Spatial-Temporal Colonization Patterns of *Azospirillum brasilense* on the Wheat Root Surface and Expression of the Bacterial *nifH* Gene during Association

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Colonization of wheat roots by *Azospirillum* was studied using the GUS reporter system. The surface distribution of *A. brasilense* on developing wheat roots was investigated using *A. brasilense* strains expressing the *gusA* gene. During the first days of association, bacteria were mainly found in the root hair zones and at the sites of lateral root emergence. Further proliferation of *Azospirillum* on the wheat root surface was dependent on the nitrogen status of the nutrient solution. Similarly, the expression of a *nifH–gusA* fusion was monitored during the association. Our results indicate that both oxygen and availability of carbon sources are limiting factors for associative nitrogen fixation.

Additional keywords: *Gramineae*, plant growth-promoting rhizosphere bacteria (PGPR), root colonization assay.

Nitrogen-fixing bacteria of the genus *Azospirillum* have been isolated from the roots of many grasses, including important crops such as maize, rice, and wheat (Tarrand *et al.* 1978). Plant growth promotion by *Azospirillum* has been demonstrated in field (Baldani *et al.* 1987) and greenhouse experiments (Gaskins *et al.* 1977) and attributed to several mechanisms, including nitrogen fixation (Von Bulow and Döbereiner 1975; Smith *et al.* 1976) and auxin production (Tien *et al.*1979).

Unlike the *Rhizobium*–legume symbiosis, the association of *Azospirillum* with plant roots does not result in the formation of an easily detectable plant structure. Largely because of the lack of a clear phenotype, the mechanisms of interaction and plant growth stimulation by *Azospirillum* are poorly understood. Precise sites of root association are not visible and, hence, the isolation and analysis of interaction mutants remains difficult.

During the last few years the study of gene expression in plants has been greatly facilitated by the use of the *E. coli gusA* (formerly *uidA*) gene as a reporter gene (Gallagher 1992). *GusA* encodes a β-glucuronidase whose activity can be easily monitored using a wide variety of glucuronides (Jefferson 1987b). Because of the absence of GUS activity in most higher plants, including wheat (Hu *et al.*1990), and the availability of sensitive histochemical substrates, we used the GUS gene fusion system to study the *Azospirillum*–wheat association. First, the wheat root colonization by *A. brasilense* was visualized on the root surface using strains constitutively expressing the *gusA* gene. Striking differences in colonization patterns were observed in time. Second, using a *nifH–gusA* fusion, the expression of the bacterial nitrogenase structural genes (*nifHDK* genes; de Zamaroczy *et al.* 1989; Fani *et al.* 1989) was monitored during this association.

RESULTS

Construction of *gusA* fusions expressed in *A. brasilense* under various physiological conditions.

An *Sau*3A gene bank of *A. brasilense* Sp7 was constructed in pFAJ31, upstream of the promoterless *gusA* gene, and conjugated to *A. brasilense* Sp245 (see Materials and Methods). Twenty dark-blue staining clones on

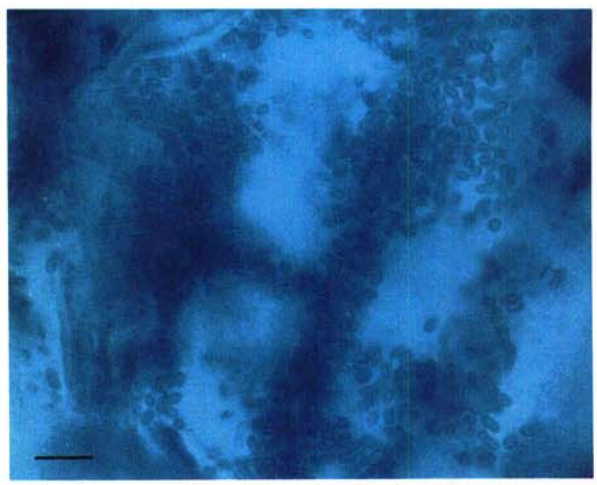

Fig. 1. A high magnification surface view of a colonization site on a wheat root, inoculated with Sp245(pFAJ31.2) and stained with X-Gluc. Bar represents 5 μm.

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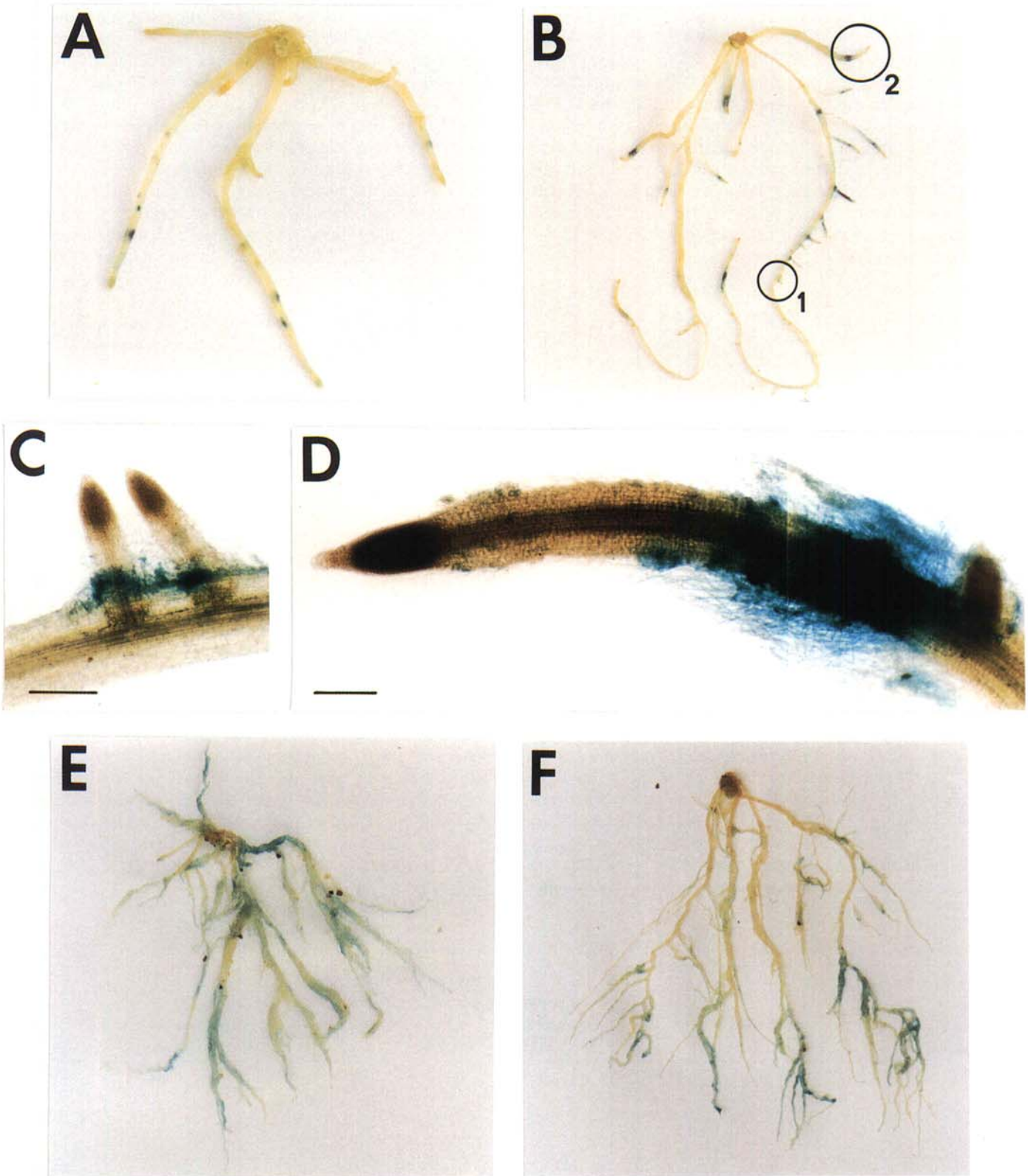

Fig. 2. Histochemical localization of *Azospirillum brasilense* Sp245, labeled with the *gusA* fusion plasmids pFAJ31.2 or pFAJ31.13 on the surface of wheat roots. **A**, **B**, Seedlings grown in MPCL supplemented sand and stained 2 days (**A**) and 4 days (**B**) after the inoculation. **C**, Enlargement of encircled area 1 in B showing the colonization at the basis of lateral roots. Bar represents 100 μm. **D**, Enlargement of encircled area 2 in B showing colonization in the root hair zone. Note absence of bacteria on the root cap, the meristematic and elongation zone. Bar represents 100 μm. **E, F**, Seedlings grown in sand cultures supplemented with MPCL (**E**) and nitrogen-free MPCL (**F**). Staining with X-gluc 21 days after inoculation. In all treatments staining with X-Gluc was for 16 hr at 37° C.

medium containing X-Gluc were further assayed quantitatively for β-glucuronidase (GUS) activity when grown aerobically in YEP medium. GUS activity of two clones, harboring the plasmids pFAJ31.2 and pFAJ31.13 and showing the highest activity under this condition was further examined under different physiological conditions. Table 1 indicates that, although variation in glucuronidase activity does exist, both fusions show substantial activity under all tested free-living and plant-associated growth conditions. Consequently, both fusions were considered suitable to localize *Azospirillum* on wheat roots in colonization assays.

Colonization of wheat roots by *A. brasilense*.

To visualize the precise sites of root colonization, wheat seedlings were inoculated with *A. brasilense* Sp245 carrying the *gusA* fusion plasmids pFAJ31.2 or pFAJ31.13 and analyzed at various times after inoculation. The plant nutrient solution was MPCL or nitrogen-free MPCL medium. Colonization was examined daily during the first 6 days after inoculation and from then on at 3-day intervals until the 27th day postinoculation. Analyses were carried out with 10 independent plant cultures for each treatment. After staining, root-colonizing bacteria were detected as blue zones on the root surface (Fig. 1). The presence and absence of bacteria in, respectively, blue-stained and nonstained zones was always confirmed by light microscopic analysis. Uninoculated roots remained white, confirming the lack of endogenous β-glucuronidase activity in wheat roots and proving the specificity of the visualization procedure.

In nitrogen-depleted as well as in nitrogen-supplemented rooting medium, primary colonization (until day 5 postinoculation) was at the sites of lateral root emergence (Fig. 2A–C) and at the root hair zone of the primary as well as of the lateral roots (Fig. 2B). The root cap and the meristematic and the elongation zones had no bacteria (Fig. 2D). In the root hair zone, bacteria were mainly localized in the zone of mature root hairs (Fig. 2D). In nitrogen-free plant nutrient solution, bacteria in this zone were located both on the root hairs, including the root hair tips, and the epidermis, whereas in the presence of combined nitrogen azospirilla were found mainly at the root hair bases and the epidermis (data not shown).

Five to seven days after inoculation, the localized colonization sites observed during the first days gradually disappeared and bacteria became more spread over the rootage. The resulting colonization patterns gradually became more dependent on the nitrogen content of the seedling nutrient solution. After 3 wk, on plants grown in MPCL medium, azospirilla were spread over the entire root surface (Fig. 2E), whereas in nitrogen-free MPCL bacteria preferentially colonized the lower parts of the root system (Fig. 2F).

As mentioned above, two different fusions were used throughout the experiments. Under no circumstances was any difference in colonization pattern observed, ruling out potential artifacts that might be attributable to the use of one particular recombinant.

Analysis of *A. brasilense nifH* expression during association with wheat roots.

In the free-living state, the nitrogen fixation process in *A. brasilense* is controlled by oxygen tension and the concentration of combined nitrogen. This control is exerted at the level of the transcription of the nitrogenase structural genes (de Zamaroczy *et al.* 1989; Liang *et al.* 1991; Milcamps and Vanderleyden 1991; Vande Broek *et al.* 1992) and at the level of nitrogenase activity (Hartmann *et al.* 1986; Hartmann and Burris 1987). To investigate whether the *A. brasilense nifH* gene is expressed during the association, the histochemical analysis was repeated with strain Sp245(pFAJ21a) (Vande Broek *et al.* 1992), carrying a *nifH–gusA* fusion plasmid. Associative *nifH* expression was examined every 2 days during the first 6 days after inoculation and from then on at 5-day intervals until the 26th day postinoculation. Analyses were carried out with five independent plant cultures. When plants were incubated in nitrogen-free MPCL medium, no β-glucuronidase activity on the root surface was observed during the first 11 days after

Table 1. Expression of the *gusA* fusions pFAJ31.2 and pFAJ31.13 in *Azospirillum brasilense* Sp245, grown under different conditions

	Glucuronidase activity[a]							
	Free-living conditions						Associative conditions[b]	
	Aerobic[c]		Microaerobic[c]		Anaerobic[c,d]			
Medium/strain	YEP	MMAB	YEP	MMAB	YEP	MMAB	MPCL	Nitrogen-free MPCL
Sp245	0	0	0	0	0	0	ND[e]	ND[e]
Sp245 (pFAJ31.2)	33.8	5.6	74.7	6.2	86.9	7.5	46.0	27.6
Sp245 (pFAJ31.13)	20.6	14.5	10.1	5.8	11.2	7.8	75.9	57.5

[a] Glucuronidase activity is expressed as nanomoles *p*-nitrophenol produced per minute per 10^8 cells. All data are the means of three independent experiments, each assayed in duplicate.

[b] Seedlings were assayed 15 days after inoculation. Reisolation of bacteria was as described in Materials and Methods.

[c] Precultures were grown aerobically to late log phase in YEP medium, washed twice and resuspended in an equal volume of sterile physiological water. 100 µl of cell suspension was used to inoculate 5 ml of the appropiate media. For aerobic incubation, cells were shaken for 24 hr at 30° C. Anaerobic incubations were for 1 wk in anaerobic jars (Gaspak, Becton-Dickinson & Co.). For microaerobic incubation cells were flushed with a gas mixture of 98.5% N_2 and 1.5% O_2 as described by Vande Broek *et al.* (1992).

[d] For anaerobic growth, media were supplemented with 100 mM KNO_3.

[e] Not determined.

inoculation. Thereafter, small blue spots appeared on the lower parts of the roots (Fig. 3A). Plants grown in MPCL medium never showed any GUS activity, conform with the inhibition of *A. brasilense nif* gene expression by combined nitrogen in the free-living state.

As a result of the observed low level of *nifH* induction, we examined whether carbon and/or oxygen concentrations are limiting factors in associative *nifH* transcription. Plants, inoculated with Sp245(pFAJ21a), were grown in nitrogen-free MPCL medium in the presence or absence of 0.1% malate or 0.1% fructose. Sixteen days after inoculation, half of the plants from each treatment were incubated microaerobically (3% O_2) for 16 hr. All root systems were subsequently examined for β-glucuronidase activity.

Under aerobic conditions, *nifH–gusA* expression was limited to the lower part of the root system either in the presence or in the absence of an additional C-source, indicating microaerobic conditions in this zone. Staining was always more intense in medium supplemented with malate or fructose as compared to the carbon-free nutrient solution (Fig. 3B). When plants were incubated microaerobically, in addition to a more intense β-glucuronidase activity at the lower part of the root system, a weak staining of the upper part of the rootage was observed (Fig. 3C,D). These data confirm earlier observations (see previous paragraph), indicating a local enrichment of the bacteria at the lower part of the root system when plants are grown in N-free rooting medium.

To corroborate these histochemical observations, we assayed associative *nifH* expression quantitatively. Plants inoculated with Sp245(pFAJ21a) were grown as described above and harvested 16 days after inoculation. Bacteria were reisolated and bacterial glucuronidase activity was determined. Data are shown in Table 2. The results are in accordance with the qualitative analysis. *nifH* induction was low when seedlings were grown in nitrogen-free medium without any additional carbon source. High levels of GUS activity were detected when the nutrient solution was supplemented with fructose or malate. In addition, *nifH* expression in all different nutrient media was approximately fourfold higher when plants were incubated microaerobically for 16 hr before analysis. No β-glucuronidase activity was measured in medium containing combined nitrogen or when bacteria were incubated under similar conditions in N-free MPCL medium in the absence of the plant.

Analysis of *A. brasilense* nitrogenase activity during the association with wheat roots.

To determine whether the observed associative *nifH* induction coincides with bacterial nitrogen fixation activity, inoculated wheat seedlings, grown in N-free MPCL, were monitored for acetylene reduction activity (ARA) at 4-day intervals during 4 wk. In accordance with data on associative *nifH* expression, ethylene production started only after day 11 of the association. In addition, ARA was determined 16 days after inoculation as a function of oxygen concentration in the presence or absence of an additional carbon source (Fig. 4). Nitrogenase activity was significantly higher when a carbon source was added to the nutrient solution. Likewise, higher acetylene reduction activities were measured when oxygen tension was lowered. Maximal activities were three- to sixfold higher than the aerobic controls and were obtained at a 3% initial oxygen concentration.

DISCUSSION

The establishment of *Azospirillum* on growing plant roots is well recognized as a critical step towards an effective plant-growth promotion (Okon 1985). Nevertheless, the molecular mechanisms of the *Azospirillum*–wheat root colonization are not yet clearly understood.

Preferential proliferation sites have been determined either by bacterial counts on root segments (Bashan *et al.* 1986; Bashan and Levanony 1989) or by direct microscopic evaluation of the root surface (Bashan *et al.* 1986; Bashan and Levanony 1989; Okon and Kapulnik 1986; Patriquin and Döbereiner 1978; Umali-Garcia *et al.* 1980). Shank *et al.* (1979) described the use of immunofluorescence for the identification of *Azospirillum* along the root surface, but the method was limited by autofluorescence and nonspecific binding of the conjugate. In the present study we describe a root colonization assay using *gusA* marked *Azospirillum* strains. Because of the complete absence of endogenous glucuronidase activity in wheat roots, this method enabled us to visibly detect in a very specific way the main association sites of *Azospirillum* on wheat roots. Besides the advantage of specificity, the technique is fast when compared with methods such as bacterial counts and direct microscopic observation, thus allowing the analysis of increased number of samples including mutant strains.

During the first days of association, colonizing azospirilla were found mainly at the basis of lateral roots and in the root hair zones of the primary as well as the lateral roots. The root elongation zone, however, remained devoid of bacteria. In the root hair zone, a strong colonization of the root hairs was only observed in the absence of combined nitrogen. Colonization of the epidermal cells, however, did not depend on the nitrogen status of the plant-growth medium. The mechanisms responsible for the preferential colonization at the sites of lateral root emergence and in the root hair zones are yet unknown but may involve chemotaxis and/or specific attachment. *Azospirillum* strains have been shown to develop significant chemotactic ability towards several amino acids, sugars, organic acids (Barak *et al.* 1983; Okon *et al.* 1980; Reinhold *et al.* 1985), components of root exudates (Heinrich and Hess 1985), and root mucilage (Mandimba *et al.* 1986). Furthermore, Bashan (1986) showed that *A. brasilense* cells migrate towards wheat roots in sand culture and in a wet soil. The attachment of *Azospirillum* to root hairs was reported by Umali-Garcia *et al.* (1980). They showed that pearl millet roots released protease sensitive, nondialyzable substances that bind to azospirilla and promote their adherence to root hairs. In their study, significantly more

azospirilla adhered to root hairs when plants were incubated in nitrogen-free nutrient solution as compared to nitrogen-supplemented rooting medium. A nitrogen-dependent root hair–*Azospirillum* association agrees with our observations.

Mutants with properties believed to be important for an efficient root colonization such as motility (Croes *et al.* 1991) and chemotaxis (van Rhyn *et al.* 1990) are now available. Moreover, a number of putative plant interaction genes have been identified indirectly by hy-

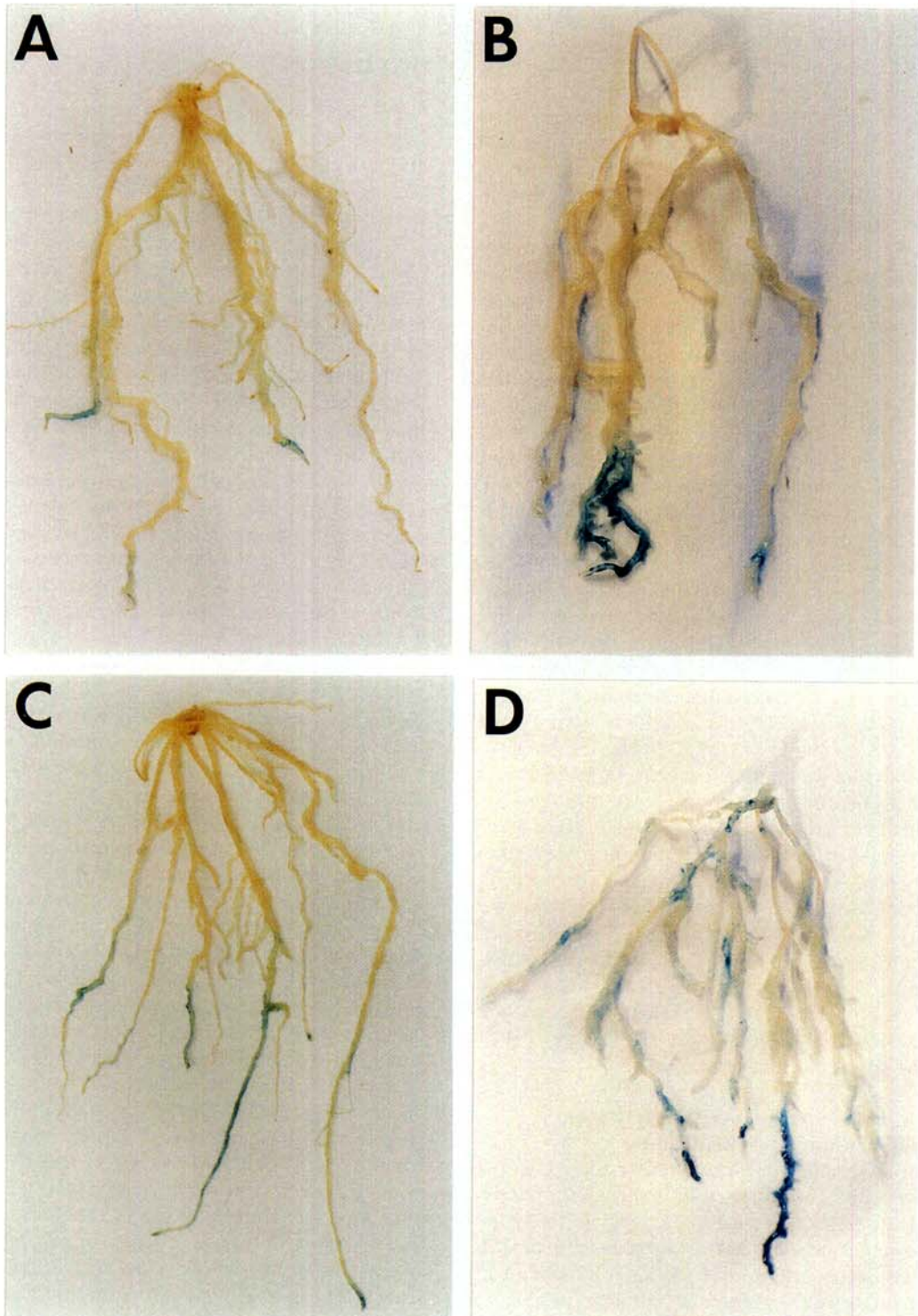

Fig. 3. Histochemical localization of β-glucuronidase activity on wheat roots, 16 days after inoculation with Sp245(pFAJ21a). Wheat seedlings were grown in sand cultures with nitrogen-free MPCL (**A**, **C**) or nitrogen-free MPCL supplemented with 0.1% of fructose (**B**, **D**) as nutrient solution. **A, B**, aerobic incubation. **C, D**, microaerobic incubation (3% O_2) for 16 hr prior to histochemical analysis. Staining with X-Gluc was for 7 hr at 37° C.

bridization with heterologous gene probes derived from *Rhizobium* (Vieille and Elmerich 1990; 1992) and *Agrobacterium tumefaciens* (Waelkens *et al.* 1987) or by genetic complementation of *Rhizobium meliloti* symbiotic mutants (Michiels *et al.* 1988). Michiels *et al.* (1990) isolated mutants deficient in the synthesis of a Calcofluor-binding polysaccharide. In short-term incubation studies, these mutants were also affected in firm anchoring to the wheat root surface (Michiels *et al.* 1991). The use of mutants and genes, in combination with the specific detection method reported here, will help us to study the bacterial determinants for *Azospirillum*–plant root interaction.

When plants were grown for 3 wk in rooting medium without combined nitrogen, bacteria gradually became enriched in the lower parts of the roots. This part corresponds to the microaerobic zone (see also further). It is likely that bacteria migrate to or specifically multiply in the zones where oxygen concentrations are low, allowing N_2 fixation to occur. Aerotaxis of *A. brasilense* to lower oxygen tensions has been demonstrated previously (Barak *et al.* 1982).

When controlled by a specific promoter, *gusA* can also be used to study bacterial gene expression during the association. Using this system, we determined associative *nifH* expression both qualitatively and quantitatively. A prerequisite for the use of a *gusA* gene fusion to visually monitor gene expression during the association is the complete absence of any background activity. This is the case in our system, since no background activity in wheat (Hu *et al.* 1990), in *Azospirillum*, or due to read-through transcription from a vector promoter in pFAJ21a (Vande Broek *et al.* 1992) was detected. In the free-living state, nitrogen fixation in *A. brasilense* occurs only in a narrow range of low oxygen tensions and in the absence of combined nitrogen. This control is exerted at the level of transcription of the nitrogenase structural genes (de Zamaroczy *et al.* 1989; Liang *et al.* 1991; Milcamps and Vanderleyden 1991; Vande Broek *et al.* 1992) as well as at the level of nitrogenase activity (Hartmann 1988). Besides the nitrogen and oxygen concentrations, the nitrogen-fixing activity in free-living conditions is also affected by the availability of energy sources (Martinez-Drets *et al.* 1984).

In accordance with gene regulation under free-living conditions, expression of the *nifH–gusA* fusion during the *Azospirillum*-wheat association was completely inhibited when combined nitrogen was present in the plant medium. In N-free rooting medium, associative *nifH* expression was low and limited to the lower parts of the rootage. Enhanced bacterial *nifH* induction was observed in the presence of an additional carbon source or when oxygen concentrations were lowered to microaerobic levels. These results were confirmed by the determination of bacterial nitrogen-fixation activity under the same conditions. From these results it appears that the availability of a C-source and the proper O_2 concentration are important factors to be taken into consideration when optimalizing associative nitrogen fixation. The oxygen tolerance of an organism is the result of different cellular mechanisms. Manipulation therefore may not be easy. Hartmann and Hurek (1988) have described the isolation of carotenoid-overproducing mutants of *A. brasilense* with a slightly enhanced oxygen tolerance. The optimum oxygen level for nitrogen fixation, however, was not extended to higher oxygen concentrations. Also, species and strain-specific differences in oxygen tolerance of *Azospirillum* and other plant associated diazotrophs have been reported (Hartmann 1988).

Numerous factors affect the association between *Azospirillum* and the plant. The study of bacterial gene expression during the interaction and the analysis of mutant strains affected in associative functions may lead to a better understanding of the factors controlling this

Table 2. Effect of the carbon and nitrogen content of the plant nutrient solution and the oxygen tension on the expression of the *A. brasilense nifH* gene during the association

	Associative *nifH* induction[a]	
Nutrient solution[b]	**Aerobic**	**Microaerobic[c]**
MPCL-N	7 (8)	32 (13)
MPCL-N + 0.1% malate	61 (20)	258 (118)
MPCL-N + 0.1% fructose	85 (38)	328 (112)
MPCL	0	ND[d]
MPCL + 0.1% malate	0	ND
MPCL + 0.1% fructose	0	ND
MPCL-N (no seedling)[e]	0	0

[a] Bacteria were reisolated from the roots and the sand 16 days after inoculation as described in Materials and Methods. β-Glucuronidase activity is expressed as nanomoles *p*-nitrophenol produced per minute per 10^8 cells. The values shown are the means of eight replicates, assayed in duplicate. Standard deviations are given within parentheses.
[b] MPCL-N is nitrogen-free MPCL medium.
[c] The oxygen concentration in the test tubes was lowered to 3% as described in Materials and Methods.
[d] Not determined.
[e] Bacteria incubated in sand cultures in the absence of a wheat seedling.

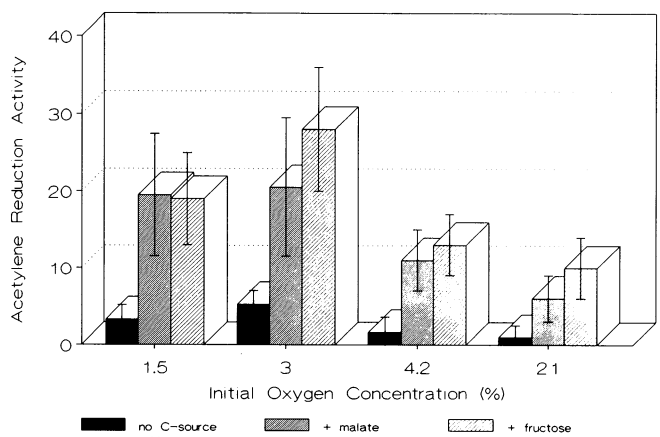

Fig. 4. Acetylene reduction activity of wheat seedlings inoculated with *Azospirillum brasilense* Sp245. Seedlings were grown in sand cultures with nitrogen-free MPCL and nitrogen-free MPCL supplemented with 0.1% malate or 0.1% fructose as nutrient solution. ARA was scored 16 days after the inoculation . Activity equals nanomoles ethylene produced per hour per 10^8 cells. Data points represent the average of 10 seedlings. Vertical bars indicate 95% confidence intervals. The oxygen concentration in the test tubes was lowered as explained in Materials and Methods.

association and may therefore constitute an important step towards an improvement of bacterial plant growth promotion.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 3. *E. coli* strains were routinely grown at 37° C in LB medium (Miller 1972). YEP medium or *Azospirillum* minimal medium (MMAB medium) (Vanstockem *et al.* 1987) was used for growing *Azospirillum* strains (30° C). When appropiate, the media were supplemented with tetracycline (Tc) at a final concentration of 10 µg/ml. Indicator plates for *Azospirillum* strains carrying *gusA* fusions contained 50 µg/ml 5bromo-4chloro-3indolyl β-D-glucuronide (X-Gluc) (Jefferson 1987a).

Construction of the *gusA promoter* probe vector pFAJ31.

To construct the promoter probe vector pFAJ31, the 2-kb *Eco*RI-*Bam*HI fragment of pBI101 (Jefferson 1987b), carrying the promoterless *E. coli gusA* gene, was cloned into the *Eco*RI-*Bam*HI digested pLAFR3 (Staskawicz *et al.* 1987). In pFAJ31 the unique restriction sites *Bam*HI, *Hin*dIII, and *Pst*I are preceding the *gusA* ATG start codon.

Isolation of recombinant plasmids expressing the *gusA* gene in *A. brasilense*.

Genomic DNA of *A. brasilense* Sp7 was partially digested with *Sau*3A and ligated into the *Bam*HI site of pFAJ31. The ligation mixture was packaged and used to transfect *E. coli* HB101 (Maniatis *et al.* 1982). Plasmids from approximately 1,000 *E. coli* clones, pooled in LB, were mobilized to Sp245, using the helper plasmid pRK2013, as described by Vanstockem *et al.* (1987). Recombinant cosmids expressing the *gusA* gene in Sp245 were directly selected as blue transconjugants on MMAB medium containing Tc and X-Gluc.

Germination, inoculation, and growth of wheat seedlings.

Wheat seeds (*Triticum aestivum* 'Fidel', Clovis-Maton, Belgium) were surface sterilized by consecutive immersions in 70% ethanol for 3 min and in 16% commercial bleach in 0.1% SDS for 1.5 hr. The seeds were rinsed three times with sterile distilled water and further incubated in the last change of water for 1 hr. The entire procedure was then repeated. The treated seeds were germinated in the dark on sterile nutrient agar plates. Three-day-old seedlings with radicles of approximately 1 cm were planted in aseptic test tubes (200 × 25 mm) containing 30 g of sterile sand and 4 ml of nutrient solution. Nutrient solution was MPCL medium (Lavigne 1987) or nitrogen-free MPCL medium (= MPCL medium without $Ca(NO_3)_2$, KNO_3,and NH_4NO_3 and supplemented with 7 mM $CaCl_2$). If required, malate or fructose was added to the nutrient solution at a final concentration of 0.1%.

The seedlings were grown in a growth chamber (12 hr day; 23° C day; 23° C night) and inoculated after 3 days with 1 ml of an overnight *Azospirillum* culture, resuspended in MPCL or nitrogen-free MPCL medium (approximately 10^8 bacteria per milliliter). Plants were irrigated weekly with 1 ml of distilled water and harvested at the indicated times.

Preparation of wheat roots for histochemical analysis.

Whole roots were carefully washed several times in phosphate-buffered saline (PBS) (0.88% [w/v] NaCl, 2.9 mM KH_2PO_4, 7.1 mM K_2HPO_4, pH 7.2) to remove any remaining sand and stained for 4–24 hr in 0.1 M phosphate buffer (pH = 7.0) containing 0.5 mg/ml X-Gluc, 0.33 mg/ml $K_3(Fe(CN)_6$, and 0.42 mg/ml $K_4(Fe(CN)_6)$.

Glucuronidase assays.

Ex planta β-glucuronidase activity was assayed spectrophotometrically using the GUS extraction buffer and

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
Azospirillum brasilense		
Sp7	Wild-type strain, isolated from *Digitaria decumbens* rhizosphere soil	Baldani *et al.* 1987
Sp245	Wild-type strain, isolated from surface sterilized wheat roots (Brazil)	Baldani *et al.* 1987
Escherichia coli		
HB101	F^- *hsd*S20 (r^-_B m^-_B) *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5 *mtl-1* sup*E*44	Maniatis *et al.* 1982
Plasmids		
pBI101	pBin19 (Bevan 1984) derivative, containing the promoterless *gusA* gene	Jefferson *et al.* 1987b
pFAJ21	Tc^r, pRK290 (Ditta *et al.* 1980) derivative contaning an *A. brasilense nifH-gusA* translational fusion cloned into the *Eco*RI site	Vande Broek *et al.* 1992
pFAJ31	Tc^r, pLAFR3 derivative containing the promoterless *E. coli gusA* gene, cloned into the *Eco*RI-*Bam*HI sites of the polylinker	This study
pFAJ31.2	Tc^r, pFAJ31 derivatives containing constitutively expressed *A. brasilense*	This study
pFAJ31.13	promoter::*gusA* fusions	
pLAFR3	Tc^r, pRK290 (Ditta *et al.* 1980) derivative with polylinker of M13mp18 (Yanish-Perron *et al.*, 1985) and lambda cos site	Staskawicz *et al.* 1987
pRK2013	Km^r, *rep* ($ColE_1$) mob^+ tra^+	Figurski and Helinski 1979

[a] Tc = tetracycline; ^{r} = resistant.

the substrate *p*-nitrophenyl-β-D-glucuronide (PNPG) as described by Jefferson *et al.* (1987a). β-Glucuronidase activity is expressed as nanomoles *p*-nitrophenol produced per minute per 10^8 cells using standards of commercially available *p*-nitrophenol.

For measuring β-glucuronidase activity during the association, plants with equally sized roots were selected. After excision of the stem and the seed, the roots together with the sand in the test tube were aseptically collected in 30 ml of sterile PBS. The samples were then vigorously shaken for 2 hr at 4° C in a hand wrist shaker (Vibromatic) to resuspend the bacteria attached to the sand particles and the roots. From each sample a 1-ml aliquot was removed and assayed for β-glucuronidase activity as described above. The cell density of each sample was determined by plate counting.

To determine the associative expression of *nifH* under a lower oxygen concentration, the test tubes containing whole plants were stoppered with rubber caps and gently flushed for 45 min with nitrogen gas. Air was subsequently injected to give the appropriate oxygen concentration and plants were further incubated for 16 hr prior to analysis. β-Glucuronidase activities were assayed as described above.

Nitrogenase assay.

Associative nitrogenase activity was assayed by the acetylene reduction method (Burris 1972). Tubes were tightly stoppered and acetylene was injected to a final concentration of 10% (v/v). Gas samples were taken after 16 hr. Ethylene production was quantified on a "PLOT fused silica" column (50 m × 0.32 mm, 5 μm of Al2O3/KCl, Chrompack, Middleburg. the Netherlands) installed in a Hewlett-Packard 5890A gas chromatograph. Propane was used as internal standard. When associative nitrogenase activity was assayed under various oxygen concentrations, plants were first flushed as described above (section glucuronidase assays). The seedlings were then incubated for 4 hr with the lowered oxygen tension prior to injection of acetylene.

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LITERATURE CITED

Baldani, V. L. D., Baldani, J. I., and Döbereiner, J. 1987. Inoculation of field-grown wheat (*Triticum aestivum*) with *Azospirillum* spp. in Brasil. Biol. Fertil. Soils 4:37-40.

Barak, R., Nur, I., Okon, Y., and Henis, Y. 1982. Aerotactic response of *Azospirillum brasilense*. J. Bacteriol. 152:643-649.

Bashan, Y. 1986. Migration of rhizosphere bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* towards wheat roots in the soil. J. Gen. Microbiol. 132:3407-3414.

Bashan, Y., and Levanony, H. 1989. Factors affecting adsorption of *Azospirillum brasilense* Cd to root hairs as compared with root surface of wheat. Can. J. Microbiol. 35:936-944.

Bashan, Y., Levanony, H., and Klein, E. 1986. Evidence for a weak active external adsorption of *Azospirillum brasilense* Cd to wheat roots. J. Gen. Microbiol. 132:3069-3073.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12:8711-8721.

Burris, R. H. 1972. Nitrogen fixation—Assay methods and techniques. Methods Enzymol. 24:419-431.

Croes, C., Van Bastelaere, E., Declercq, E., Eyers, M., Vanderleyden, J., and Michiels, K. 1991. Identification and mapping of loci involved in motility, adsorption to wheat roots, colony morphology and growth in minimal medium on the *Azospirillum brasilense* Sp7 90MDa plasmid. Plasmid 26:83-93.

De Zamaroczy, M., Delorme, F., and Elmerich, C. 1989. Regulation of transcription and promoter mapping of the structural genes for nitrogenase (*nifHDK*) of *Azospirillum brasilense* Sp7. Mol. Gen. Genet. 220:88-94.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Fani, R., Allotta, G., Bazzicalupo, M., Ricci, F., Schipani, C., and Polsinelli, M. 1989. Nucleotide sequence of the gene encoding the nitrogenase iron protein (*nifH*) of *Azospirillum brasilense* and identification of a region controlling *nifH* transcription. Mol. Gen. Genet. 220:81-87.

Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA 76:1648-1652.

Gallagher, S. R. 1992. GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression. S. R. Gallagher, ed. Academic Press, New York.

Gaskins, M. H., Umali-Garcia, M., Tien, T. M., and Hubbell, D. H. 1977. Nitrogen fixation and growth substance produced by *Spirllum lipoferum* and their effects on plant growth. Plant Physiol. 59 (Suppl.):128.

Hartmann, A. 1988. Ecophysiological aspects of growth and nitrogen fixation in *Azospirillum* spp. Plant Soil 110:225-238.

Hartmann, A., and Burris, R. H. 1987. Regulation of nitrogenase activity by oxygen in *Azospirillum brasilense* and *Azospirillum lipoferum*. J. Bacteriol. 169:944-948.

Hartmann, A., Fu, H., and Burris, R. H. 1986. Regulation of nitrogenase activity by ammonium chloride in *Azospirillum* spp. J. Bacteriol. 165:864-870.

Hartmann, A., and Hurek, T. 1988. Effect of carotenoid overproduction on oxygen tolerance of nitrogen fixation in *Azospirillum brasilense* Sp7. J. Gen. Microbiol. 134:2449-2455.

Heinrich, D., and Hess, D. 1985. Chemotactic attraction of *Azospirillum lipoferum* by wheat roots and characterization of some attractants. Can. J. Microbiol. 31:26-31.

Hu, C. H., Chee, P. P., Chesney, R. H., Zhou, J. H., Miller, P. D., and O'Brien, W. T. 1990. Intrinsic GUS-like activities in seed plants. Plant Cell Rep. 9:1-5.

Jefferson, R. A. 1987a. Assaying chimeric genes in plants: The GUS gene fusion system. Plant. Mol. Biol. Rep. 5:387-405.

Jefferson, R. A. 1987b. GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Lavigne, C. 1987. Contribution à l'étude du système racinaire du bananier. Mise au point de rhizotrons et premiers resultats. Fruits 42:265-271.

Liang, Y. Y., Kaminski, P. A., and Elmerich, C. 1991. Identification of a *nifA*-like regulatory gene of *Azospirillum brasilense* Sp7 expressed under conditions of nitrogen fixation and in the presence of air and ammonia. Mol. Microbiol. 5:2735-2744.

Madimba, G., Heulin, T., Bally, R., Guckert, A., and Balandreau, J. 1986. Chemotaxis of free-living nitrogen fixing bacteria towards maize mucilage. Plant Soil 90:129-136.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Martinez-Drets, G., Del Gallo, M., Burpee, C., and Burris, R. H. 1984. Catabolism of carbohydrates and organic acids and ex-

pression of nitrogenase by azospirilla. J. Bacteriol. 159:80-85.
Michiels, K., Croes, C., and Vanderleyden, J. 1991. Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. J. Gen. Microbiol. 137:2241-2246.
Michiels, K., Vanderleyden, J., Van Gool, A., and Signer, E. 1988. Isolation and characterization of *Azospirillum brasilense* loci that correct *Rhizobium meliloti exoB* and *exoC* mutants. J. Bacteriol. 170:5401-5404.
Michiels, K., Verreth, C., and Vanderleyden, J. 1990. *Azospirillum lipoferum* and *Azospirillum brasilense* surface polysaccharide mutants that are affected in flocculation. J. Appl. Bacteriol. 69:705-711.
Milcamps, A., and Vanderleyden, J. 1991. *In vitro* construction of *lacZ* gene fusions with the *nifHDK* operon of *Azospirillum brasilense*. FEMS Microbiol. Lett. 77:79-84.
Miller, J. H.1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Pages 354-358.
Okon, Y. 1985. *Azospirillum* as a potential inoculant for agriculture. Trends Biotechnol. 3:223-228.
Okon, Y., Gakmakgi, L., Nur, I., and Chet, I. 1980. Aerotaxis and chemotaxis of *Azospirillum brasilense*. Microbiol. Ecol. 6:277-280.
Okon, Y., and Kapulnik, Y. 1986. Development and function of *Azospirillum*-inoculated roots. Plant Soil 90:3-16.
Patriquin, D. G., and Döbereiner, J. 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Can. J. Microbiol. 24:734-742.
Reinhold, B., Hurek, T., and Fendrik, I. 1984. Strain-specific chemotaxis of *Azospirillum* spp. J. Bacteriol. 162:190-195.
Shank, S. C., Smith, R. L., Weiser, G. C., Zberer, D. A., Bouton, J. A., Queenberry, K. H., Tyler, M. E., Milam, J. R., and Littell, R.C. 1979. Fluorescent antibody technique to identify *Azospirillum brasilense* associated with roots of grasses. Soil Biol. Biochem. 11:287-295.
Smith, R. L., Bouton, J. H., Schank, S. C., Quesenberry, K. H., Tyler, M. E., Milam, J. R., Gaskins, M. H., and Litell, R. C. 1976. Nitrogen fixation in grasses inoculated with *Spirillum lipoferum*. Science 193:1003-1005.
Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.
Tarrand, J. J., Krieg, N. R., and Döbereiner, J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with description of a new genus, *Azospirillum* gen. nov., and two species *Azospirillum lipoferum* (Beijerinck) sp. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. 24:967-980.
Tien, T. M., Gaskins, M. H., and Hubbell, D. H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl. Environ. Microbiol. 37:1016-102.
Umali-Garcia, M., Hubbell, D. H., Gaskins, M. H., and Dazzo, F. B. 1980. Association of *Azospirillum* with grass roots. Appl. Environ. Microbiol. 39:219-226.
Vande Broek, A., Michiels, J., de Faria, S. M., Milcamps, A., and Vanderleyden, J. 1992. Transcription of the *Azospirillum brasilense nifH* gene is positively regulated by NifA and NtrA and is negatively controlled by the cellular nitrogen status. Mol. Gen. Genet. 232:279-283.
Van Rhyn, P., Vanstockem, M., Vanderleyden, J., and De Mot, R. 1990. Isolation of behavioral mutants of *Azospirillum brasilense* by using Tn*5-lacZ*. Appl. Environ. Microbiol. 4:990-996.
Vanstockem, M., Michiels, K., Vanderleyden, J., and Van Gool, A. 1987. Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*, physical analysis of Tn*5* and Tn*5*-mob insertion mutants. Appl. Environ. Microbiol. 53:410-415.
Vieille, C., and Elmerich, C. 1990. Characterization of two *Azospirillum brasilense* Sp7 plasmid genes homologues to *Rhizobium meliloti nodPQ*. Mol. Plant-Microbe Interact. 3:389-400.
Vieille, C., and Elmerich, C. 1992. Characterization of an *Azospirillum brasilense* Sp7 gene homologues to *Alcaligenes eutrophus phbB* and to *Rhizobium meliloti nodG*. Mol. Gen. Genet. 231:375-384.
Von Bulow, J. G. D., and Döbereiner, J. 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proc. Natl. Acad. Sci. USA 72:2389-2393.
Waelkens, F., Maris, M., Verreth, C., Vanderleyden, J., and Van Gool, A. 1987. *Azospirillum* DNA shows homology with *Agrobacterium tumefaciens* chromosomal virulence genes. FEMS Microbiol. Lett. 43:241-246.
Yanish-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.