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Effect of Oxygen on the Free-Living Nitrogen Fixation Activity and Expression of the *Azospirillum brasilense NifH* Gene in Various Plant-Associated Diazotrophs

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Abstract

As demonstrated previously (Vande Broek et al., 1993), the ambient oxygen tension can severely reduce associative N_2 fixation by plant-associated diazotrophs such as *Azospirillum brasilense*. To identify associative N_2 fixers that possess a more oxygen-tolerant nitrogen-fixation system, we analyzed the expression of an *Azospirillum brasilense nifH-gusA* fusion and the acetylene reduction activity as a function of the oxygen concentration in eight aerobic associative diazotrophs, namely *Acetobacter diazotrophicus, Alcaligenes faecalis, Azoarcus indigens, Azorhizophilus paspali, Azospirillum brasilense, Azospirillum irakense, Burkholderia vietnamiensis* and *Herbaspirillum seropedicae*. On the basis of the maximum oxygen concentration at which activation of the *nifH* fusion and acetylene reduction were still observed, these organisms were classified into three groups. In order of increasing oxygen tolerance, these groups are: (i) *Acetobacter diazotrophicus, Alcaligenes faecalis, Azospirillum brasilense, Azospirillum irakense, Burkholderia vietnamiensis* and *Herbaspirillum seropedicae* (maximum oxygen tension for acetylene reduction between 2.0 and 3.0%); (ii) *Azoarcus indigens* (maximum oxygen tension for acetylene reduction 6.5%); and (iii) *Azorhizophilus paspali* (maximum oxygen tension for acetylene reduction >8.5%).

Keywords: Associative nitrogen fixation, *NifH* induction, nitrogenase activity, oxygen tolerance

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1. Introduction

Biological nitrogen fixation is catalyzed by the nitrogenase enzyme complex which consists of two subunits: dinitrogenase (MoFe protein), containing the active site of dinitrogen reduction, and dinitrogenase reductase (Fe protein), supplying the reducing power to dinitrogenase. Because both proteins are irreversibly damaged by oxygen, biological nitrogen fixation requires intracellular microaerobiosis for optimal functioning of the nitrogenase enzyme. In the symbiotic rhizobial legume association, the nodule free oxygen concentration is constantly kept very low by the presence of a variable oxygen diffusion barrier in the nodule parenchyma and bacterial respiration in the central tissue (Witty et al., 1986). In this way, the nitrogenase enzyme is protected against oxygen damage, allowing nitrogen fixation to occur in a wide range of external oxygen tensions. In contrast, due to the extreme oxygen sensitivity of the nitrogen fixation system of plant-associated diazotrophs such as *Azospirillum brasilense* (Hartmann and Hurek, 1988), the environmental oxygen concentration is a major factor affecting associative nitrogen fixation by these N_2 fixers (Vande Broek et al., 1993). Therefore, the identification of bacterial strains that are able to fix nitrogen within a broad range of oxygen concentrations (large oxygen tolerance) is essential in attempts to enhance associative nitrogen fixation activity.

Here, we aimed at comparing the oxygen tolerance of several plant-associated diazotrophs. Oxygen tolerance of nitrogen fixation is generally determined in a closed system with a constant dissolved oxygen concentration. Although very accurate, such an approach makes it cumbersome to analyse large numbers of bacteria under different oxygen concentrations. Therefore, we developed a method to analyze oxygen tolerance for nitrogen fixation that allows testing of many samples.

The regulation of nitrogen fixation genes has not yet been studied in most plant-associated bacteria, except in *Azospirillum brasilense* (for a review see Michiels et al., 1992; Vande Broek and Vanderleyden, 1995). However, mechanisms of *nifH* expression have been shown to be strongly conserved among nitrogen-fixers (for a review see Merrick, 1992; Fisher, 1994). In all nitrogen-fixing Gram-negative proteobacteria studied, the *nifH* gene is always preceded by a conserved -24/-12 type of promoter. This type of promoter is recognized by the alternative RNA polymerase sigma factor, σ^{54}. Transcription of the *nifH* gene by the -24/-12 promoter-bound σ^{54} polymerase holoenzyme complex is activated by the transcriptional activator protein NifA. The NifA protein has been shown to bind a DNA motif, called UAS (upstream activator sequence), with the consensus sequence TGT-N_{10}-ACA that is generally located between 80 and 150 base pairs upstream from the *nifH* transcription initiation

site. Based on the conservation of this regulatory system, we monitored the induction of an *A. brasilense nifH-gusA* translational fusion as a function of the oxygen concentration in eight different associative aerobic nitrogen fixers as a first indication of their oxygen tolerance. These experiments were further extended by the analysis of the acetylene reduction activity of the selected strains.

2. Materials and Methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1.

Table 1. Bacterial strains used in this study.

Strain	Isolated from	Reference or source
Acetobacter diazotrophicus LMG 7603	Roots and stems of sugarcane, Brazil	Gillis et al., 1989
Alcaligenes faecalis LMG 10652	Rhizosphere of rice, China	You et al., 1983
Azoarcus indigens LMG 9092	Roots and stems of Kallar grass (*Leptochloa fusca*), Pakistan	Reinhold-Hurek et al., 1993
Azorhizophilus paspali LMG 3864	Rhizosphere of the grass *Paspalum notatum*	Thompson and Skernan, 1979
Azospirillum brasilense Sp245	Surface sterilized wheat roots, Brazil	Baldani et al., 1986a
Azospirillum irakense LMG 10653	Roots and rhizosphere of rice, Iraq	Khammas et al., 1989
Burkholderia vietnamiensis LMG 10927	Rhizosphere of rice, Vietnam	Van Tran et al., 1994
Herbaspirillum seropedicae Z67	Rice roots, Brazil	Baldani et al., 1986b

Media

Azospirillum minimal medium (MMAB) (Vanstockem et al., 1987) was used to grow *Alcaligenes faecalis, Azoarcus indigens, Azospirillum brasilense, Azospirillum irakense, Burkholderia vietnamiensis* and *Herbaspirillum seropedicae. Azorhizophilus paspali* was cultured in MMAB with 0.5% (w/v) sucrose instead of malate. For *Acetobacter diazotrophicus,* malate was also replaced by 5% sucrose and the pH of the medium was adjusted to 5.5. *Azoarcus indigens* cultures were supplied with 20 µg/l *p*-aminobenzoic acid. All strains were grown at 30°C.

The nitrogen-free media used in the derepression experiments were the media described above without NH_4Cl.

Transfer of pFAJ21 into the various diazotrophs

The *nifH-gusA* fusion plasmid pFAJ21 (Vande Broek et al., 1992) was transferred from *E. coli* strain S17.1 (Simon et al., 1983) into the various diazotrophs by a biparental mating. In pFAJ21, a 1.8 kb DNA fragment carrying the UAS for NifA mediated activation, the -24/-12 promoter and the first 71 codons of the *A. brasilense nifH* gene, is inserted upstream of the promoterless *gusA* gene. Transconjugants were selected on the appropriate minimal media supplemented with 10 µg/ml tetracycline (Tc) except for *B. vietnamiensis* for which a Tc concentration of 150 µg/ml was used. To verify the presence of pFAJ21 in the tetracycline resistant transconjugants, plasmid DNA was extracted and controlled by *Eco*RI restriction analysis.

Effect of oxygen on the expression of the A. brasilense nifH gene in the various diazotrophs

Exponential cultures of the various plant associated diazotrophs containing pFAJ21 were harvested, washed twice with 0.85% NaCl and finally dissolved in the appropriate nitrogen-free medium at a cell density of 10^7 cells/ml except where stated otherwise in the text. Portions (1 ml) of these suspensions were then injected with a sterile syringe and needle into 25 by 200 mm test tubes (total volume 80 ml) that were tightly stoppered with rubber caps and contained a specific gas mixture of N_2 and O_2. To introduce the gas mixture in the test tubes, the tubes were first flushed for 20 minutes with nitrogen and then, using a syringe, air was readmitted to give the appropriate oxygen tensions. The oxygen content in the headspace was monitored by an oxygen electrode (ABISS, Vak 10). Cultures were vigorously shaken on a rotary shaker at 200 rpm at 30°C to provide rapid equilibration between the gas and liquid

phase. After 20 hours of incubation, the final oxygen concentration in the test tubes was measured using an oxygen electrode and the glucuronidase activity of the culture was determined quantitatively using the substrate *p*-nitrophenyl-β-D-glucuronide as described by Jefferson (1987). Three independent cultures were always tested for each oxygen tension and each culture was assayed in duplicate. Units are as described by Miller (1972) for β-galactosidase activity.

Effect of oxygen on the acetylene reduction activity in the various diazotrophs

Wild-type cultures were incubated at various oxygen tensions in nitrogen-free minimal medium as described above. After 4 hours of incubation at 30°C, acetylene was injected in the test tubes to a final concentration of 10% (v/v). After an additional incubation of 16 hours, ethylene production was quantified on a 'Plot fused silica' column (50 m × 0.32 mm, 5 μm Al_2O_3/KCl, Chrompack) installed in a Hewlett Packard 5890A gas chromatograph. Propane was used as internal standard. The final oxygen tension in the test tube was determined with an oxygen electrode.

3. Results

Optimization of the oxygen tolerance assay

Our objective was to determine *nifH* expression and nitrogenase activity in various plant-associated diazotrophs (Table 1) at different constant oxygen tensions. Due to cellular respiratory activity, cultures incubated in stoppered test tubes will constantly decrease the prevailing oxygen tension. This decrease in oxygen concentration by respiration depends on the bacterial species cultured, the number of bacteria present in the tube and the volume of the headspace. In our assay, a headspace of 80 times the liquid volume was used (see materials and methods). The tubes were vigorously shaken to ensure a rapid equilibrium between the oxygen concentration in the gas and liquid phases and to avoid an oxygen concentration gradient in the liquid phase. A similar approach has been described previously for *A. brasilense* (Okon et al., 1976) and for *A. paspali* (Abbass and Okon, 1993). To estimate bacterial respiratory activity in our assay, we determined the decrease in the oxygen concentration in the headspace when different cell densities of *A. brasilense* strain Sp245(pFAJ21) were incubated for 20 hours in the 80 ml test tubes in N-free MMAB medium at different oxygen tensions. When 10^8 cells were injected, the maximum oxygen consumption observed over this period was 2%; with 10^7 cells the decrease in the oxygen tension was 0.2%, whereas when 10^6 cells were used no reduction in

the oxygen concentration could be measured (sensitivity of the O_2-electrode is 0.1%). These different reductions in the prevailing oxygen concentration due to bacterial respiration are responsible for the shift in the optimal initial oxygen tension for *A. brasilense nifH* induction. The oxygen concentration with maximal *nifH* induction increased from 0.3% for 10^6 cells in the tubes, to 0.5% for 10^7 cells, to 2% for 10^8 cells (Fig. 1.). In subsequent experiments, 10^7 cells were used instead of 10^6 cells since expression analysis of the *A. brasilense nifH-gusA* expression was not accurate in low cell density assays. In the comparative experiments, initial as well as final oxygen concentrations were always determined in each experiment to monitor strain dependent respiratory activity.

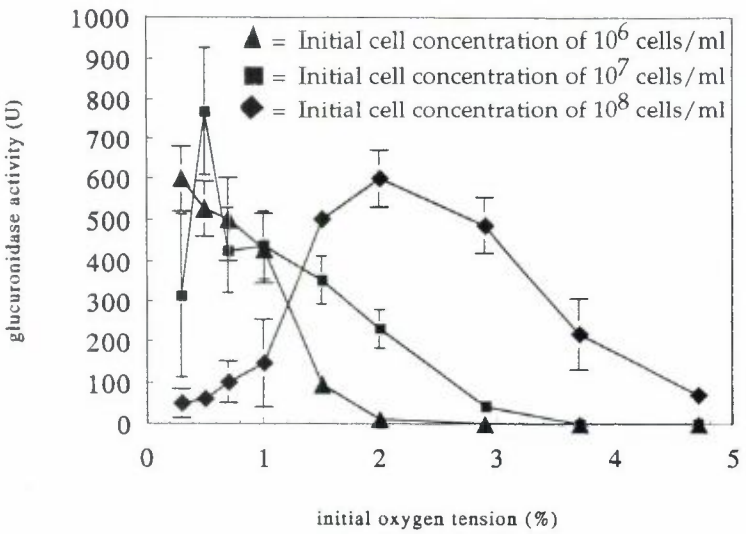

Figure 1. Expression of the *A. brasilense nifH-gusA* fusion plasmid pFAJ21 in *A. brasilense* Sp245 as a function of the initial oxygen concentration. Cells were incubated for 20 hours in nitrogen-free minimal medium at different initial oxygen tensions as described in Materials and Methods. β-glucuronidase activities (U) were determined at different initial cell concentrations.

Effect of oxygen on the expression of the A. brasilense nifH gene

Expression of the *A. brasilense nifH-gusA* fusion was evaluated in different plant-associated diazotrophs for several reasons. First, this method is easier and faster than acetylene reduction measurements and therefore can be used as a first selection criterion in the screening of different strains for their oxygen

tolerance. The first level of control of nitrogenase activity is *nifHDK* gene activation. If the *nifHDK* genes are not induced at 'high' oxygen concentrations, such a condition will not result in nitrogen fixation. Secondly, this method may give insight in the regulation of nitrogen fixation genes in the bacterium under study.

The oxygen profiles of pFAJ21 induction in the eight plant-associated N_2 fixers are shown in Fig. 2. The optimal and maximal oxygen concentrations at which activation of the *A. brasilense nifH* gene was observed are summarized in Table 2. The *A. brasilense nifH-gusA* fusion is active and induced microaerobically in all species tested. Oxygen concentrations for maximal *nifH* expression are less than 2.5% (v/v) for *A. diazotrophicus, A. indigens, A. brasilense, A. irakense, B. vietnamiensis* and *H. seropedicae;* and <3.7% for *A. faecalis* and between 3.0 and 6.5% for *A. paspali.* With the exception of *A. faecalis, A. indigens* and *A. paspali,* expression of the fusion is shut off between 3.7 and 6.1% oxygen. High levels of expression of pFAJ21 were still observed in *A. faecalis, A. indigens* and *A. paspali* at 8.5% oxygen.

Table 2. Optimal and maximum oxygen tensions of *nifH* expression and acetylene reduction activity in various plant-associated diazotrophs.

Strain	Optimal oxygen tension* (%)		Maximum oxygen tension** (%)	
	Expression pFAJ21	ARA	Expression pFAJ21	ARA
A. diazotrophicus	<1.5	0.5–1.5	4.8	3.0
A. faecalis	<3.7	0.3–0.7	>8.5	2.9
A. indigens	0.3–1.5	0.3–0.9	>8.5	6.5
A. paspali	3.0–6.5	2.5–6.5	>8.5	>8.5
A. brasilense	0.3–0.7	0.3–1.0	3.7	2.0
A. irakense	0.3–0.9	0.3–0.7	6.1	2.5
B. vietnamiensis	1.0–2.0	0.7–1.5	3.7	3.0
H. seropedicae	0.5–2.5	<1.0	4.7	2.5

*The optimal initial oxygen concentration is contained within the indicated range and differs statistically from both limits of the range. In case only one number preceded by the symbol < is given, the optimal initial oxygen concentration is lower than this number.
**The lowest initial oxygen tension tested at which no *nifH* induction and acetylene reduction were observed, >8.5% indicates that the maximum initial oxygen tension for *nifH* induction and acetylene reduction is higher than 8.5% oxygen.

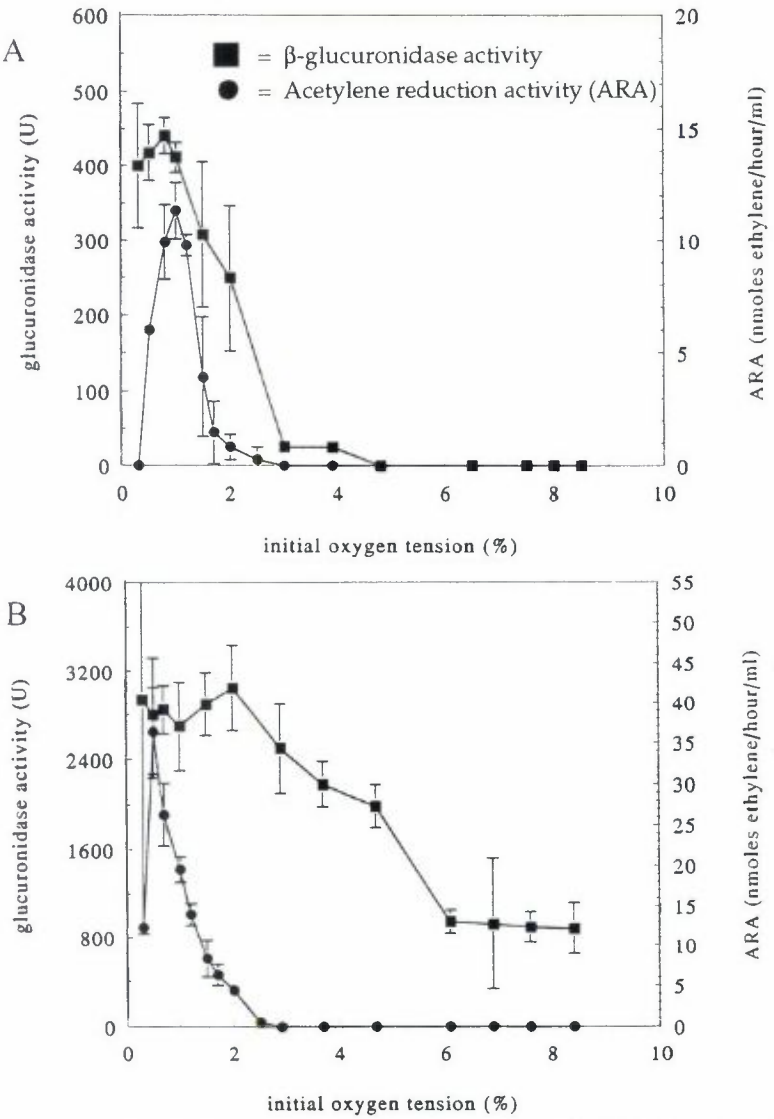

Figure 2. Expression of the *A. brasilense nifH-gusA* fusion plasmid pFAJ21 and acetylene reduction activity in several plant-associated diazotrophs as a function of the initial oxygen concentration. β-glucuronidase activities (U) are indicated on the left axis, acetylene reduction activities (ARA, nmoles ethylene/hour/ml) on the right axis. The strains that were analyzed are *A. diazotrophicus* (A), *A. faecalis* (B), *A. indigens* (C), *A. paspali* (D), *A. brasilense* (E), *A. irakense* (F), *B. vietnamiensis* (G), and *H. seropedicae* (H). Maximum oxygen respiration levels (initial minus final oxygen concentration) observed were 0.4% in *A. diazotrophicus*, 0.2% in *A. faecalis*, 0.6% in *A. indigens*, 1% in *A. paspali*, 0.2% in *A. brasilense*, 0.2% in *A. irakense*, 0.2% in *B. vietnamiensis*, and 0.2% in *H. seropedicae*. Vertical bars indicate the 95% confidence interval for the mean.

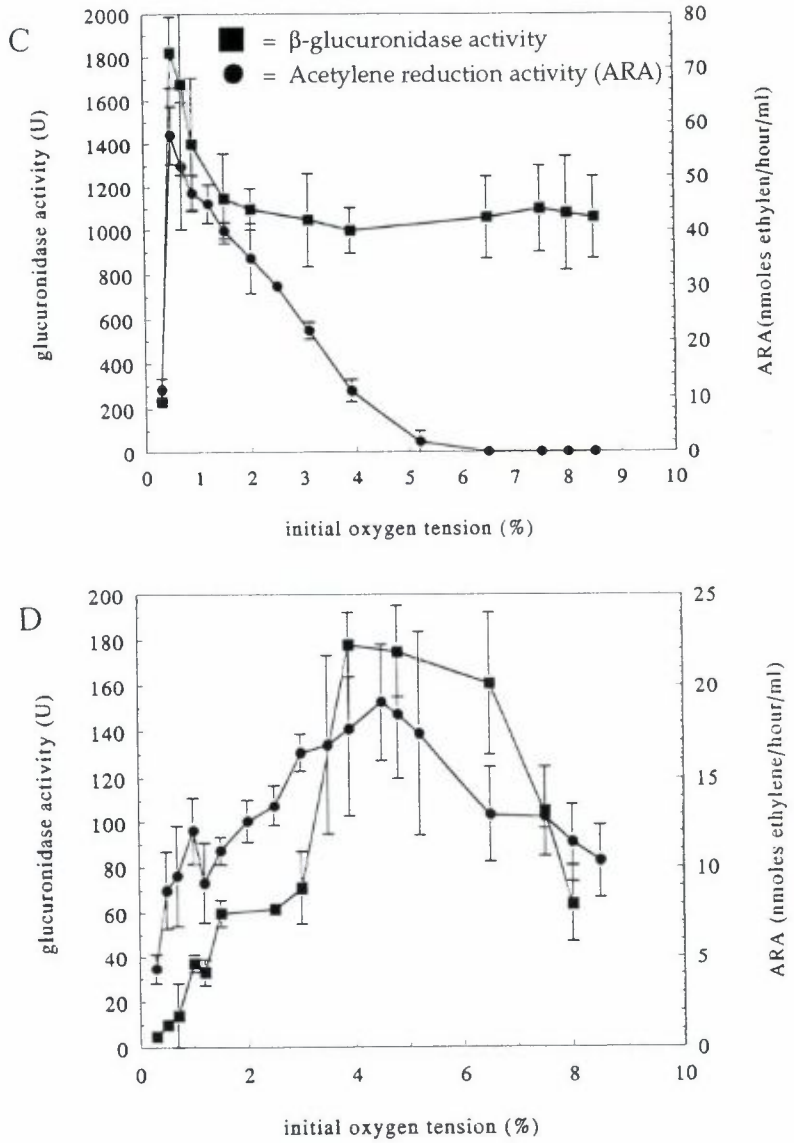

Figure 2. Continued. *A. indigens* (C), *A. paspali* (D).

Effect of oxygen on acetylene reduction activity

In a second experiment, acetylene reduction activity (ARA) of the bacterial species was measured as a function of the oxygen concentration. The results of

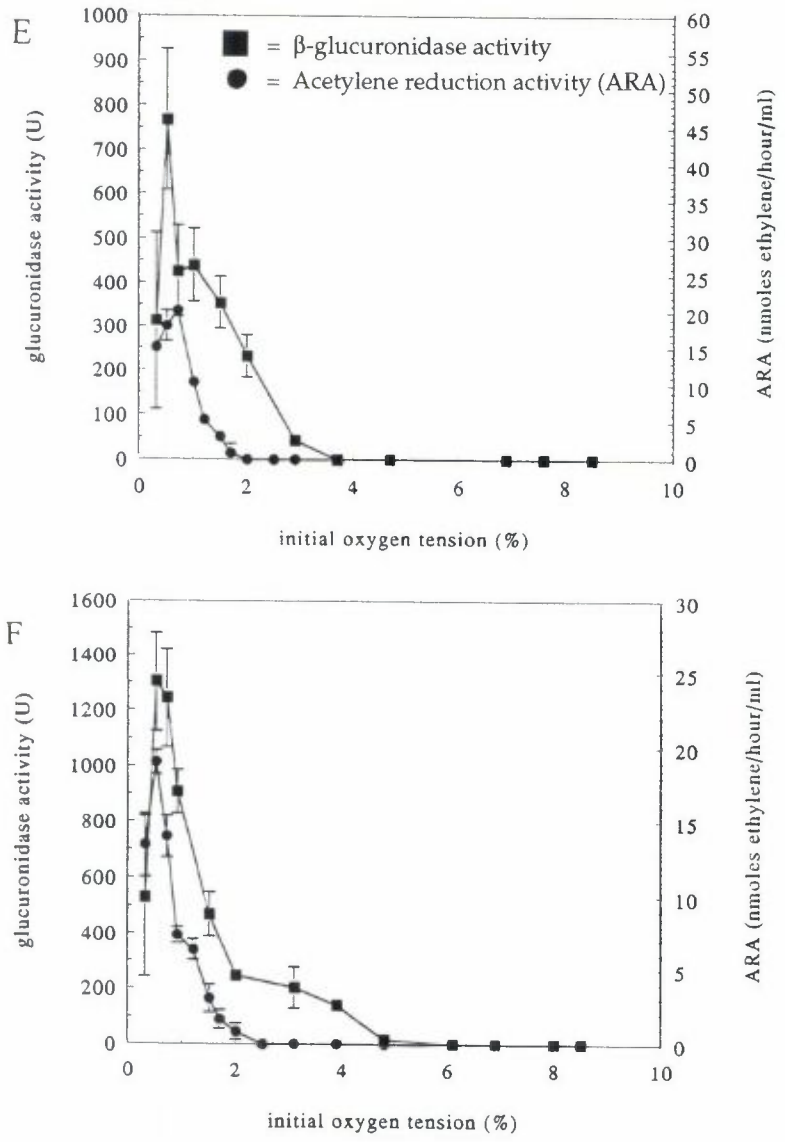

Figure 2. Continued. *A. brasilense* (E), *A. irakense* (F).

these tests are given in Fig. 2. The optimal and maximal oxygen concentrations for acetylene reduction activity in the different species are presented in Table 2. Optimal oxygen concentrations for ARA coincide quite well with optimal oxygen concentrations for the induction of pFAJ21. Maximum ARA for *A.*

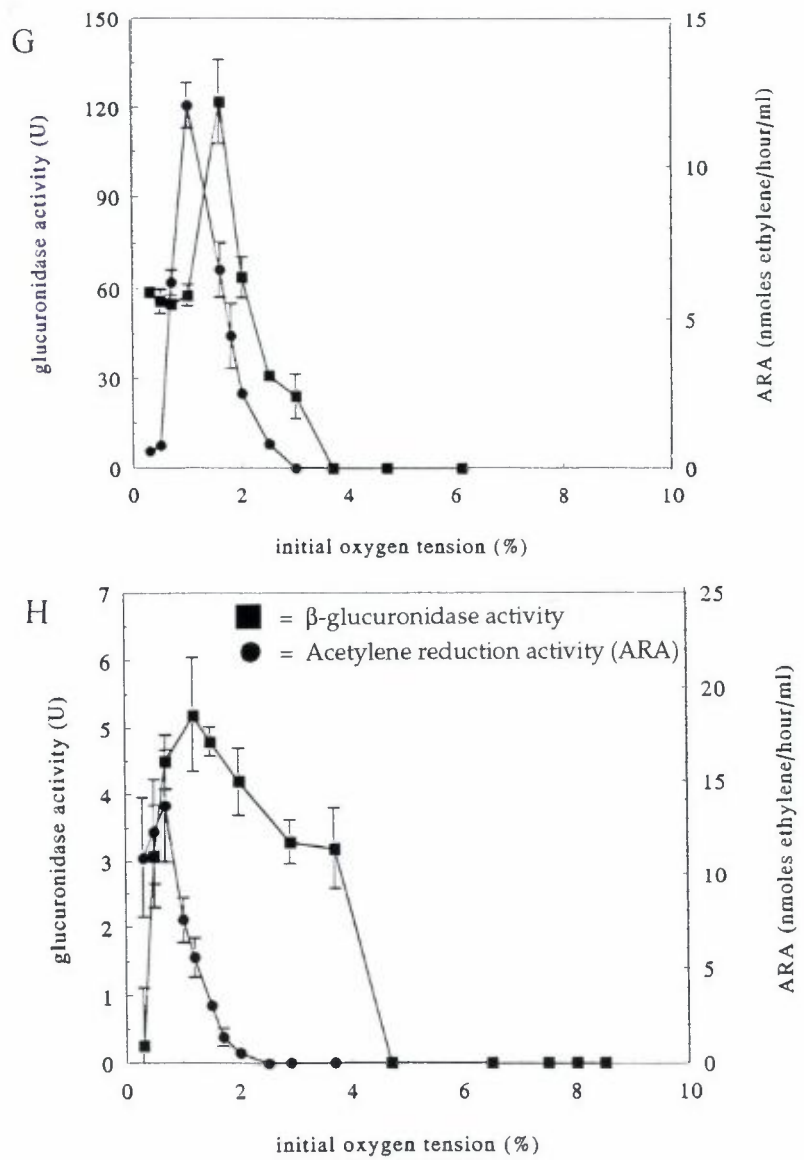

Figure 2. Continued. *B. vietnamiensis* (G), and *H. seropedicae* (H).

diazotrophicus, A. faecalis, A. indigens, A. brasilense, A. irakense, B. vietnamiensis and *H. seropedicae,* occurred at oxygen concentrations lower than 1.5% and between 2.5 and 6.5% in *A. paspali*. ARA reached the zero level between 2.0 and 3.0% oxygen in *A. diazotrophicus, A. faecalis, A. brasilense, A.*

irakense, *B. vietnamiensis* and *H. seropedicae*, at 6.5% in *A. indigens* and at >8.5% in *A. paspali*.

4. Discussion

We have analyzed the oxygen dependence of *nifH* expression and nitrogen fixation in various plant-associated aerobic diazotrophs. In aerobic nitrogen fixers optimal oxygen concentrations for nitrogen fixation result from two opposite processes. On one hand, oxygen is required for the production of reducing power and ATP to meet the energy demands of the nitrogen fixation process. On the other hand, too high oxygen levels can severely inhibit nitrogenase activity.

The oxygen concentration affects nitrogenase activity both at the transcriptional and the protein levels. The transcription of most nitrogen fixation genes, including the nitrogenase structural genes *nifHDK*, is induced only at low oxygen tensions by the gene product of *nifA*. Activity of the NifA protein itself is controlled at the transcriptional and/or posttranslational levels depending on the bacterial species (for a review see Merrick, 1992; Fisher, 1994). Within the group of eight associative diazotrophs analyzed in this work, the regulation of nitrogen fixation genes has only been studied in *A. brasilense*. In this species, *nifA* is constitutively transcribed but the level of transcription varies according to the cellular oxygen status and the NifA protein itself is oxygen sensitive (Liang et al., 1991).

The mechanisms involved in the regulation of nitrogenase activity depend on the oxygen concentration and on the bacterial species. In anaerobiosis, the *A. brasilense* and *A. lipoferum* nitrogenases are rapidly and reversibly inactivated by a covalent modification (ADP-ribosylation) of the Fe-protein (Hartmann and Burris, 1987). In *Azotobacter* spp., photosynthetic bacteria, cyanobacteria, *Azospirillum* spp. and *K. pneumoniae*, nitrogenase activity is reversibly inhibited at intermediate oxygen concentrations (Hochman et al., 1987). This phenomenon, termed nitrogenase switch-off, was initially described in *Azotobacter chroococcum* and was correlated in this species with the presence of a 2Fe-2S redox protein (Shethna protein) (Moshiri et al., 1994). However, no Shethna protein was detected in the other organisms such as *K. pneumoniae*, *Azospirillum* spp. and *Rhodospirillum rubrum* also displaying a reversible inhibition of nitrogenase activity (Hochman et al., 1987). It is suggested that at intermediate oxygen concentrations, instead of supporting nitrogen fixation activity, electrons are diverted from the nitrogenase towards respiration (Goldberg et al., 1987). Such a mechanism results in a shortage of reducing power for the nitrogenase and concomitantly in repression of

nitrogenase activity. Finally at high oxygen concentrations, the nitrogenase is irreversibly inactivated by the oxidation of the metal-S-centers of the protein.

In a first experiment, we have analyzed the activity of an *A. brasilense nifH-gusA* fusion in *A. diazotrophicus, A. faecalis, A. indigens, A. paspali, A. brasilense, A. irakense, B. vietnamiensis* and *H. seropedicae*. The *A. brasilense nifH-gusA* fusion is expressed in all diazotrophs tested. This is probably due to the high conservation of the mechanism of *nifH* expression in nitrogen fixing organisms (see Introduction). However, considerable differences in the level of expression are observed between the different species. Maximal β-glucuronidase activities observed in the different species range from 5 to 3000 units. Promoter sequences for maximal *nifH* activation are therefore likely to differ between the species analyzed in this work. Nucleotides that may be important for maximal promoter activation of *nifH* in one species, such as those surrounding the -24/-12 GG/GC base pairs and the NifA-binding site, may be absent in the *A. brasilense nifH* promoter. This in turn may lead to a low level of expression of the *A. brasilense nifH* gene in other species.

In all species tested, induction of the *A. brasilense nifH-gusA* fusion was shown to be oxygen-dependent. Except for *A. indigens, A. faecalis* and *A. paspali*, β-glucuronidase activities were maximal in the 0.3 to 2.5% oxygen range, decreased at higher oxygen tensions and reached the zero level at oxygen concentrations of 3.7 to 6.1%. In *A. indigens, A. faecalis* and *A. paspali* activation of the *A. brasilense nifH-gusA* fusion was still observed at an oxygen concentration of 8.5%. Activation of the *nifH* gene at this oxygen concentration indicates that the mechanism of NifA-mediated activation of *nifH* in these three organisms is less oxygen-sensitive than in the other species analyzed. Therefore, both *nifA* transcription and activity of the NifA protein in *A. indigens, A. faecalis* and *A. paspali* are more oxygen-tolerant.

In a second experiment, nitrogen fixation activity of the eight different diazotrophs was determined as a function of the oxygen concentration. On the basis of the *nifH* induction tests, *A. brasilense, A. irakense, B. vietnamiensis, H. seropedicae* and *A. diazotrophicus* can be classified as oxygen-sensitive. In agreement with this classification, the upper limit for nitrogenase activity in these organisms was between 2 and 3% oxygen. The *A. brasilense nifH-gusA* fusion was actively transcribed in *A. indigens, A. faecalis* and *A. paspali* at an 8.5% initial oxygen concentration. On the basis of acetylene reduction tests, these organisms can be further subdivided into three classes. The upper limit for nitrogen fixation in *A. faecalis, A. indigens* and *A. paspali* is 2.9, 6.5 and >8.5% oxygen, respectively. Therefore *A. faecalis* can be classified, together with *A. brasilense, A. irakense, B. vietnamiensis, H. seropedicae* and *A. diazotrophicus* in the group of organisms with a low degree of oxygen-tolerance, *A. indigens* possesses medium oxygen tolerance and *A. paspali* is

highly tolerant to oxygen. Recently, Hurek et al. (1994) described for the *Azoarcus* strain BH72 a phenomenon called 'hyperinduction' that is characterized by high rates of respiration and efficient N_2 fixation at very low oxygen concentrations and that is thought to enable these bacteria to create niches of low oxygen concentrations at high oxygen fluxes.

Besides the maximum oxygen concentration at which an organism is able to fix nitrogen, the optimum oxygen concentration for nitrogen fixation also merits attention. In all strains tested except *A. paspali*, the oxygen tension at which *nifH* expression was maximal ranged from 0.3 to 2.5% (*A. faecalis* <3.7%), the oxygen tension with maximal ARA was between 0.3 and 1.5% oxygen. In *A. paspali*, maximal *nifH* expression and ARA were observed between 3.0 to 6.5% and 2.5 to 6.5% oxygen, respectively. Clearly, *A. paspali* possesses besides a high oxygen tolerance for nitrogen fixation also a high optimal oxygen concentration for *nifH* expression and ARA.

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