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Living a Grounded Life: Growth and Nitrogenase Activity of *Gluconacetobacter diazotrophicus* on Solid Media in Response to Culture Conditions

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Abstract

We have previously shown that colony structure of *Gluconactobacter diazotrophicus* grown on solid media is important in protecting nitrogenase from inhibitory effects of O_2. In this paper we develop a model based on empirical measurements to estimate the diffusion coefficient of the colony mucilage for O_2 (D_{O2}) and characterize the effect of various conditions of solid media culture on growth and nitrogenase activity of *G. diazotrophicus*. Based on colonies growing at 20 kPa O_2 at 22°C, we estimated the flux (F) of O_2 to the bacterial zone in the colony to be 0.0246 nmol O_2 mm^{-2} s^{-1}. Based on this value of F, D_{O2} was calculated to be 0.000919 mm^2 s^{-1}, a value 44% of the diffusion coefficient of O_2 in water. The media pH and temperature optima for nitrogenase activity (5.5 and 30°C, respectively) of *G. diazotrophicus* grown on solid media are similar to those reported for liquid and semi-solid media. Although the response to ammonium concentration in the media is similar to that reported for liquid media, nitrate concentrations 0.5 mM were inhibitory to nitrogenase activity. The range of sucrose concentrations supportive of nitrogenase activity was broad (0.5 to 15%), but growth and nitrogenase

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activity were much lower when glucose and fructose were the carbon sources. We speculate that good growth and nitrogenase activity by *G. diazotrophicus* on solid media requires sucrose for levansucrase-mediated production of the fructan components of the colony mucilage.

Keywords: *Gluconactobacter diazotrophicus*, growth, nitrogenase activity, O_2 diffusion, solid media

1. Introduction

Gluconacetobacter diazotrophicus (previously known as *Acetobacter diazotrophicus* (Gillis et al., 1989; Yamada et al., 1997)) is an N_2-fixing endophyte originally isolated from sugarcane (Cavalcante and Döbereiner, 1988). Studies using ^{15}N have shown that N_2 fixation by *G. diazotrophicus* can contribute up to 65% of the N accumulated by sugarcane (Boddey et al., 1991), however field measurements in Brazil suggests that sugarcane more commonly obtains 25 to 45% of its N from fixation (Boddey et al., 2001), presumably largely due to *G. diazotrophicus*.

Many aspects of the growth and physiology of *G. diazotrophicus* cultured in liquid media have been well characterized (Stephan et al., 1991; Burris, 1994; Alvarez and Mertinez-Drets, 1995; Flores-Encarnacion et al., 1999; Hernandez et al., 1999; Luna et al., 2000). Maximum nitrogenase activity occurred when the partial pressure of oxygen (pO_2) in liquid media was at equilibrium with 0.2 kPa O_2 (approximately 1% of the pO_2 of ambient air) in the gaseous phase of the culture (Zhang et al., 1991; Reis and Döbereiner, 1998; Luna et al., 2000). Nitrogenase activity is higher with 10% sucrose in the media, compared to 1% sucrose or glucose (Reis and Döbereiner, 1998), and the higher sucrose concentration also appears to make nitrogenase activity more resilient to the inhibitory effects of ammonium, sodium chloride, and certain amino acids.

The growth and physiology of *G. diazotrophicus* on solid media has not been well characterized. Dong et al. (1994) provided microscopic evidence that the bacterium lives in mucilaginous microcolonies in intercellular spaces of sugarcane apoplast and that colonies of the bacterium on solid media had nitrogenase activity at ambient pO_2 in the surrounding atmosphere. Later, Dong et al. (1995) showed that some basic biochemical characteristics of *G. diazotrophicus* on solid media were the same as those for the species on semi-solid media (Cavalcante and Döbereiner, 1988). More recently, Pan and Vessey (2001) have shown that nitrogenase activity by *G. diazotrophicus* colonies is very adaptable to changes in atmospheric O_2 and tolerant of very high concentrations atmospheric O_2 (up to 60 kPa). Dong et al. (2002) provide

microscopic and physiological evidence that the position of *G. diazotrophicus* cells within the colony is influenced by the external atmospheric pO_2. They proposed that the bacteria use the path-length of mucilage between themselves and the surface of the colony as a resistance to O_2 diffusion, thereby providing an optimal O_2 flux for bacterial respiration without inhibiting nitrogenase activity.

The objectives of the current study were two fold. Firstly, we utilize the knowledge on gas exchange (Pan and Vessey, 2001) and structure of *G. diazotrophicus* colonies (Dong et al., 2002) to model the flux of O_2 through the mucilage of a *G. diazotrophicus* colony and to estimate the O_2 diffusion coefficient of the mucilage. Secondly, we report on nitrogenase activity of *G. diazotrophicus* colonies on solid media in response to media pH, sugar concentration and composition, mineral-N form and concentration and to culture temperature. Some of the bacterium's responses to these culture conditions are similar to those reported for liquid culture and some are very different.

2. Materials and Methods

O_2 flux model development: equations and assumptions

The flux of O_2 from the surface of a colony of *G. diazotrophicus* to the bacterial zone within the colony was modelled to estimate the diffusion coefficient of the colony mucilage. Colony morphometric parameters for the model were taken from Dong et al. (2002). The shape of the colony was assumed to be a spherical cap (i.e. the portion of a sphere cut off by a plane) (Coolidge, 1971) (Fig. 1) and the surface area (S) of the colony was calculated using the formula:

$$S = 2\pi rh \qquad (1)$$

where, r equals the radius of the sphere and h equals the height of the spherical cap. The radius of the sphere was calculated from h and r_1, the radius of the base of the spherical cap, using:

$$r = (h^2 + r_1^2)/(2h) \qquad (2)$$

Simplifying the observations of Dong et al. (2002) for the purpose of this modelling exercise, it was assumed that at 20 kPa pO_2, *G. diazotrophicus* are positioned as a spherical cap of bacterial cells within the colony 0.1 mm below the surface of the colony (Fig. 1). Given this assumption, the surface area of the bacterial zone (S_b) was calculated applying the formulae in Eq. 3 and Eq. 4,

with the height (h_b), the radius of the base (r_{1b}) of the spherical cap, and the calculated radius of the sphere (r_b) of the bacterial zone:

$$S_b = 2\pi r_b h_b \quad (3)$$

$$r_b = (h_b^2 + r_{1b}^2)/(2h_b) \quad (4)$$

The flux of O_2 from the surface of the colony to the bacterial zone and the diffusion coefficient of the bacterial mucilage was modelled using Fick's law of diffusion (West, 1995):

$$D_{O2} = F(\Delta c/\Delta x) \quad (5)$$

where, D_{O2} is the diffusion coefficient of the bacterial mucilage, F is the flux rate of O_2 across a plane midway between the surface of the colony and the bacterial zone within the colony, Δc is the difference in dissolved O_2 concentration between the surface of the colony and the bacterial zone, and Δx is the distance between the surface of the colony and the bacterial zone (i.e., $h - h_b$). The concentration of dissolved O_2 at the surface of the colony was assumed to be equal to that of a saturated aqueous solution at 22°C, approximately 270 µM. We have measured the water content of *G. diazotrophicus* colony biomass grown under these conditions at approximately 85%, therefore assuming the dissolved O_2 content within the colony mucilage as equivalent to that of an aqueous environment is reasonable. As per the interpretations of Dong et al. (2002), it is assumed that *G. diazotrophicus* solely dependent on N_2 as an N source position themselves in the colony to optimize dissolved O_2 concentrations for growth and N_2 activity. Consequently, the concentration of dissolved O_2 in the bacterial zone was assumed to be 2.7 µM. This value is based upon measurements of Burris et al. (1991), Reis and Döbereiner (1998), and Luna et al. (2002) who found that nitrogenase activity of *G. diazotrophicus* in liquid culture was maximized when the concentration of dissolved O_2 in the media was at equilibrium with 2.0 kPa O_2 in the gas phase of their culture systems. Given Henry's law, that the concentration of a solute gas in a solution is directly proportional to the partial pressure of that gas above the solution (West, 1995), it is assumed here that the concentration of dissolved O_2 for optimal nitrogenase activity by *G. diazotrophicus* is a pO_2 of 2.0 kPa or approximately 2.7 µM O_2 at 22°C.

The flux rate of O_2, F, was calculated from estimates of the O_2 uptake rate (OUR) by the bacterial population of a colony, and the surface area of a plane midway between the surface of the colony and the bacterial zone:

$$F = OUR/((S + S_b)/2) \quad (6)$$

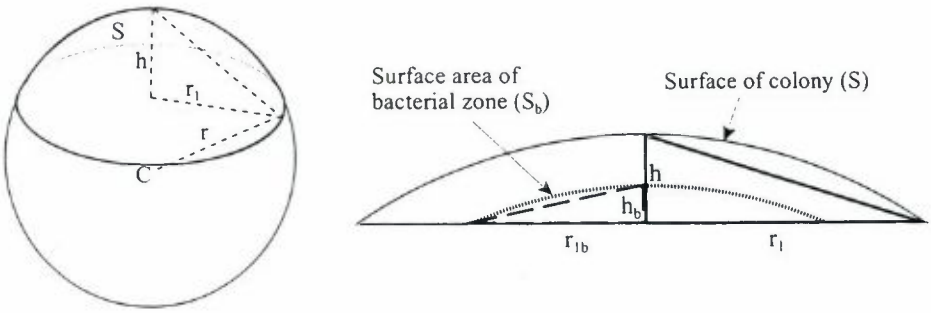

Figure 1. The geometry of a spherical cap (left) and diagrammatic representation of *G. diazotrophicus* colony as a spherical cap, with the bacterial zone forming a spherical cap 0.1 mm below the surface of the colony (right). S = surface area of the colony; r_1 = the radius of the base of the colony; h = the height of the colony; r = the radius of the sphere defined by the colony's dimensions; C = the centre of the sphere defined by the colony's dimensions; S_b = surface area of the bacterial zone; r_{1b} = the radius of the base of the bacterial zone; h_b = the height of the bacterial zone. See Table 1 for values of these parameters.

The OUR of the bacterial population in colonies grown at 20 kPa O_2 at 22°C was estimated from the measurement of the rate of CO_2 evolution from *G. diazotrophicus* colonies by Pan and Vessey (2001) assuming 10^7 bacteria per colony and a respiratory quotient (RQ) of 1.0. Respiratory quotients calculated from data of Luna et al. (2000) indicate a range of RQ for *G. diazotrophicus* from 0.928 to 1.013 depending upon C and N source for the bacterium.

Finally, it is assumed that there are no gaseous inclusions in the colony [there is no evidence of "gas pockets" in the images of *G. diazotrophicus* colonies in Dong et al. (2002)], which would dramatically affect the diffusion rate of O_2 through the colony mucilage (the diffusion rate of O_2 in air is 10,000 times that in water).

Growth and nitrogenase activity of G. diazotrophicus in response to culture condition

G. diazotrophicus, strain PAL5 (ATCC 49037), obtained from American Type Culture collection, was cultured for 2 days at 30°C, shaken at 150 rpm in LGI-P liquid medium (Pan and Vessey, 2001), modified by omitting the 5 ml l^{-1} of sugarcane juice. To test nitrogenase activity in response to culture conditions, diluted cells from the liquid cultures were streaked on to solid modified LGI-P

agar medium (15 g agar l^{-1} plus 50 mg Difco yeast extract l^{-1}) in Petri dishes. The standard media contained 10% sucrose (i.e. refined table sugar, Rogers Sugar Ltd., Vancouver, Canada) but was modified according to the treatment being tested (see below).

Six experiments were carried out to study nitrogenase activity of *G. diazotrophicus* in response to culture conditions. Nitrogenase activity was measured in *G. diazotrophicus* grown on modified LGI-P solid medium containing: (1) 0.1, 0.5, 1.0, 5.0, 10.0, or 15.0% sucrose; (2) buffered pH of 4.5, 5.5, 6.5 and 7.5 (pH adjusted with McIlvaine-Lillie buffer, Clark, 1981); (3) 0.0, 0.1, 0.5, 1, 5 and 10 mM KNO_3; (4) 0.0, 0.1, 0.5, 1, 5, and 10 mM $(NH_4)_2SO_4$; and (5) 5% sucrose, 5% fructose, 5% glucose, or 2.5% glucose plus 2.5% fructose (chemical grade glucose and fructose acquired from Acros Organics, New Jersey, USA). The sixth experiment tested nitrogenase activity on cells grown on modified LGI-P media (10% sucrose) at varying temperatures (15, 22 and 30°C). In experiments 3 and 4, different concentrations of K_2SO_4 were used to balance as much as possible the variability in K^+ and SO_4^{2-}, respectively, in the media. In Experiments 1 to 5, *G. diazotrophicus* was grown at 30°C and the pO_2 of the atmosphere in all experiments was ambient (i.e. approximately 21 kPa).

Nitrogenase activity and the electron allocation coefficient (EAC) for nitrogenase (Edie and Phillips, 1983) was measured by H_2 evolution in atmospheres of air and argon (Ar:O_2, 80:20). Gas-exchange measurements were conducted on intact *G. diazotrophicus* colonies on Petri plates as described in Pan and Vessey (2001). Gas-exchange measurements were done when the colonies had reached a standard size of 1 to 2 mm in diameter. In most experiments colonies had reached this size after 5 days of culture on solid media, however, in the experiments with media pH buffered at 4.5 and 7.5, incubation temperatures of 15 and 20°C, and growth on glucose and fructose, longer time periods (up to 14 days) were needed for colonies to reach the standard size. For each experiment, gas-exchange measurements of 20 to 40 Petri plate cultures per batch were replicated at least four times.

To determine the effect of culture conditions on population growth rate of *G. diazotrophicus* in colonies and to normalize measurements of nitrogenase activity made from colonies of different ages, the number of viable *G. diazotrophicus* cells per colony were enumerated. After the gas-exchange measurements, 10 colonies per replicate were randomly sampled and titre of *G. diazotrophicus* was determined by serial dilution and plate counting as detailed in Pan and Vessey (2001). Nitrogenase activity is reported as μmoles of H_2 evolved per 10^{10} cells per hour.

Data were analysed using the GLM model of the SAS Statistical Package (SAS Institute, Cary, NC), assuming a completely randomized design, and mean separation was tested using the LSD procedure.

3. Results and Discussion

Model of O_2 flux in G. diazotrophicus colonies

Based on observations of colony structure (Dong et al., 2002) and gas-exchange characteristics (Pan and Vessey, 2001) of *G. diazotrophicus* grown on solid media, and on the optimal dissolved O_2 concentration for nitrogenase activity for the bacterium (Burris et al., 1991; Reis and Döbereiner, 1998; Luna et al., 2000), the flux of O_2 between the surface of the colony to the bacterial zone was modelled to estimate the diffusion coefficient of the colony mucilage. In this model, the bacterial zone is assumed to be a spherical cap within the spherical cap of the colony mucilage, which is consistent with the observations of Dong et al. (2002).

Based on Eqs. 1 to 6 and the parameters defined in Table 1, F was calculated to be 0.0246 nmol O_2 mm^{-2} s^{-1}. Based on this value of F and according to Fick's law of diffusion (Eq. 5), the diffusion coefficient (D_{O2}) of the bacterial mucilage was calculated to be 0.000919 mm^2 s^{-1}. Given that the diffusion coefficient of water ($D_{O2\text{-}H2O}$) at 22 C is 0.00211 mm^2 s^{-1} (Lango et al., 1996), the estimated D_{O2} of the bacterial mucilage is 44% of that of water.

The facilitated diffusion of O_2 by leghemoglobin through gels (Bergersen, 1993) and the infected zone of legume nodules (Hunt et al., 1988; Layzell et al., 1988) has been well characterized. However, the authors could not find any reports of the measure of O_2 flux to aerobic diazotrophs existing in colonies. The D_{O2} of gels are dependent upon the size and orientation of the component molecules of the gel. In gels where the structural integrity is due to 2% agar (w/v), D_{O2} has been measured empirically as low as 70% of $D_{O2\text{-}H2O}$ (Sato and Toda, 1983). However, others estimate the D_{O2} of 2% agar gels on to be much higher, in the range of 97 to 99% of $D_{O2\text{-}H2O}$ (Ven der Meeren et al., 2001). Mucoid gels of biological origin appear to have much larger effects on D_{O2} than simple agar gels. Although reports of D_{O2} of biological mucoid gels are rare, Saldena et al. (2000) found that the D_{O2} of isolated mucosa of rat colon was significantly decreased from 50% of $D_{O2\text{-}H2O}$ to 27.3 % when the mucus gel layer associated with the mucosa was included in the measurements.

The importance of understanding F and D_{O2} of the mucilage produced by *G. diazotrophicus* is integral to our understanding of how the bacterium protects itself from the inhibitory effects of O_2. Pan and Vessey (2001) and Dong et al. (2002) provide evidence that the path length of mucilage between atmospheric O_2 and the bacterium is used to help provide an optimal pO_2 for nitrogenase activity and respiration. Our estimate of the D_{O2} of the colony mucilage being 44% of $D_{O2\text{-}H2O}$ suggests that this would make an effective resistance to O_2 diffusion and supports the importance of the observations of Dong et al. (1994) that *G. diazotrophicus* exists embedded in mucilage in the intercellular spaces

Table 1. Values for parameters in Eqs. 1–6 used to model the flux of O_2 from the surface of a *G. diazotrophicus* colony to the bacterial zone within the colony.

Model component	Parameter identity*	Parameter value
Surface area of colony	h	0.20 mm
	r_1	0.60 mm
	r	1.00 mm
	S	1.26 mm^2
Surface area of bacterial zone	h_b	0.10 mm
	r_{1b}	0.40 mm
	r_b	0.90 mm
	S_b	0.57 mm^2
Flux of O_2	Δc	267.3 µM O_2
	Δx	0.1 mm
	OUR	0.08 µmol O_2 $colony^{-1}$ h^{-1}

*See text of Material and Methods for definition of symbols.

Table 2. Growth and nitrogenase activity of *G. diazotrophicus* populations within colonies in response to sucrose concentration, pH and temperature of solid media cultures.

Culture variable	Treatment	Days of growth*	Growth rate (CFU d^{-1})**	Nitrogenase activity (µmol H_2 10^{10} $cells^{-1}$ d^{-1})	EAC+
pH	4.5	9	1.14×10^6 d++	0.23 c	0.56 a
	5.5	5	3.04×10^6 b	0.88 a	0.70 a
	6.5	5	3.45×10^6 a	0.71 ab	0.71 a
	7.5	8	2.34×10^6 c	0.56 b	0.73 a
Temperature (°C)	15	14	1.61×10^5 c	0.28 b	0.52 a
	22	8	2.75×10^6 b	0.59 a	0.62 a
	30	5	5.01×10^6 a	0.75 a	0.67 a
Sucrose (%)	0.1	5	5.84×10^4 e	0.09 d	0.24 c
	0.5	5	4.80×10^6 a	0.32 c	0.45 bc
	1	5	5.01×10^6 a	0.52 b	0.68 ab
	5	5	4.25×10^6 b	0.79 a	0.71 a
	10	5	2.75×10^6 c	0.65 ab	0.63 a
	15	5	2.10×10^6 d	0.52 b	0.58 ab

*Days of growth required for colonies to reach a standard size (1 to 2 mm in diameter). **CFU = colony forming units; growth rate = (CFU/colony)/(days of growth). +EAC = electron allocation coefficient of nitrogenase. ++Values within the same culture variable followed by different letters are significantly different at p = 0.05.

Table 3. Growth of *G. diazotrophicus* populations within colonies in nitrate and ammonium concentration of solid media cultures.

Culture variable	Treatment	Days of growth*	Growth rate (CFU d^{-1})**	Nitrogenase activity (μmol H_2 10^{10} cells^{-1} d^{-1})	EAC+
Nitrate (mM)	0	5	2.89×10^6 a++	0.66 a	0.64 a
	0.1	5	2.43×10^6 b	0.76 a	0.69 a
	0.5	5	2.14×10^6 bc	0.43 b	0.58 a
	1	5	2.08×10^6 bc	0.25 bc	0.59 a
	5	5	2.01×10^6 c	0.03 c	0.55 a
	10	5	1.19×10^6 d	0.03 c	0.68 a
Ammonium (mM)	0	5	3.03×10^6 a	0.72 a	0.64 a
	0.2	5	2.96×10^6 a	0.84 a	0.61 a
	1	5	2.62×10^6 b	0.80 a	0.65 a
	2	5	2.16×10^6 c	0.61 ab	0.63 a
	10	5	2.00×10^6 c	0.51 bc	0.63 a
	20	5	1.66×10^6 d	0.32 c	0.61 a

*Days of growth required for colonies to reach a standard size (1 to 2 mm in diameter). **CFU = colony forming units; growth rate = (CFU/colony)/(days of growth). +EAC = electron allocation coefficient of nitrogenase. ++Values within the same culture variable followed by different letters are significantly different at $p = 0.05$.

of sugarcane vascular parenchyma. The pO_2 of airspaces inside sugarcane tissue or dissolved in intercellular fluids is unknown. However, given that Dong et al. (1994) found that 4% of the intercellular spaces were air-filled and that even in quite dense tissues, such as the center of potato tubers (Abdul-Baki and Solomos, 1994), the pO_2 is quite high (approximately 10 to 17 kPa) and nonlimiting to aerobic respiration, it is highly unlikely that the sugarcane tissue itself represents a significant resistance to O_2 flux.

The accuracy of any model is only as good as its underlying assumptions (see Materials and Methods). An important assumption made in our model of O_2 flux into *G. diazotrophicus* colonies is that the metabolically-active, N_2 fixing bacteria exist as a single layer of bacteria within the colony. Dong et al. (2002) actually found two populations of bacterial cells within *G. diazotrophicus* colonies, an "upper" population whose position varied with the external pO_2 of the surrounding atmosphere, and a "lower" static population. In the current study, we assume that the upper population accounts for all the metabolically-active, N_2 fixing bacteria in the colony. We speculate that the lower population is actually dead or metabolically inactive cells. However, if the lower population of cells are actually respiring and fixing N_2, then Δx in our

model is underestimated and consequently D_{O2} of the colony mucilage is greater than we estimated.

Growth and nitrogenase activity of G. diazotrophicus in response to culture condition

Culture conditions affected *G. diazotrophicus* growth rate, nitrogenase activity, and EAC (Tables 2 and 3). Growth was optimal when the pH of the buffered media was set at pH 5.5 and 6.5 (Table 2). For colonies grown on media with a buffered pH of 7.5, the population growth rate decreased by approximately 30% from the optimal rate. With a media pH of 4.5, the growth rate was markedly lower. Although the pH of the media was buffered to an initial set point, it is not possible to know what the pH was inside the colonies. Nonetheless, the trends in growth response of *G. diazotrophicus* to buffered pH of solid media are similar to those reported in Stephan et al. (1991) for *G. diazotrophicus* grown in liquid media. In liquid media growth was optimal at pH 5.5, however still higher at pH 4.5, but almost totally inhibited at pH 7.5. Hence, growth on solid media appears to shift the range for growth to slightly higher pH compared to that in liquid media. Optimal nitrogenase activity was achieved with a media pH of 5.5; increasing media pH to 6.5 and 7.5 decreased nitrogenase activity by approximately 20% with each step. Nitrogenase activity at a media pH of 4.5 was very much lower than the optimal. The EAC was approximately 0.7 at media pH range from 5.5–7.5, meaning that at these pH approximately 70% of the total electron flow through nitrogenase was being used to reduce N_2 and 30% was being used to reduce protons to H_2. For comparison, these values of EAC are similar to those (0.6 to 0.7) commonly reported for rhizobia in legume nodules in which EAC is measured by H_2 evolution (Hunt and Layzell, 1993). Only at a media pH of 4.5 was EAC negatively affected by acidity of the medium. Cavalcante and Döbereiner (1988) found optimal nitrogenase activity in *G. diazotrophicus* grown on semi-solid media in the pH 4 to 5 range. Stephan et al. (1991) found good levels of nitrogenase activity as low as pH 2.5 in liquid media. Again, it appears that growth on solid media shifts the pH range for nitrogenase activity to slightly higher levels compared to liquid and semi-solid media.

Cavalcante and Döbereiner found the optimal temperature for growth of *G. diazotrophicus* to be 30°C. We are interested in the adaptation of *G. diazotrophicus* to more temperate regions, so we were particularly interested in the effect of decreasing temperature below the optimum. Decreasing the culture temperature from 30 to 22 and 15°C had particularly severe effects on the measure parameters (Table 2). At 15°C, growth rate, nitrogenase activity, and EAC were 4, 37 and 76%, respectively, of the values at 30°C. We could find no

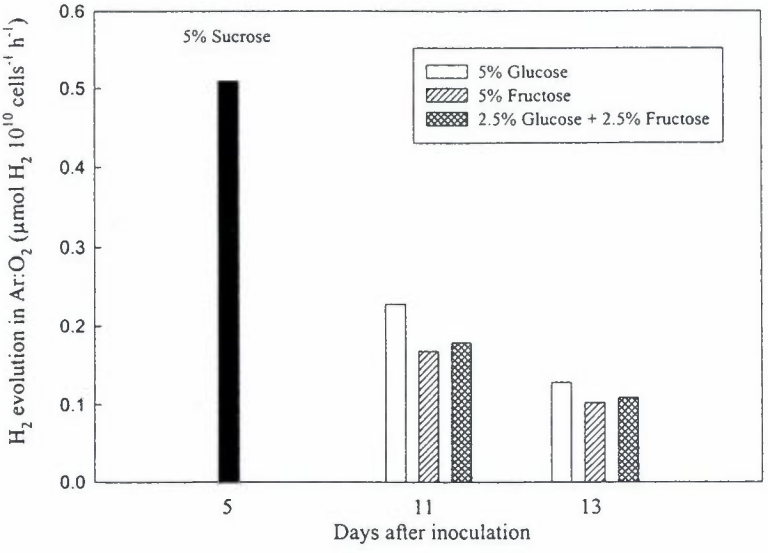

Figure 2. Nitrogenase activity of *G. diazotrophicus* in response to sugar composition (sucrose, glucose, fructose, or glucose + fructose) of solid media. The sugar concentration of the media in all treatments was 5% (w/v). Nitrogenase activity in response to sugar source was measured when colonies had reached 1 to 2 mm in diameter which took a longer time (11 and 13 days) when glucose and/or fructose were the sources of sugar.

similar measures of the effect of lowering culture temperature on these parameters in *G. diazotrophicus*, however, Hernandez et al. (1999) report that increasing liquid culture temperature from the optimal of 30°C to 35°C resulted in a complete inhibition of growth.

The concentration of sucrose in liquid and semi-solid culture media has previously been shown to effect growth and nitrogenase activity of *G. diazotrophicus* (Calvalcante and Döbereiner, 1988; Reis and Döbereiner, 1998). Grown in colonies on solid media, *G. diazotrophicus* had good levels of growth (i.e. $>2.0 \times 10^6$ CFU d^{-1}) from 0.5 to 15% sucrose; only at 0.1% sucrose was growth severely decreased (5.8×10^4 CFU d^{-1}) (Table 2). However, nitrogenase activity and EAC were more sensitive to sucrose concentration of the media. Nitrogenase activity and EAC were maximized at 5% sucrose; at 0.5 % sucrose these parameters had decreased by 59 and 37%, respectively, which would result in a considerable decline in N_2 fixation capacity. Reis and Döbereiner (1998) found nitrogenase activity of PAL5 (the same strain used in this study) and PAL3 were not significantly different with 1 or 10% sucrose in liquid media,

whereas Cavalcante and Döbereiner (1988) found a 47% reduction in nitrogenase activity in *G. diazotrophicus* when sucrose concentration of semi-solid media was reduced from 10 to 1%.

G. diazotrophicus is known to grow and fix N_2 well with glucose as the major carbon source in liquid culture (Stephan et al., 1991; Flores-Encarnacion et al., 1999; Luna et al., 2000). Although *G. diazotrophicus* PAL3 could use fructose as a carbon source, respiration rate was 30 to 50% of that with glucose as the carbon source (Alvarez and Martinez-Drets, 1995). Much to our surprise, we found that growth rate of colonies and nitrogenase activity of *G. diazotrophicus* dependent on glucose, fructose, or a combination of the two, were quite depressed compared to sucrose (Fig. 2).

Although *G. diazotrophicus* has been shown to grow on a number of carbon sources in liquid (Stephan et al., 1991), in semi-solid (Cavalcante and Döbereiner, 1988), and on solid media (Dong et al., 1995), the carbon source and concentration optimal for nitrogenase activity in each of these media types are not clear. *G. diazotrophicus* is known to grow and fix N_2 well with sucrose as the sole carbon source, however, Alvarez and Martinez-Drets (1995) reported that the bacterium was unable to transport sucrose. *G. diazotrophicus* has been show to excrete large amounts of levansucrase (Hernandez et al., 1995), an enzyme which extracellularly hydrolysis sucrose, releasing the glucose moiety into the media and utilizing the frustose moiety to build high molecular weight fructans. *G. diazotrophicus* has also been shown to contain a membrane-bound pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GDH) (Galar and Boiardi, 1995) which oxidizes glucose to gluconate (Attwood et al., 1991). Gluconate appeared to be a minor substrate for oxidase activity in the bacterium compared to glucose (approximately one-tenth) (Flores-Encarnacion et al., 1999), however Luna et al. (2000) found the growth yield (i.e. g DW of cells per mol C source] of *G. diazotrophicus* dependent upon gluconate and N_2 fixation to be 70% of the that when the cells were dependent upon glucose and N_2 for growth.

From our observations of the critical role of colony mucilage on the ability of *G. diazotrophicus* to fix N_2 on solid media (Pan and Vessey, 2001; Dong et al., 2002), and the need for sucrose (Table 1) and the inability of glucose or fructose to support good growth and nitrogenase activity on solid media (Fig. 2), we speculate that sucrose is a necessary carbon source for sufficient mucilage production by the bacterium to protect nitrogenase activity. The composition of the abundant mucilage of *G. diazotrophicus* colonies is not clear. A variety of exopolysaccharides are known to be produced by members of the genus *Gluconacetobacter* (e.g. Couso et al., 1987; Tajima et al., 1997; Ridout et al., 1998; Sakairi et al., 1998); likewise, a number of capsular polysaccharides and lipopolysaccharides of *G. diazotrophicus* have been characterized (Fontaine et al., 1995; Stephan et al., 1995; Previato et al., 1997). Given that levansucrase

represents more than 70% of the extracellular protein secreted from *G. diazotrophicus* (Hernandez et al., 1995), that strains of the genus *Gluconacetobacter* are known to have sucrose-dependent production of high molecular weight fructans (Tonouchi et al., 1998), and that fructo-oligosaccharide production by *G. diazotrophicus* levansucrase is dependent upon sucrose availability (Tambara et al., 1999), it is reasonable to hypothesize that fructans are a major component of the mucilage of *G. diazotrophicus* colonies and that absence of sucrose from a medium could interfere with the production of this component of the mucilage. This hypothesis is consistent with the findings of Hernandez et al. (2000) who showed that targeted disruption of the levansucrase (*lsdA*) gene in *G. diazotrophicus* resulted in bacteria that produced "non-mucous" colonies and were unable to grow in a media with sucrose as the sole C source.

In previous experiments utilizing liquid (Stephan et al., 1991) and semi-solid (Cavalcante and Döbereiner, 1988) culture, nitrogenase activity by *G. diazotrophicus* has shown to be tolerant of up to 10 mM NO_3^- in the media and it has been concluded that the bacterium is nitrate reductase deficient. In contrast, Muthukumarasamy et al. (1999) found that nitrogenase activity of some isolates of *G. diazotrophicus* was negatively affected by nitrate concentration of the media. In our experiments, we found that nitrogenase activity by *G. diazotrophicus* PAL5 grown on solid media was maximized at 0.1 mM NO_3^- and negatively affected by increasing concentrations, with only marginal activity at >5.0 mM NO_3^- (Table 3). The reason for this discrepancy between our study and previous reports of nitrate tolerance in liquid and semi-solid media is unclear. Clearly the physiological functioning of bacteria can vary between liquid, semi-solid, and solid culture systems. Many bacterial nitrate reductases are non-constitutive systems induced by various growth conditions (e.g. Arendsen et al., 1999; Oremland et al., 1999; Spector et al., 1999). It is possible that a some conditions or factors in a colony induces nitrate reductase activity in *G. diazotrophicus*. Alternatively, it is possible that the differentials effects of nitrate (Moir and Wood, 2001) versus ammonium (Dommelen et al., 2001) on the pH balance of the bacterial colony might be involved in the differential effects of mineral N source on colony nitrogenase activity.

Nitrogenase activity and growth of *G. diazotrophicus* was tolerant of ammonium concentrations in the solid media up to 2 mM NH_4^+ (Table 3). Above this concentration, ammonium was partially inhibitory. This findings are similar to previous findings in liquid culture (Stephan et al., 1991; Reis and Döbereiner, 1998). Note that in cultures with no ammonium, nitrogenase activity was high (Table 3). This appears to be in contrast to the work of Cavalcante and Döbereiner (1988) and Stephan et al. (1991) where a starter level of ammonium was required in media to initiate nitrogenase activity in *G.*

diazotrophicus. However these data are not in conflict; our solid media contains 50 mg yeast extract l^{-1} which Muthukumarasamy et al. (1999) have shown to support nitrogenase activity in various strains of *G. diazotrophicus*. Hence low concentrations of yeast extract can act as an N-starter dose in *G. diazotrophicus* similarly to starter levels of ammonium.

Overall our study indicates that growth and nitrogenase activity by *G. diazotrophicus* on solid media can be quite different from that reported for the bacterium in liquid or semi-solid culture. It is very interesting that sucrose appears to be a necessary component of solid media to enable good growth and nitrogenase activity. We suggests that sucrose-dependent production of fructans by levansucrase is an important component of the colony mucilage formation, a process putatively required for O_2 protection of nitrogenase activity by the bacterium on solid media.

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