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 pO_2 (10, 20 and 30 kPa). Optimal nitrogenase activity by *G. diazotrophicus* colonies occurred at or slightly above (i.e. +10 kPa) the O_2 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 O_2 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 O_2 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 pO_2, 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 O_2, and N_2 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 pO_2 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 pO_2 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 subtending 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 subtending 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), rinsed (3 × 10 min each) in methanol on ice, post-fixed with 1 % OsO_4 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 microcopy 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 H_2 evolution in the presence of Ar/O_2 (Hunt & Layzell, 1993) in a flow-though 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/O_2 (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/O_2 mixture. Hydrogen evolution by the disrupted colonies was recorded after 1 h and reported as nmol H_2 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 H_2

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 H_2 evolution rate per cell number (i.e. μmol 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. diazotrophicus* colonies on solid media have nitrogenase activity at 2 and 20 kPa O_2. Further to this, Pan & Vessey (2001) recently showed that nitrogenase activity by *G. diazotrophicus* in colonies adapts to long-term changes in atmospheric pO_2. The current study supports the hypothesis that colony structure is important in the ability of nitrogenase activity by *G. diazotrophicus* to adapt to long-term changes in pO_2.

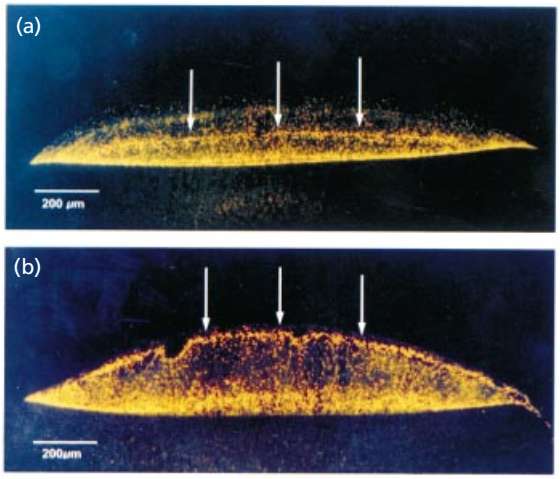

Fig. 2. Mid-colony, transverse sections through fixed colonies of *G. diazotrophicus* grown at an atmospheric pO_2 of 20 kPa (a) and 2 kPa (b) for 5 days. Cells were stained with bromothymol blue absorbed from the culture medium and viewed using darkfield microscopy. Arrows indicate the level of the uppermost population of bacteria within the colonies. Note that the highest density of the uppermost population is deeper in the colony grown at 20 kPa (a) than in that at 2 kPa O_2 (b).

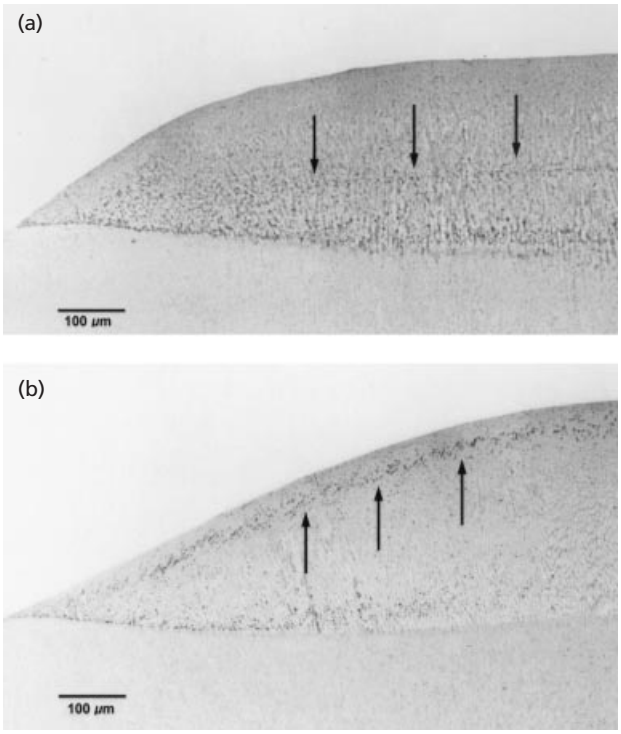

Fig. 1. Mid-colony, transverse sections through fixed colonies of *G. diazotrophicus* grown at an atmospheric pO_2 of 20 kPa (a) and 2 kPa (b) for 5 days. Sections were stained with toluidine blue and viewed using phase-contrast microscopy. Arrows indicate the level of the uppermost population of bacteria within the colony matrix. Note that the highest density of the uppermost population is deeper in the colony grown at 20 kPa (a) than in that at 2 kPa O_2 (b).

Influence of pO_2 on colony structure

Colonies grown for 4 days under 2 and 20 kPa 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 pO_2 treatment. At 20 kPa 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 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. diazotrophicus* colonies (Fig. 2), at slightly lower magnification than those in Fig. 1, confirm that 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 O_2 were located deeper in the colony matrix (Fig.

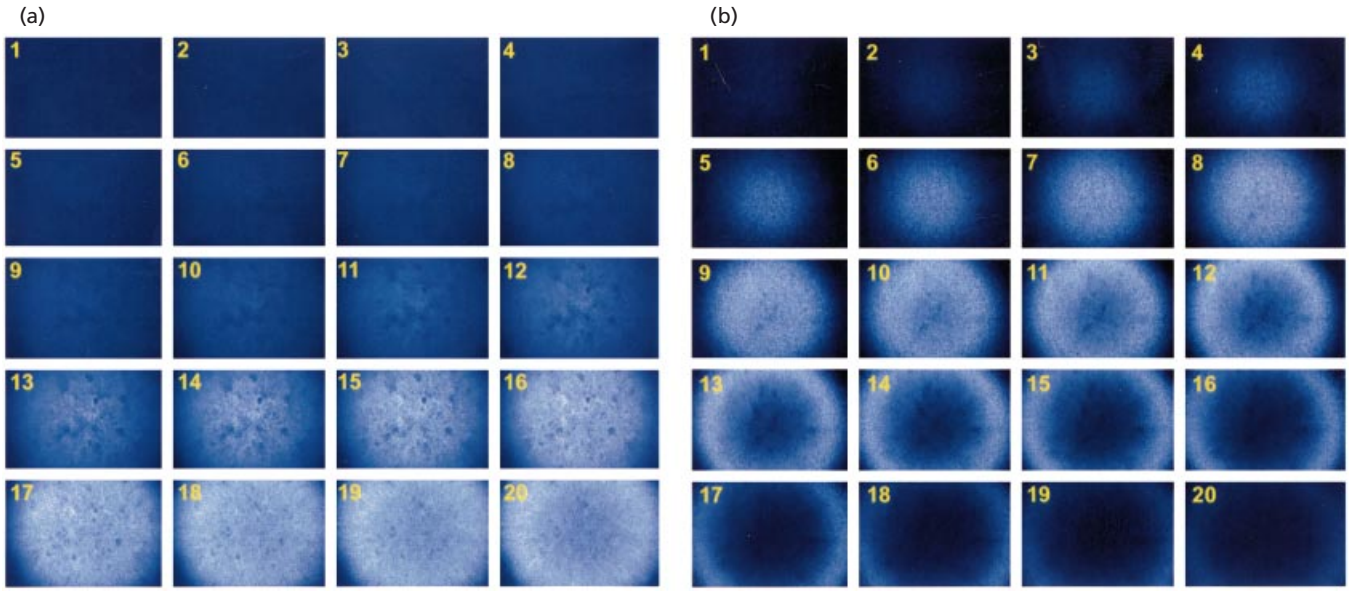

Fig. 3. Serial optical sections (Z series) at low magnification down through intact, live colonies of *G. diazotrophicus* grown at an atmospheric pO_2 of 20 kPa (a) and 2 kPa (b) for 4 days. Reading from left to right and top to bottom, optical sections start at the colony surface (section 1) and each 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 O_2 (a) and only 45–60 μm from the surface (sections 9–12) of the colony grown under 2 kPa O_2 (b).

2a) than the uppermost population of the colonies grown at 2 kPa O_2 (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 Fig. 1.

Because fixation processes have the potential of disrupting the structure of what is being observed, live colonies grown at 2 and 20 kPa O_2 were also observed by CLSM. The difference in location of the upper population of bacteria between the pO_2 treatments was also evident in intact colonies of *G. diazotrophicus* (Fig. 3). Each panel in these images represent 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 florescence indicates the presence of bacteria due to laser-induced excitation of bromothymol blue bound to bacterial capsular material. For colonies grown at 20 kPa O_2, 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 O_2, 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 O_2 is located deeper in the colony matrix than that of colonies grown at 2 kPa O_2. Since nitrogenase activity by *G. diazotrophicus* colonies is known to adapt in the long term to changes in atmospheric pO_2 (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 O_2 diffusion and achieve a proper flux of O_2 for aerobic respiration without inhibiting nitrogenase activity. Bacterial mucilage is known to decrease the rate of O_2 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 O_2; however if grown at 5 kPa O_2, the bacterium forms large, highly mucilaginous colonies which fix N_2 (Hill, 1971; Hill *et al.*, 1972). The motile diazotroph *Azospirillum brasilense* (Zhulin *et al.*, 1996) displays aerotaxis within suspensions to achieve the appropriate O_2 environment for N_2 fixation. *G. diazotrophicus* is also motile (Gillis *et al.*, 1989); however it is unknown at this time whether, if

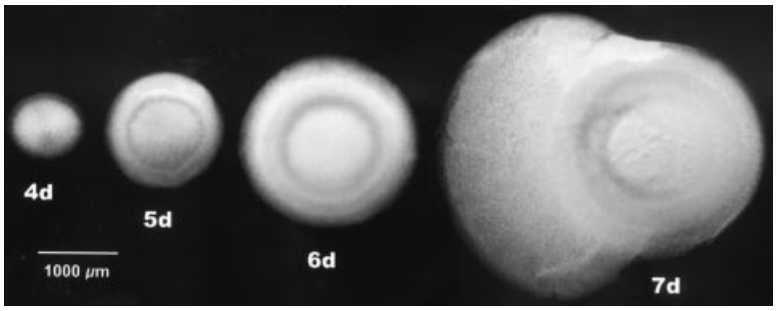

Fig. 4. Time series of a *G. diazotrophicus* colony illustrating the morphological changes in the colony as it develops from 4 to 7 days (d) after inoculation (DAI). Colonies were grown on solid medium at 30 °C at an atmospheric pO_2 of 20 kPa. Note that by 7 DAI, the colony has 'slumped' and breakdown in colony structure is coincident with a decline in nitrogenase activity (see text for details).

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 pO_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 $1{\cdot}14 \pm 0{\cdot}07$ 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 $0{\cdot}038 \pm 0{\cdot}006$ 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.

Colony structure of *G. diazotrophicus* changes 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 proportion 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 $<0{\cdot}2$ 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

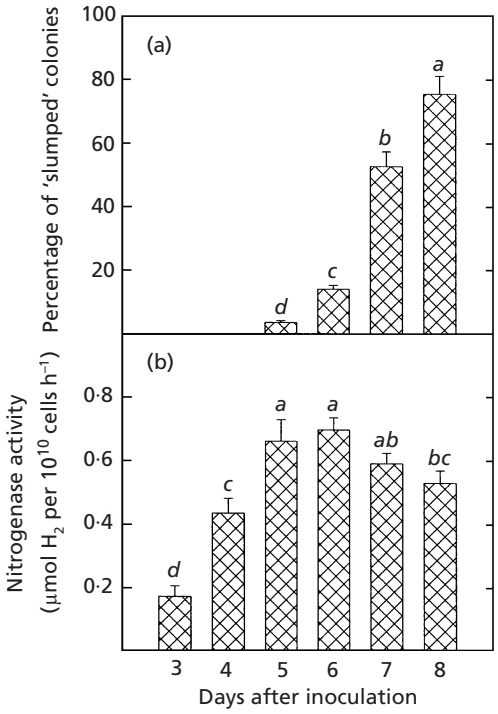

Fig. 5. Proportion of slumped colonies (a) and nitrogenase activity (b) of *G. diazotrophicus* colonies from 3 to 8 DAI. Colonies were grown on solid media at 30 °C at an atmospheric pO_2 of 20 kPa. Bars represent ± SEM and values in columns marked by different letters are significantly different at $P \leqslant 0{\cdot}05$.

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 & Döbereiner (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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