Use of *lacZ* Fusions to Study the Expression of *nif* Genes of *Azospirillum brasilense* in Association with Plants

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A simple protocol has been developed to detect *Azospirillum* carrying *lacZ* fusions in association with wheat. *In situ* staining of the bacteria with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a chromogenic substrate and examination by light microscopy revealed bacteria localized at the surface of the root and also within the cortex. Determination of β-galactosidase activity allowed a comparison of the expression of *nif* promoters in the plant-associated bacteria. Plasmid-borne *nifA*-, *nifB*-, and *nifH-lacZ* fusions carried by the wild-type *A. brasilense* strain Sp7 were significantly expressed in association with the plants, compared to the basal level observed with bacteria lacking a *lacZ* fusion or carrying a *nifH-lacZ* fusion in *nifA*- and *glnB*-mutated backgrounds. A chromosomally borne *nifH-lacZ* fusion was also expressed. Colonization efficiency was examined with a partially constitutive *nifA-lacZ* fusion or with a *lacZ* fusion to a *kan* promoter. The colonization efficiency of a *nifA*-Tn*5* mutant strain was identical to that of the wild type, while glutamine and tryptophan auxotrophs showed less colonization. As the protocol is applicable to a range of *A. brasilense* wild-type and mutant strains, it can be used to compare gene expression in free-living bacteria and those in association with plants and to screen for hyperperforming strains in association with plants.

Additional keywords: in situ detection of bacteria, *nif-lacZ* fusions, wheat colonization.

Azospirillum bacteria are gram-negative, vibrioid, aerobic chemoorganotrophs isolated from the roots of grasses. Under free-living conditions these bacteria fix nitrogen at low oxygen tension in a medium devoid of ammonia. The process of root colonization by *Azospirillum* has been reviewed by Patriquin *et al.* (1983), Okon (1985), Michiels *et al.* (1989), Elmerich *et al.* (1992), and Kennedy and Tchan (1992). The bacteria induce morphological changes in the root system, with an increase of the number of root hairs and lateral roots (Tien *et al.* 1979; Kapulnik *et al.* 1985; Jain and Patriquin 1985; Okon and Kapulnik 1986), and they appear to be anchored to the root surface by fibrillar material (Umali-Garcia *et al.* 1980; Gafny *et al.* 1986; Bashan *et al.* 1991). Surface polysaccharides and the polar flagellum have been shown to play a role in attachment to roots (Croes *et al.* 1991, 1993; Michiels *et al.* 1990, 1991). Colonization of intercellular spaces between the epidermis and the cortex has been reported (Schank *et al.* 1979; Bashan *et al.* 1986; Levanony *et al.* 1989). However, only sparse information on the expression of bacterial genes involved in nitrogen fixation during the association with the host plant is yet available (Vande Broek *et al.* 1993).

Two DNA regions containing *nif* genes have been characterized in the wild-type *A. brasilense* strain Sp7. The first region contains *nifHDK*, the structural genes for nitrogenase, as well as *nifNE*, *nifUS*, and *fixABC* homologs (Galimand *et al.* 1989), and the second one contains *nifA* and *nifB* (Liang *et al.* 1991). A third region, containing the *ntrBC* genes, has also been characterized (Liang *et al.* 1993). Mutants in *nifA*, *ntrB*, and *ntrC* have been constructed (Liang *et al.* 1991, 1993). The *nifA* mutants were Nif^-, whereas *ntrBC* mutants were Nif^+. The expression of the *nifH*- and *nifB-lacZ* fusions, controlled by σ^{54}-dependent promoters, was abolished in *nifA* but not in *ntrBC* mutants, suggesting that NifA positively controls the expression of the other *nif* operons, while NtrC does not (Liang *et al.* 1991, 1992, 1993). A *nifA-lacZ* fusion was expressed from its own promoter not only under conditions favorable for nitrogen fixation but also in the presence of ammonia and/or air (Liang *et al.* 1991, 1992). As the presence of oxygen and ammonia are not compatible with nitrogen fixation or with the expression of a *nifH-lacZ* fusion, it has been proposed that the *nifA* product is synthesized in an inactive form under these conditions (Liang *et al.* 1991). Properties of a *glnB* mutant strain, which displayed a strict Nif^- phenotype, suggested that the *glnB* product, P_{II}, might be involved in modulation of NifA activity under conditions of nitrogen fixation (Liang *et al.* 1992; de Zamaroczy *et al.* 1993).

In order to study the regulation of *nif* gene expression in *Azospirillum* in association with wheat, a protocol was developed for *in situ* detection and determination of the β-galactosidase activity of bacteria carrying a *nifA*-, *nifB*-, or *nifH-lacZ* fusion. The protocol was applied to *A. brasilense* wild-type and mutant strains.

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RESULTS

In situ detection of bacteria on roots by X-Gal staining.

The rationale of the experiments was to develop an easy procedure for studying the colonization process by *in situ* staining of *Azospirillum* displaying β-galactosidase activity. In preliminary experiments, *in situ* X-Gal staining of wheat root segments not inoculated with bacteria revealed some blue color due to the endogenous β-galactosidase activity of the plant. Fixation of the plant tissue with 2% glutaraldehyde,

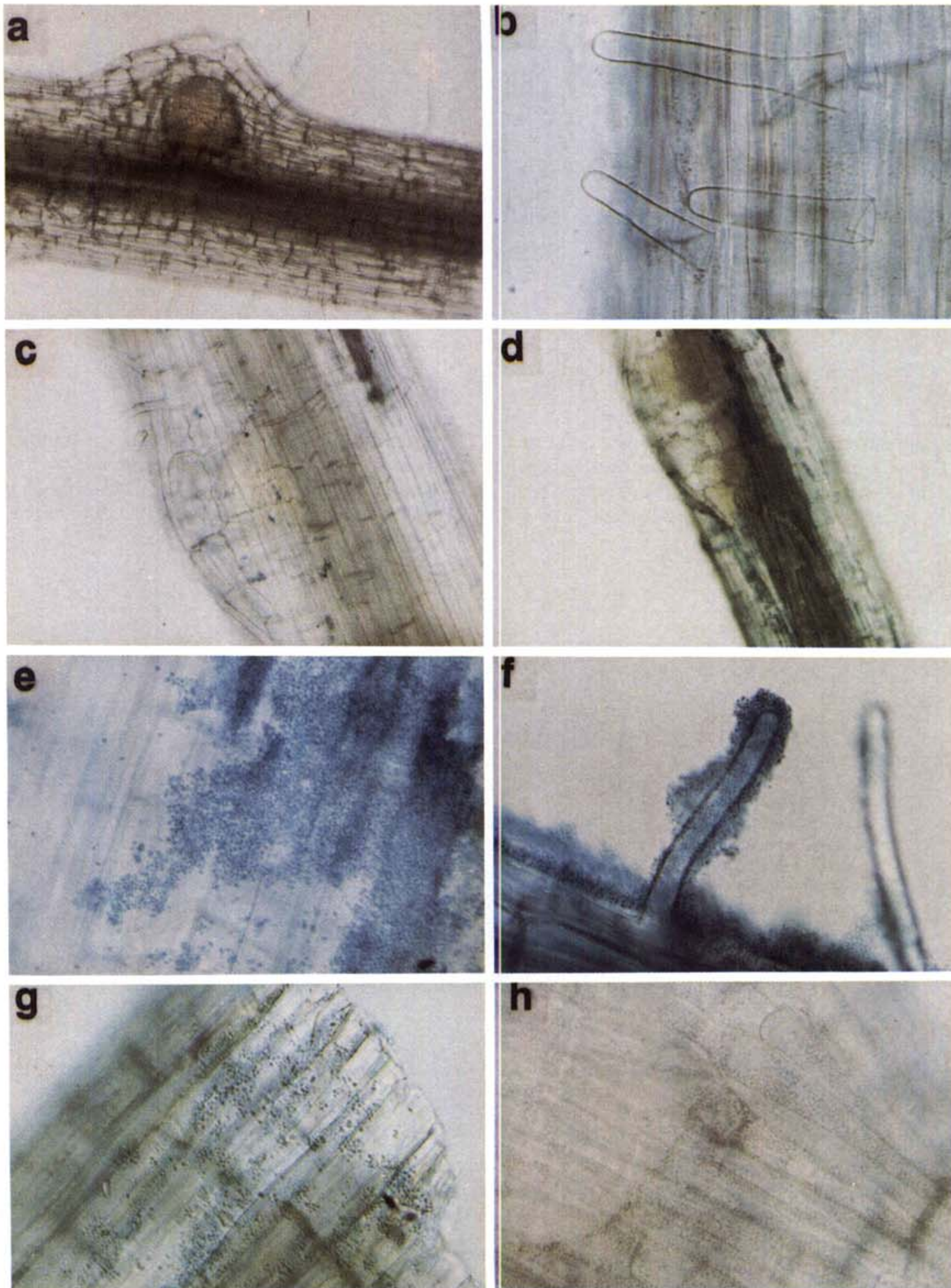

Fig. 1. *In situ* detection of β-galactosidase activity on wheat root segments. All samples were stained after 10 days, except **C**, which was stained after 20 hr. **A,** Uninoculated control (×53). **B,** Root inoculated with *Azospirillum brasilense* Sp7 (×315). **C–E,** Root inoculated with Sp7 (pAB576) (*nifA-lacZ*) (×106, 53, and 315, respectively). **F,** Root inoculated with 7067 (*nifA*-Tn*5* mutant strain) containing pAB576 (315×). **G,** Root inoculated with Sp7 (pAB358) (*nifH-lacZ*) (×315). **H,** Root inoculated with 7067 (*nifA*-Tn*5* mutant strain) containing pAB358 (×315).

as described in Materials and Methods, abolished this background activity, and root samples remained uncolored in the absence of colonization, even when observed by light microscopy (Fig. 1A). Similarly, no blue coloration was detected in root samples inoculated with *Azospirillum* which did not contain a *lacZ* plasmid, and bacteria detected at high magnification were not stained (Fig. 1B). By contrast, when the inoculation was performed with strain Sp7 containing the plasmid-borne *nifA-lacZ* (pAB576) or *nifH-lacZ* (pAB358) fusion (Table 1) it was possible to distinguish zones of heavy colonization, where bacteria were stained blue. It should be noted, however, that the staining of the specimen carrying the *nifH-lacZ* fusion was less intense and more scattered (Fig. 1G), associated with clusters of bacteria, with other more lightly stained bacteria also visible on the root surface. Other available *lacZ* fusions (Table 1) have also been successfully used, including *ntrC-lacZ* (pAB792), *nodG-lacZ* (pAB538), and *nifB-lacZ* (pAB135) (data not shown).

The *nifA-lacZ* fusion, which is expressed over a wide range of conditions in bacteria grown in the free-living state, including conditions of nitrogen fixation, ammonia assimilation, microaerobiosis, and aerobiosis (Liang *et al.* 1991, 1992), was taken as standard to study the extent of colonization. Bacteria were detected on the root surface after 20 hr of incubation (Fig. 1C), and stronger colonization was observed after 10 days on the surface (Fig. 1D and E) and around root hairs (Fig. 1F). It was possible to distinguish bacteria not only on the surface but also in the first layers of the cortex. It was also observed that the bacteria were preferentially localized in the lower part of the root system (data not shown). As shown in Figure 1E, no diffusion of the stain from the bacteria to the adjacent tissue occurred.

The *nifH-lacZ* fusion was taken as standard to determine whether nitrogen fixation genes are transcribed or not. Thus, *in situ* detection of the blue color with the *nifH-lacZ* fusion should reflect transcription of the nitrogenase structural genes of *Azospirillum* in association with the plant. This was indeed the case with samples of roots inoculated with strain Sp7 (pAB358) (Fig. 1G). To be sure that the blue color could be correlated with the expression of nitrogenase structural genes, strain 7067, a *nifA*-Tn*5* mutant containing pAB358, was used as a control. Under free-living conditions the *nifH-lacZ* fusion is not expressed in a *nifA*-mutated background (e.g., 7067), even under conditions compatible with nitrogen fixation (Liang *et al.* 1991). The bacteria were hardly stained in association with the plant (Fig. 1H), in contrast with the wild-type strain with the same fusion (Fig. 1G). It was verified that 7067 (pAB576) stained as blue as the wild type with the same *nifA-lacZ* fusion (Fig. 1F).

Correlation between staining sensitivity, number of associated bacteria, and β-galactosidase activity.

The *in situ* detection of bacteria associated with wheat seedlings stained with X-Gal revealed differential expression

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Reference
Escherichia coli		
S17-1		Simon *et al.* 1983
Azospirillum brasilense		
Sp7	Wild type	Tarrand *et al.* 1978
Sp245	Wild type	Baldani *et al.* 1983
Sp246	Wild type	Baldani *et al.* 1983
Sp107	Wild type	Baldani *et al.* 1983
7067	*nifA*-Tn*5* mutant of Sp7	Liang *et al.* 1991
7148	*ntrC*-Tn*5* mutant of Sp7	Liang *et al.* 1993
7194	*ntrB*-Tn*5* mutant of Sp7	Liang *et al.* 1993
7028	*glnA* mutant, Gln^-, Nif^c of Sp7	Gauthier and Elmerich 1977
7606	*glnB-kan* mutant of Sp7	de Zamaroczy *et al.* 1993
7853	*trpD*-Tn*5* mutant of Sp7	Zimmer *et al.* 1991
7854	*trpC*-Tn*5* mutant of Sp7	Zimmer *et al.* 1991
7358	Nif^+, derivative of Sp7 containing the *nifH-lacZ* fusion recombined into the chromosome, Km^r, Spe^r	This work
70671	Nif^-, derivative of 7067 containing the *nifH-lacZ* fusion recombined into the chromosome, Km^r, Spe^r	This work
Plasmids		
pAB576	*nifA-lacZ* transcriptional fusion, Tc^r, Km^r, pVK100 derivative[b]	Liang *et al.* 1991
pAB358	*nifH-lacZ* transcriptional fusion, Tc^r, Km^r, pRK290 derivative[b]	Liang *et al.* 1991
pAB135	*nifB-lacZ* translational fusion, Tc^r, pGD926 derivative[b]	Liang *et al.* 1992
pAB538	*nodG-lacZ* translational fusion, Tc^r, pGD926 derivative	Vieille and Elmerich 1992
pAB792	*ntrC-lacZ* transcriptional fusion, Tc^r, Km^r, pLA2917 derivative[b]	Liang *et al.* 1993
pSUP202	Amp^r, Tc^r, Cm^r	Simon *et al.* 1983
pAB359	*nifH-lacZ* transcriptional fusion, Amp^r, Spe^r, Km^r, pSUP202 derivative	This work
pLA-*lacZ*	*lacZ-kan* cartridge cloned into pLA2917 vector, Tc^r, Km^r, Lac^+	This work
pAB3	Derivative of pSUP202 containing the *nifHDK* genes on a 6.7-kb *Eco*RI fragment, Amp^r, Tc^r	Perroud *et al.* 1985
pAB57	Derivative of pVK100 containing the *nifA* gene on a 2.6-kb *Sal*I fragment, Tc^r	Liang *et al.* 1991
pAB53	Same as pAB57 but containing the cloned fragment in the opposite orientation	Liang *et al.* 1991
pHP45Ω	Amp^r, source of the *Spe-Sm* interposon	Prentki and Krisch 1984
pKOK5	Amp^r, source of the *lacZ-kan* cartridge	Kokotek and Lotz 1989

[a] Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Spe^r, spectinomycin resistance; Tc^r, tetracycline resistance.
[b] pVK100, pRK290, pGD926, and pLA2917 are low-copy, broad-host-range vector derivatives of RK2 that can be transferred by conjugation from *E. coli* S17-1 to *Azospirillum* recipients.

of *nifA*- and *nifH-lacZ* fusions, depending on the bacterial background. Thus, it was of interest to correlate the data with 1) the number of bacteria associated with the root system and 2) a quantitative assay of β-galactosidase activity. Because the *nifA-lacZ* fusion was partially constitutive, it cannot be known whether the fusion is fully expressed in association with plants. For this reason a constitutive *lacZ* fusion, designated pLA-*lacZ*, in which *lacZ* is under the control of the *kan* promoter of the vector pLA2917, was constructed and used in the following experiments. In order to demonstrate that the number of bacteria associated with the root system was related to the blue color, we performed an experiment using, as inoculum, mixtures containing different proportions of wild-type Sp7 cells and Sp7 carrying pLA-*lacZ* (Table 2). Enumeration of the two types of bacteria was performed at the time of inoculation and after 10 days, and the relative proportion remained roughly constant (Table 2), indicating no segregation of the plasmid and that the plasmid did not impair the colonization efficiency. A strong reduction in the color was observed when the inoculum contained only 10% of the constitutive fusion, and no color was observed with 1% or with plants inoculated with *Escherichia coli* strains (Table 2). This is an indication that a reduction in colonization efficiency of 90% in this range would be readily detected. It is worth mentioning that by light microscopy it was possible to distinguish between the blue-stained and the unstained bacteria as described above (see Fig. 1).

Similarly, β-galactosidase activity of the inoculum and of bacteria on plants was determined after 10 days of incubation, by means of the optimized protocol described in Materials and Methods. As shown in Table 2, there was a good correlation between β-galactosidase activity and the proportion of *lacZ*-containing cells at the time of inoculation and after colonization. The relative β-galactosidase activity was also correlated with the number of lac^+ bacteria on the plant. However, the most-probable-number method may not be very precise in determining the number of bacteria in this particular type of sample, in which the bacteria are strongly anchored on the root surface (see Table 4). Calculation of the specific activity per 10^6 tetracycline-resistant (Tc^r) bacterial cells showed that the β-galactosidase values on plants were regularly higher than expected from data obtained from free-living bacteria. Such results indicate that the number of bacteria determined to be present on the plant was an underestimate of the true number. This probably reflects the difficulty of separating individual cells from each other and from plant tissue, even after thorough grinding of the roots. Moreover, as a heat treatment to remove the plant background was included in the assay, the determinations of β-galactosidase activity in the plant extracts and in free-living cultures are not strictly comparable. Since the largest source of error is considered to be the degree of disruption of plant tissue, the data for activity on plants are therefore expressed relative to soluble plant proteins.

Quantification of the β-galactosidase activity of strains carrying *nif-lacZ* fusions in association with wheat.

Table 3 shows that heat treatment considerably reduced the background β-galactosidase activity of the plant. When inoculated with the wild-type Sp7, roots exhibited β-galactosidase activity roughly identical to that of the controls. Significantly increased activity was obtained with inoculants containing *nifA*-, *nifB*-, and *nifH-lacZ*. For each fusion, the mean number of viable bacteria per plant after 10 days was in

Table 2. β-Galactosidase activity and intensity of X-Gal staining of *Azospirillum brasilense* wild type and auxotrophs and of *Escherichia coli* carrying a plasmid containing a *lacZ* fusion to the *kan* promoter[a]

Strain[b]	β-Galactosidase activity: Free-living cultures[c]	β-Galactosidase activity: Plant extract[d]	X-Gal staining[e]	MPN (10^6) (% Tc^r colonies)[f]
Sp7/Sp7(pLA-*lacZ*) mixtures with pLA-*lacZ* content (%) of:				
100	11,033 ± 931	1,268 ± 508	++++	2.1
10	1,225 ± 68	159 ± 50	+	8.4 (13.8)
1	112 ± 78	40 ± 13	–	1.2 (2.1)
0	43 ± 13	17 ± 4	–	1.2 (<0.1)
E. coli S17-1 (pLA-*lacZ*)	4,026 ± 1,094	24 ± 12	–	0.3
Auxotrophs				
7028 (pLA-*lacZ*)	10,624 ± 4,304	293 ± 212	++	0.8
Sp7 (pLA-*lacZ*) (+ Gln)	11,879 ± 5,215	1,277 ± 324	++++	2.1
7853 (pLA-*lacZ*)	9,953 ± 2,147	677 ± 349	+++	...[g]
7854 (pLA-*lacZ*)	11,385 ± 2,727	587 ± 429	+++	1.2
Sp7 (pLA-*lacZ*) (+ Trp)	11,294 ± 2,746	1,699 ± 249	++++	3.2

[a] *A. brasilense* was grown overnight in minimal lactate medium containing 10 mM ammonia. *E. coli* was grown overnight in Luria-Bertani medium. Plants were inoculated with 0.1 ml of culture (for *A. brasilense,* this was about 5×10^6 bacteria per milliliter of hydroponic solution; for *E. coli,* about 2×10^7 bacteria per milliliter of hydroponic solution). Mixtures of Sp7 and Sp7(pLA-*lacZ*) were made at the time of plant inoculation. Glutamine (200 μg/ml) or tryptophan (20 μg/ml) was added to the overnight culture when required, and control plants were also inoculated with Sp7(pLA-*lacZ*) cultures, pregrown with the same amount of amino acids. The data are averages for five plants.

[b] Sp7: wild-type *A. brasilense;* 7028: Gln auxotroph; 7853 and 7854: Tn*5*-induced Trp auxotrophs in *trpC* and *trpD,* respectively; pLA-*lacZ:* pLA2917 vector containing a *lacZ* cartridge under the control of the *kan* promoter of the vector.

[c] Miller units · min^{-1} · mg^{-1} of protein, estimated from the bacterial cultures at the time of inoculation.

[d] Miller units · min^{-1} · mg^{-1} of plant protein, estimated after 10 days of growth on plants.

[e] X-Gal staining was performed on five or six 1-cm root segments excised from the same plant, starting from the tip. ++++, Segments stained deep blue along most of their length; ++ and +++, intermediate staining; +, weak staining; –, no stain visible.

[f] MPN, most probable number of bacteria associated with an entire root. Tc^r, tetracylince-resistant. The percentage of Tc^r colonies was estimated by plating appropriate dilution samples on minimal medium containing tetracycline (10 μg/ml) and on medium not containing the antibiotic.

[g] Not determined.

the range from 1×10^6 to 2×10^7, depending on the series, which reflects good colonization, as discussed above. Similar counts were obtained with Sp7 devoid of a *lacZ* fusion. In agreement with the absence of *in situ* detection, the *nifA*-Tn*5* mutant 7067 containing the *nifH-lacZ* fusion (pAB358) did not display β-galactosidase activity greater than that of the background when tested in association with wheat (Table 3). It was verified that the difference of expression between the wild-type strain and the *nifA* mutant strain containing *nifH-lacZ* was not due to a difference in the stability of the plasmid or to a difference in the number of bacteria anchored on the root surface (data not shown; see Table 4 for the *nifA-lacZ* fusion). It was also verified that the *nifH* expression observed with the wild-type Sp7 could not be due to the occurrence of a constitutive mutation of the *nifH* promoter, since Sp7 (pAB358) reisolated from the roots displayed wild-type repression of *nifH* transcription when cultured in the presence of air and ammonia.

Umali-Garcia *et al.* (1980) showed that the number of bacteria firmly adhering to grass root hairs was influenced by the age of the bacterial culture serving as the inoculum. More recently, it was reported that the first step in adsorption was mediated through the polar flagellum (Michiels *et al.* 1991). For this reason, the cultures were not centrifuged or washed prior to inoculation. Different conditions of plant inoculation were tested with the Sp7 strain carrying the *nifH-lacZ* fusion. The highest β-galactosidase activity associated with roots was obtained with plants inoculated with overnight bacterial cultures grown aerobically in minimal lactate medium containing 10 mM ammonia (Table 3). Counting bacteria associated with each root system showed that the highest β-galactosidase activity was always correlated with the highest number of bacteria (data not shown). It was verified that after overnight growth in the minimal lactate medium no β-galactosidase activity higher than the background level could be detected, suggesting that *nifH* was not derepressed prior to inoculation of the plant system. After overnight growth the ammonia concentration of the medium was about 4.5 mM. Thus, the ammonia added with the inoculum (30 μM) was insufficient to support significant growth of the bacteria or of the plant.

These results show that expression detected with the plasmid-borne *nifH-lacZ* fusion in the wild-type strain reflects significant transcription of the nitrogenase structural genes in association with the plant. Similarly, expression of *nifB* strongly suggests that other genes controlled by *nifA* are transcribed.

Table 3. β-Galactosidase activity in wheat inoculated with *Azospirillum brasilense* wild-type and mutant strains and in uninoculated wheat[a]

Treatment	Heated to 50° C for 15 min[b]	None[b]
No inoculation	17 ± 7 (12)	60 ± 28 (8)
Sp7	17 ± 6 (14)	69 ± 12 (5)
Sp7 (pAB576)	490 ± 206 (14)	625 ± 202 (10)
Sp7 (pAB358)	211 ± 118 (25)	254 ± 139 (10)
Sp7 (pAB135)	144 ± 85 (10)	...
7067 (pAB358)	26 ± 17 (10)	...
7358	23 ± 17 (19)	...
70671	15 ± 9 (19)	...
70671 (pAB53)	112 ± 40 (10)	...
70671 (pAB57)	128 ± 42 (9)	...

[a] Plants were inoculated with 0.1 ml of culture grown overnight in minimal lactate medium containing 10 mM ammonia. Sp7: wild-type *Azospirillum brasilense;* 7067: *nifA*-Tn*5;* pAB576: *nifA-lacZ* plasmid; pAB358: *nifH-lacZ* plasmid; pAB135: *nifB-lacZ* plasmid; 7358 and 70671: Sp7 and 7067 containing the *nifH-lacZ* fusion recombined into the chromosome, respectively; pAB53 and pAB57: *nifA*-containing plasmids.

[b] β-Galactosidase activity was estimated after 10 days and is expressed in Miller units · min^{-1} · mg^{-1} of plant protein. The number of plants tested is given in parentheses.

Comparison of β-galactosidase activity produced and colonization by Sp7 and various regulatory mutants.

The colonization efficiency of the wild type and the Nif^- mutant (7067) was studied. Enumeration of bacterial cells associated with an entire root and determination of β-galactosidase activity were performed 20 hr after inoculation and 10 days after inoculation with the two strains carrying a *nifA-lacZ* fusion (Table 4). In the *nifA*-Tn*5* mutant, the fusion was expressed at the same rate as in the wild type in free-living conditions (Liang *et al.* 1991). After 20 hr, about 7% of the inoculated bacteria were attached to roots. It was verified that the sum of the number of bacteria associated with the root plus those remaining free in the hydroponic solution corresponded roughly to the number of bacteria inoculated (data not shown). Ten days after inoculation, the increase in β-galactosidase activity was greater than the increase in bacteria associated with the root. This can be a result of partial derepression of the *nifA-lacZ* fusion in association with wheat, but more probably it reflects the difficulty of precisely determining the number of bacteria. Similar results were obtained with the *nifA* mutant strain, showing that a functional *nifA* gene is not required in the colonization process. To be sure that bacteria were strongly anchored, roots were washed by being shaken in a 0.9% NaCl solution for 1 hr. No significant difference in β-galactosidase activity or in the number of bacteria was observed with these strains, compared to the unwashed samples.

Colonization efficiency was also tested in various auxotrophs, which were compared with the wild-type strain (Table 2). The pLA-*lacZ* plasmid was introduced into the *glnA* strain 7028 and into two Trp auxotrophs, strains 7853 and 7854, which carry a Tn*5* insertion in *trpD* and *trpC*, respectively. As indicated in Table 2, the lower β-galactosidase activity of

Table 4. β-Galactosidase activity produced and colonization by *Azospirillum brasilense* strain Sp7 and *nifA*-Tn*5* mutant strain 7067 containing a *nifA-lacZ* fusion (pAB576)[a]

Time after inoculation	Sp7 (pAB576)	7067 (pAB576)
20 hr		
β-Gal[b]	62 ± 24	62 ± 36
MPN[c]	7.1×10^6	7.7×10^6
10 days		
β-Gal	485 ± 190	697 ± 143
MPN	2.7×10^7	1.9×10^7
10 days + washing		
β-Gal	571 ± 87	632 ± 193
MPN	3.7×10^7	1.9×10^7

[a] The data are means of results from five plants, except the data from washed samples 10 days after inoculation, which are means of results from three plants.

[b] β-Galactosidase activity, expressed in Miller units · min^{-1} · mg^{-1} of plant protein.

[c] Most probable number of bacteria associated with an entire root.

these strains was correlated with a reduced number of enumerated bacteria and also with a reduction in the extent of staining with X-Gal. Zones of highest coloration, in that case, were localized more frequently at points of emergence of secondary roots.

The expression of a *nifH-lacZ* fusion relative to that of a *nifA-lacZ* fusion was compared in several regulatory mutants (Table 5). In the free-living state, the two fusions were similarly expressed in *nifA*-Tn*5* and in *glnB-kan* mutants, both of which are Nif^- and prototrophs. Comparable data were also obtained in the plant, as reported in Table 5. The *ntrB*-Tn*5* and *ntrC*-Tn*5* mutants (7194 and 7148, respectively) had a Nif^+ phenotype but were impaired in nitrate utilization and in the nitrogenase switch-off by ammonia (Liang *et al.* 1993). In association with wheat, they displayed slightly reduced β-galactosidase activity, compared to the wild type. No major difference in the colonization patterns of the *ntrBC*, the *nifA*-Tn*5*, and the *glnB-kan* mutant strains and the wild-type strain was detected by X-Gal staining. The glutamine auxotroph mutant *glnA* (7028) is also a regulatory mutant which expressed nitrogenase activity in the presence of ammonia, under free-living conditions. In that case, low β-galactosidase activity was detected (Table 5), in agreement with the poor colonization also observed with the pLA-*lacZ* fusion (Table 2).

Application of the procedure to other strains of *A. brasilense*.

The *nifH*- and *nifA-lacZ* fusions were transferred by conjugation to several *A. brasilense* wild-type strains. Among them, *A. brasilense* Sp245 and Sp107 were chosen because they have been reported to be good wheat colonizers (Jain and Patriquin 1984). It was verified that the expression pattern of the *nifH*- and *nifA-lacZ* fusions, under nitrogen-fixing and non-nitrogen-fixing free-living conditions, were identical to those of strain Sp7 (data not shown). As reported in Table 5, significant expression of the *nifH-lacZ* (pAB358) fusion was observed with the different wild-type strains in inoculated wheat. Relative β-galactosidase activity detected with the *nifA-lacZ* fusion (pAB576) was much higher with strain Sp7 and Sp107 than with Sp245 and Sp246 (Table 5). This could reflect a difference in colonization efficiency. Although the number of bacteria was not estimated, less colonization by the two latter strains was observed after X-Gal staining.

Table 5. β-Galactosidase activity of several wild-type and mutant strains of *Azospirillum brasilense* carrying *nifH-lacZ* (pAB358) or *nifA-lacZ* (pAB576) fusions in association with wheat

Strain	pAB358 (*nifH-lacZ*)	pAB576 (*nifA-lacZ*)
Sp7 (wild type)	220 ± 112	421 ± 209
7067 (*nifA*-Tn*5*) (Nif^-)	26 ± 17	408 ± 235
7606 (*glnB-kan*) (Nif^-)	9 ± 4	194 ± 176
7148 (*ntrC*-Tn*5*) (Nif^+)	140 ± 47	254 ± 202
7194 (*ntrB*-Tn*5*) (Nif^+)	161 ± 57	312 ± 173
7028 (*glnA*) (Nif^c)	67 ± 61	46 ± 25
Sp245	67 ± 15	98 ± 65
Sp246	48 ± 27	75 ± 25
Sp107	183 ± 89	238 ± 118

[a] β-Galactosidase activity is expressed in Miller units · min^{-1} · mg^{-1} of plant protein. The data are averages of results of at least two independent experiments with five plants, except for the data on strains Sp245, Sp246, and Sp107, which are averages of results of one experiment with five plants for each strain.

Expression of the *nifH-lacZ* fusion recombined into the host chromosome.

As all experiments were performed with plasmid-borne fusions, it was important to determine whether similar results would be observed when *nifH-lacZ* was chromosomally borne. For that purpose, pAB359 (Fig. 2), a derivative of pSUP202 containing the *nifH-lacZ* fusion, was constructed and integrated into the chromosome of strains Sp7 and 7067 (*nifA*-Tn*5*), as described in Materials and Methods, yielding strains 7358 and 70671, respectively. As expected, the chromosomally borne fusion was expressed under the same physiological conditions as the plasmid-borne one in the free-living state, i.e., in the absence of ammonia at low oxygen tension (nitrogen fixation conditions). Under these conditions, in the wild type, the β-galactosidase activity of the chromosomally borne fusion (strain 7358) was about 40% of that of the plasmid-borne fusion (1,450 ± 185 vs. 3,100 ± 1,950 Miller units per milligram of protein), a result in agreement with a difference in the relative copy number of the two fusions. This indicates that the plasmid vector is present in at least two or three copies per chromosome. In the presence of ammonia the *lacZ* expression was absent, as well as in the *nifA*-Tn*5* (70671) mutant, which did not display β-galactosidase activity even in the absence of ammonia. It was verified that a *nifA*-containing plasmid (pAB57 or pAB53) restored *nifH* expression under conditions of nitrogen fixation when introduced into 70671 (data not shown). Moreover, as shown in Figure 3, the expression of both fusions is oxygen-regulated. In agreement with the number of *nifH* gene copies per cell, the expression of the plasmid-borne *nifH-lacZ* fusion was higher than that of the chromosomally borne one, which was hardly above the background level with oxygen tension

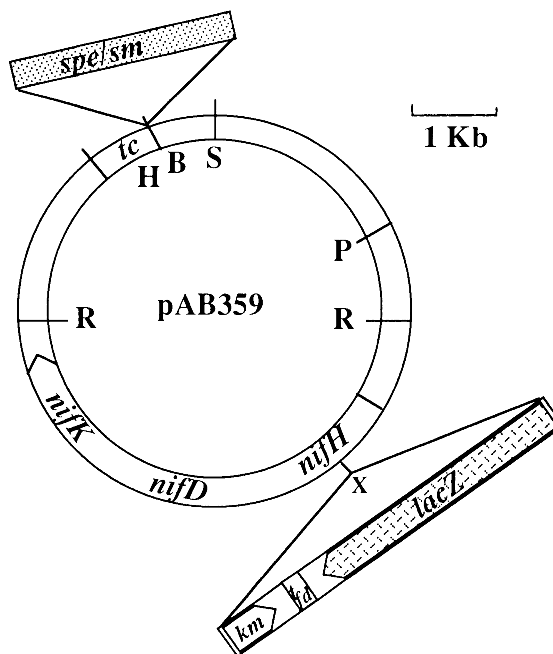

Fig. 2. Schematic representation of pAB359. Restriction sites: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I. The circular map corresponds to pAB3, the orientation of the *nifHDK* genes is indicated by the arrow, and the *spe/sm* interposon and the *lacZ-km* cartridge are represented at their insert locations. t_{fd}, terminator of fd phage.

of 4% in the gas phase. It is also worth noting that nitrogenase activity is totally abolished above 2% oxygen.

In association with wheat, as indicated in Table 3, no β-galactosidase activity above the background was detected with the chromosomal fusion carried either by the wild type (7358) or the *nifA* mutant (70671). However, significant activity was restored when the *nifA*-containing plasmids pAB53 and pAB57 were introduced into strain 70671 (Table 3). The activity detected was lower than when the *nifH-lacZ* is carried by pAB358, in agreement with the difference in copy number. It was verified that similar counts of bacteria associated with the root system were obtained in the presence and in the absence of spectinomycin, suggesting that chromosomal integration of the *nifH-lacZ* fusion was stable. The reason why no activity above the background was detected with strain 7358 remains obscure.

DISCUSSION

The data on the constitutive *lacZ* fusions (pLA-*lacZ* and *nifA-lacZ*) reported above confirm previous reports on the colonization of the root system by azospirilla (Patriquin *et al.* 1983; Okon 1985; Michiels *et al.* 1989) and are in agreement with the results of Vande de Broek *et al.* (1993), who used *gus* fusions. An enhanced proliferation of the root system and of root hairs was noted in plants inoculated with bacteria, compared to uninoculated controls (Jain and Patriquin 1984; Kapulnik *et al.* 1985). After 20 hr, about 7% of the inoculated bacteria were anchored on the surface, a figure in agreement with reports of Michiels *et al.* (1991). At this early stage of the colonization, bacteria were found around the region of emergence of secondary roots, which might be sites of greater exudation, as proposed by Bilal *et al.* (1993) for Kallar grass. After 10 days, bacteria had preferentially colonized the lower part of the roots and were located on the surface or in the first

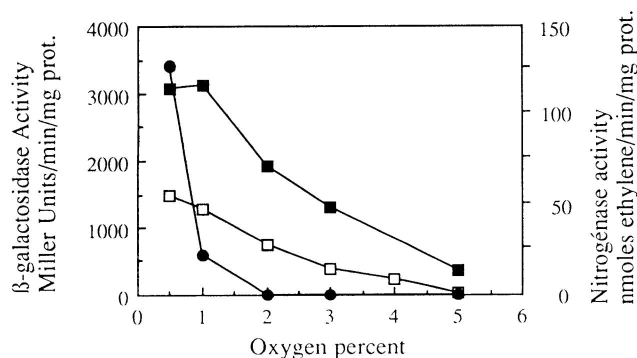

Fig. 3. Effect of the oxygen tension on the expression of the chromosomal and plasmid-borne *nifH-lacZ* fusions and on nitrogenase activity, in the free-living state. Bacteria grown overnight in rich medium were inoculated in nitrogen-free minimal medium in an argon atmosphere containing oxygen at the concentration indicated. β-Galactosidase and nitrogenase activities were determined after 4 hr of incubation. ■, β-Galactosidase activity of strain Sp7 carrying pAB358 (plasmid-borne *nifH-lacZ* fusion). □, β-Galactosidase activity of strain 7358 carrying chromosomally borne *nifH-lacZ* fusion. ●, Nitrogenase activity of strain Sp7. Similar curves were obtained with 7358 and Sp7 (pAB358) strains. At 0.5% oxygen, the acetylene reduction rate of strains 7358 and Sp7 (pAB358) were 50 and 75%, respectively, of that of the wild-type strain. These results are the means of three independent experiments.

layer of the cortex, in agreement with previous reports (Levanony *et al.* 1989; Bilal *et al.* 1993; Vande Broek *et al.* 1993).

Although the bulk of this work was focused on verifying the expression of *nif* fusions on wheat plants, it is worth noting that it can be extended to any other available fusions, such as *ntrC* or *nodG*, also tested here. In preliminary experiments, it was also successfully applied to examine the colonization of rice, sorghum, and pearl millet (data not reported). As a general rule, β-galactosidase activity determinations and X-Gal staining with fully or partially constitutive fusions (pLA-*lacZ* and pAB576) could be correlated with the extent of colonization, as also reported by Vande Broek *et al.* (1993). We have shown here (Fig. 1 and Tables 2 and 4) that this may be more reliable and less tedious than direct counting of bacteria. Since positive samples can easily be identified by their blue color, the method could be used as a rapid screening protocol in a genetic approach to the identification of mutants in colonization efficiency, to differentiate the potential of various wild-type strains to colonize the root systems of different wheat genotypes or other plants. This was verified here in Sp7 wild type and mutants, including auxotrophs, other *A. brasilense* strains (Tables 2, 3, and 5) and, by the same protocol, *A. lipoferum* wild-type strains (Katupitiya *et al.*, in press). Higher expression of β-galactosidase activity was observed with strains Sp7 and Sp107, compared to the other wild-type strains used (Table 5), in agreement with the frequency of observation of cells with X-Gal staining. This could reflect differences in wheat genotypes or better adaptation of strain Sp7 to the particular plant culture conditions. Similarly, Katupitiya *et al.* (in press) found *A. lipoferum* Br17 and 596 to be more efficient than other *A. brasilense* strains on the same wheat genotype.

As the potential for nitrogen fixation by *Azospirillum* in association with forage grasses and cereal crops is often controversial, it was of special interest to determine whether *nif* genes were expressed in the plant model system designed here, in a fashion similar to that observed for cells in free culture. Significant activity was detected with plasmid-borne *nifA*-, *nifB*-, and *nifH-lacZ* fusions. Indeed, the cultures containing the *nifA-lacZ* fusion already expressed *nifA* when inoculated, at about 30% of the maximal rate, although the NifA product is inactive (Liang *et al.* 1991). Therefore, it was expected that significant expression of *nifA* would be detected in the plant after 10 days. By contrast, cultures containing the transcriptional *nifH*- or the translational *nifB-lacZ* fusion did not display β-galactosidase activity at the time of plant inoculation. Our data suggest that NifA (encoded by the chromosomal *nifA* gene) can be converted into its active form on the plant, thus activating the expression of other *nif* genes. As stressed above for pLA-*lacZ*, because of uncertainty regarding the total number of cells, the relative expression of *nifH* or *nifB* in association with the plant cannot be directly compared to that in the free-living state. Also, the maximal rate of expression in association with the plant has not been determined. Using *A. brasilense* strain Sp245 with a *nifH-gusA* plasmid-borne fusion to inoculate wheat grown in sand, Vande Broek *et al.* (1993) showed that the expression of *nifH* as well as nitrogenase activity in association with wheat was greatly increased by limiting oxygen concentration and by adding a carbon source such as malate or fructose. In the

present case, significant expression of *nifA* and *nifH* was detected without the addition of an external carbon source or modification of the oxygen tension, and a chromosomal *nifH-lacZ* fusion was significantly expressed in a *nifA*-mutated background (7061) when a source of NifA was provided with the pAB53- and pAB57-containing wild-type *A. brasilense nifA* gene.

As shown in Tables 4 and 5, the colonization process and plant response were not affected in Nif$^-$ mutants (*nifA*-Tn*5* and *glnB-kan*) or in mutants impaired in nitrate assimilation (*ntrBC*-Tn*5* mutant), suggesting that neither nitrogen reduction capacity nor nitrate assimilation is essential for colonization of wheat roots. By contrast, a glutamine auxotroph (7028) and tryptophan auxotrophs (7853 and 7854) colonized less (Table 2), possibly for nutritional reasons. As glutamine is a compound usually found in wheat root exudates (McDougall 1970), it is not known whether the behavior of the mutant 7028 reflects only a defect in nutritional conditions. This raises the question of the role of bacterial glutamine synthetase in the time course of the association with the plant. This will be further explored with other mutants and the recently constructed *glnB*- and *glnA-lacZ* fusions (de Zamaroczy *et al.* 1993).

Thus the model system developed here offers to provide an efficient tool to analyze at the genetic level which steps are limiting in the expression of nitrogen fixation genes, in nitrogenase activity, and in the transfer of fixed nitrogen in associations with host plants. It also provides a means, when appropriate *lacZ* fusions are used, of studying the efficiency of colonization of various plant cultivars by various bacterial strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and mating procedure.

The bacterial strains and plasmids used in this work are listed in Table 1. Complete media were Luria-Bertani broth for *E. coli* and nutrient broth (Difco) for *A. brasilense.* Minimal lactate medium was as described by Galimand *et al.* (1989). Antibiotics were used at the following concentrations: tetracycline, 5 μg/ml; kanamycin, 20 μg/ml; and spectinomycin, 100 μg/ml. Transfer of the plasmids containing the *lacZ* fusions into *Azospirillum* recipients was performed by conjugation using *E. coli* S17-1 as a donor. Transconjugants were selected on minimal lactate medium containing 20 mM ammonia and tetracycline as described by Galimand *et al.* (1989). Glutamine (200 μg/ml) or tryptophan (20 μg/ml) was added to the minimal medium when required. Ammonia was assayed with Berthelot reagent according to the protocol of Chaykin (1969).

Cultivation of plants and inoculation of seedlings with bacteria.

Wheat seeds (*Triticum aestivum* spring cultivar Miskle) were surface-sterilized, germinated, and transferred aseptically to tubes containing 15 ml of hydroponic solution, as described by Zeman *et al.* (1992). Plants were incubated at 22° C with a day-night cycle of 16 and 8 hr. After 1 week of growth, the hydroponic solution was inoculated with 0.1 ml of an overnight culture, approximately 5×10^6 bacterial cells per milliliter of hydroponic solution. In most experiments, the bacteria were grown overnight in minimal medium containing sodium lactate (5 g/L) and 10 mM NH_4Cl (see Results). *In situ* colonization after X-Gal staining and assay for β-galactosidase activity were determined in general 10 days after inoculation. Uninoculated plant tubes and tubes inoculated with bacteria without the *lacZ* fusion were routinely included as controls.

In situ detection of β-galactosidase activity in root segments inoculated with *Azospirillum*.

Staining of root segments with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was performed by a modification of the protocol of Boivin *et al.* (1990). A few 1-cm root segments placed in microfuge tubes were fixed with 2% glutaraldehyde solution in Z buffer (Miller 1972) devoid of β-mercaptoethanol and adjusted to pH 7.4 (Teeri *et al.* 1989) under vacuum for 30 min at room temperature and then at atmospheric pressure for 60 min. The glutaraldehyde solution was discarded, and the specimens were washed twice with Z buffer for 15 min. X-Gal solution prepared in Z buffer containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and X-Gal (850 μg/ml) was added to each tube, and the root samples were incubated overnight at room temperature, in the dark. Specimens were washed with Z buffer and distilled water and treated with 12% sodium hypochlorite for 40 sec, as described by Truchet *et al.* (1989), and then washed twice with distilled water. The root segments were then examined by light microscopy with an Olympus BHA or a Nikon X microscope.

Assay for β-galactosidase activity.

β-Galactosidase was assayed essentially as described by Miller (1972). Determination of activity in free-living cultures of *A. brasilense* was performed with cells incubated in minimal lactate medium containing or not containing ammonia, under microaerobic conditions (99.5% A, 0.5% O_2) or air as described by Liang *et al.* (1991). Results were expressed in Miller units $\cdot$ $min^{-1} \cdot mg^{-1}$ of protein. Determination of activity on roots was performed according to the following protocol. Roots were ground in 2 ml of Z buffer (Miller 1972) devoid of β-mercaptoethanol. The plant-endogenous β-galactosidase was inactivated by heating the suspension for 15 min at 50° C; then β-mercaptoethanol was added to a final concentration of 35 μM, and the bacterial cells were lysed by adding 20 μl of 0.1% sodium dodecyl sulfate and 40 μl of chloroform and vortexing. The main debris was eliminated by centrifugation at low speed. Assay for β-galactosidase activity was performed as usual; the plant extract with Na_2CO_3 added before *o*-nitrophenyl-β-D-galactopyranoside and without incubation was used as a blank. Protein concentration was determined with Bio-Rad protein assay reagent. The results are expressed in Miller units $\cdot$ $min^{-1} \cdot mg^{-1}$ of plant protein.

Enumeration of bacteria associated with plant tissues.

Counts of bacteria associated with the root system were estimated by the most-probable-number method, on nutrient broth medium inoculated with ground root extract in Z buffer. Segregation of plasmids pAB358 and pAB576 after cultivation of the plant was estimated by plating appropriate dilutions on minimal lactate medium devoid of tetracycline and the same medium containing tetracycline. The difference in

titer in the presence and in the absence of tetracycline was estimated. The same protocol was used to assay the stability of strains 7358 and 70671 on minimal medium containing or not containing spectinomycin.

Construction of strains 7358 and 70671, containing the *nifH-lacZ* fusion integrated into the chromosome, and construction of pLA-*lacZ*.

Plasmid construction, DNA-mediated transformation, extraction of total DNA, and hybridization by the technique of Southern were performed according to conventional methods (Sambrook *et al.* 1989). Plasmid pAB3 is a derivative of pSUP202, which carries the *nifHDK* operon of strain Sp7 and cannot replicate in *Azospirillum*. A spectinomycin derivative of pAB3 was obtained by cloning the *spe/sm* interposon purified from pHP45Ω into the *Bam*HI site of the Tc^r gene of the plasmid. Then a *Sal*I fragment containing the *lacZ-kan* cartridge of pKOK5 was inserted at the *Xho*I site into the *nifH* coding sequence, in the same orientation as *nifH*, to yield pAB359 (Fig. 2). The resulting plasmid was introduced by transformation into *E. coli* S17-1 and transferred by conjugation into strains Sp7 and 7067. Transconjugants were selected for spectinomycin resistance and kanamycin resistance. Correct recombination was checked by hybridization of total DNA of the transconjugants with *lacZ* and *nifH* probes (data not shown).

To construct an IncP plasmid derivative which expressed β-galactosidase activity from a plasmid promoter, a *Bam*HI fragment carrying the *lacZ-km* cartridge of pKOK5 was cloned into the *Bgl*II site of the pLA2917 vector in the two possible orientations. The ligation mixture was used to transform strain S17-1, and the recombinant plasmids were selected on medium containing tetracycline and X-Gal. Three types of colonies were obtained: unstained, light blue, and deep blue. As the *Bgl*II site is located within the Km^r gene of the vector, the deep blue colonies corresponded to the cloning of the *lacZ* cartridge under the *kan* promoter. The resulting plasmid was named pLA-*lacZ*.

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