Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense*

Ann Vande Broek, Mark Lambrecht and Jos Vanderleyden

Bacteria of the genus *Azospirillum* are able to colonize plant roots. Using the β-glucuronidase (GUS) reporter system, various *Azospirillum* mutants, including mutants affected in chemotactic motility or extracellular polysaccharide biosynthesis, were investigated for their capacity to initiate wheat root colonization at the root hair zones. Only non-flagellated mutants and a generally non-chemotactic mutant exhibited a strongly reduced colonization ability as compared to the wild-type. No role of the *Azospirillum* calcofluor-binding polysaccharide in primary wheat root colonization could be observed. This is the first report demonstrating directly, by using different motility mutants, the requirement of bacterial motility in the establishment of the *Azospirillum*-plant root association.

**Keywords**: GUS reporter system, plant-growth-promoting rhizobacteria (PGPR), plant root association, *Azospirillum brasilense*

**INTRODUCTION**

Nitrogen-fixing bacteria of the genus *Azospirillum* are known to colonize the rhizosphere of economically important crops and to exert beneficial effects on plant growth under certain environmental conditions (for a review see Okon & Labandera-Gonzalez, 1994; Okon & Vanderleyden, 1997). Unlike the *Rhizobium*-legume symbiosis and the interaction of plant pathogens with their host plants, the association of *Azospirillum* with plant roots does not result in the formation of an easily detectable plant phenotype. Mainly due to the absence of a clear plant phenotype indicative of a successful interaction, the mechanisms involved in the *Azospirillum*-plant association process are still poorly understood (Vande Broek & Vanderleyden, 1995a).

To facilitate the molecular analysis of the *Azospirillum*-plant association, we previously designed a strategy to visualize the *Azospirillum*-plant interaction by using *Azospirillum* strains expressing the *Escherichia coli* gusA gene (Vande Broek *et al.*, 1993). Because of the complete absence of endogenous β-glucuronidase (GUS) activity in wheat roots, this method enabled us to determine in a very specific way the main colonization sites of *Azospirillum* on wheat roots. Colonization patterns were shown to differ as a function of time. During the first days of the association, we observed a root colonization restricted to the sites of lateral root emergence and the root hair zones (Vande Broek *et al.*, 1993). Further proliferation of *Azospirillum* on the wheat root surface depends on the nitrogen content of the rooting medium. The mechanisms responsible for the localized bacterial accumulation observed during the first days of the association are not known but may involve specific attachment sites, specific chemotaxis and/or a higher bacterial proliferation at these sites (Vande Broek & Vanderleyden, 1995b).

*Azospirilla* have a mixed pattern of flagellation: during growth in liquid medium, a single polar flagellum is synthesized whilst, in addition to the polar flagellum, lateral flagella are induced during growth on solidified media (Hall & Krieg, 1983). The polar flagellum is primarily used for swimming, i.e. motility in liquid media, whereas the lateral flagella are responsible for swarming of the bacteria on solid surfaces (Moens *et al.*, 1995). *Azospirillum* strains were demonstrated to exhibit positive chemotaxis in vitro towards several attractants, including sugars, amino acids, organic acids (Okon *et al.*, 1980; Barak *et al.*, 1982; Reinhold *et al.*, 1985; Zhulin & Armitage, 1993) and aromatic compounds (Lopez-De-Victoria *et al.*, 1993), as well as towards root exudates (Heinrich & Hess, 1985) and root

**Abbreviations**: GUS, β-glucuronidase; PNP, p-nitrophenyl β-o-glucuronide; PS, polysaccharide; X-Gluc, 5-bromo-4-chloro-3-indolyl β-o-glucuronide.
mucilage (Mandimba et al., 1986). Furthermore, migration of *Azospirillum brasilense* cells towards roots of wheat seedlings has been shown in sand cultures and in a wet soil (Bashan, 1986a).

Using two short-term *in vitro* assays, two modes of attachment of *A. brasilense* to plant roots could be distinguished and a two-step attachment mechanism was proposed (Michiels et al., 1991). The first step, termed the adsorption step, consists of a rapid and weak binding of the bacteria to the plant root surface and was found to be mediated by the polar flagellum of the bacteria (Croes et al., 1993). The second step, termed the anchoring step, only occurs in medium with a high C/N ratio and is mediated by a bacterial calcifluor-binding polysaccharide (PS). By means of this PS, the bacteria become firmly attached to the plant root and additional free bacteria are entrapped to form large clusters on the root surface.

In this study, to examine the specific role of bacterial motility and wheat root attachment in the *Azospirillum*-plant root interaction, we analysed by means of gusA fusion plasmids (Vande Broek et al., 1993) the primary colonization of the root hair zones of wheat by *A. brasilense* mutants deficient in either one of the above processes. Several additional mutants defective in PS biosynthesis and an *A. brasilense* strain cured of its 115 MDa megaplasmid (p115) were included in the analysis.

**METHODS**

**Bacterial strains, plasmids and media.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* S17-1 was maintained on LB agar (Miller, 1972) and grown at 37 °C in LB broth. *Azospirilla* were maintained on YEP agar (per liter: 100 g Bacto Peptone, 50 g NaCl, 10 g Yeast Extract, 150 g agar) and cultured at 30 °C in YEP broth or in *Azospirillum* minimal MMAB malate medium (Vannstockem et al., 1987). When appropriate, the media were supplemented with the antibiotics kanamycin and tetracycline at a final concentratio of 5 μg ml⁻¹ and 10 μg ml⁻¹, respectively. D agar plates, used for bacterial conjugations, contained per liter: 8.0 g Bacto Nutrient Broth, 0.25 g MgSO₄·7H₂O, 10 g KCl, 0.01 g MnCl₂.

**Biparental bacterial matings.** Portions (1 ml) of exponential cultures of the acceptor *Azospirillum* strain and of the donor strain *E. coli* S17-1 harbouring the mobilizable plasmid (pFAJ31.13 or pFAJ31.2) were centrifuged (6000 r.p.m., 5 min), washed with 0.85% NaCl and resuspended in 100 μl 0.85% NaCl. Thirty microliters of both suspensions were mixed and spotted on a D agar plate. After overnight incubation at 30 °C, the mating mixture was scraped from the agar surface, resuspended in 1 ml 0.85% NaCl and serial dilutions of this suspension were plated on selective medium.

**Stability of pFAJ31.13 in A. brasilense.** *A. brasilense* Sp7 (pFAJ31.13) and 7030 (pFAJ31.13) were cultured in YEP medium without tetracycline with five repetitions for each strain. After 48 h growth at 30 °C (at least 10 generations), serial dilutions were plated on YEP agar plates (without tetracycline) and incubated for 2 d at 30 °C. One hundred individual colonies originating from each starter culture were then streaked on YEP medium with and without tetracycline. The percentage of tetracycline-resistant clones was determined; the mean and the standard deviation of the five replicates are presented.

**Ex planta GUS assay.** GUS activity in bacterial cultures was measured using the GUS extraction buffer and the substrate p-nitrophenyl β-D-glucuronide (PNPG) (0.4 mg ml⁻¹) as described by Jefferson (1987). Units were calculated as defined by Miller (1972) for β-galactosidase. The mean and standard deviation of four replicates are presented.

To evaluate the relationship between GUS activity and bacterial cell numbers, serial dilutions of an Sp7 (pFAJ31.13) overnight culture were tested for GUS activity as follows. Samples (100 μl) of these dilutions were added to 0.9 ml GUS extraction buffer containing 0.4 mg PNPG ml⁻¹ and incubated at 37 °C. Aliquots (100 μl) were removed at regular time intervals and transferred to microtitre plates filled with 35 μl 1 M Na₂CO₃ to stop the GUS reaction. Optical densities (OD₄₂0, OD₃₈₅) were measured using an automatic microtitre plate reader (Bio Rad Laboratories, 3530-UV). The cell density of the culture was determined by spotting 25 μl of appropriate dilutions on YEP agar.

**Plant culture assay.** Wheat seeds (*Triticum aestivum* cv. Soissons) were surface-sterilized as described by Vande Broek et al. (1993). After 4 d incubation in the dark on nutrient agar plates (per liter: 8.0 g Bacto Nutrient Broth, 150 g agar) at 23 °C, sterile germinated seeds with radicles approximately 1 cm long were aseptically transferred into cotton-plugged 25 mm x 150 mm test tubes containing 15 ml nitrogen-free MPCL nutrient solution (pH 6.0) (Lavigne, 1987). The seeds were placed on a metal grid positioned at the surface of the nutrient solution. Tubes were placed in a growth chamber (at a constant temperature of 23 °C and 12 h light per day). The next day, an exponential culture of *A. brasilense* was harvested by centrifugation (6000 r.p.m., 5 min), washed twice with sterile 0.85% NaCl and resuspended in 0.85% NaCl to a final cell density of 10⁸ c.f.u. ml⁻¹. Portions (1 ml) of this suspension were inoculated directly into the nutrient solutions of the wheat seedlings. Where indicated, fructose or malate was added to the plant nutrient solution to a final concentration of 0.1%.

**Staining of inoculated roots with X-Glc.** Roots were excised and gently washed three times by consecutive immersion in 0.85% NaCl for 1 min without agitation. Washed roots were transferred to 0.1 M sodium phosphate buffer (pH = 7.0) containing 0.5 mg X-Glc ml⁻¹, 0.33 mg K₃Fe(CN)₆ ml⁻¹ and 0.42 mg K₄Fe(CN)₆ ml⁻¹ and incubated in the dark for 16 h at 37 °C. For each *Azospirillum* strain at least 10 independent seedlings were stained. After staining, the roots were rinsed three times in phosphate buffer and examined for specific colonization sites by eye and by using a light microscope.

**Quantitative assay for GUS activity on inoculated roots.** Seedlings with equal-sized root systems were harvested 3 or 4 d after inoculation (10 seedlings for each mutant and each growth condition). Roots were excised, gently washed as described above and transferred into separate Eppendorf tubes filled with 1 ml GUS extraction buffer (Jefferson, 1987) containing 0.4 mg PNPG ml⁻¹. Tubes were incubated at 37 °C. Aliquots (100 μl) were removed from each tube after different times of incubation and transferred to microtitre plates filled with 35 μl 1 M Na₂CO₃ to arrest GUS activity. The intensity of the yellow staining, which is proportional to the amount of p-nitrophenol produced and to the number of bacteria colonizing the root, was measured as OD₄₁₇ using an automatic microtitre plate reader. As a control, 10 non-inoculated seedlings were treated in the same way as the
**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>Azospirillum brasilense</em></td>
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<tr>
<td>Sp7</td>
<td>Wild-type strain (ATCC 29145)</td>
<td>Tarrand et al. (1978)</td>
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<tr>
<td>AB7001</td>
<td>Km*; Tn5-induced mutant of Sp7, Tn5 is inserted in a gene that complements the <em>exoC</em> mutation of <em>Sinorhizobium meliloti</em></td>
<td>Michiels et al. (1988)</td>
</tr>
<tr>
<td>AB7002</td>
<td>Km*; Tn5-induced mutant of Sp7, Tn5 is inserted in a gene that complements the <em>exoB</em> mutation of <em>Sinorhizobium meliloti</em></td>
<td>Michiels et al. (1988)</td>
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<tr>
<td>AB7200</td>
<td>Sp7 spontaneously cured of the 115 MDa megaplasmid Sm*; non-motile derivative of Sp7, Ads* Fla* Mot* Swarm*</td>
<td>Croes et al. (1991)</td>
</tr>
<tr>
<td>Sp7p90A84</td>
<td>Km*; Tn5-induced mutant of Sp7, Tn5 is inserted in a gene that complements the <em>exoB</em> mutation of <em>Sinorhizobium meliloti</em></td>
<td>Croes et al. (1991)</td>
</tr>
<tr>
<td>7030</td>
<td>Km*; Tn5-induced mutant of Sp7</td>
<td>Franche &amp; Elmerich (1981)</td>
</tr>
<tr>
<td>7030Tn5-23</td>
<td>Km*; Tn5-induced mutant of Sp7</td>
<td>Michiels et al. (1990)</td>
</tr>
<tr>
<td>7030Tn5-101</td>
<td>Km*; Tn5-induced mutant of Sp7</td>
<td>De Troch et al. (1992)</td>
</tr>
<tr>
<td>NM304</td>
<td>Km*; Tn5-induced mutant of Sp7</td>
<td>van Rhijn et al. (1990)</td>
</tr>
<tr>
<td>NM313</td>
<td>Km*; Tn5-induced mutant of Sp7</td>
<td>van Rhijn et al. (1990)</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>pro thi hsdR hsdM-recA, Rp4-2-Tc::Mu-Km::Tn7 integrated in the chromosome</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>S17-1</td>
<td>pro thi hsdR hsdM-recA, Rp4-2-Tc::Mu-Km::Tn7 integrated in the chromosome</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pFAJ31.2</td>
<td>Tc*; pLAFR3 derivative containing a constitutively expressed <em>A. brasilense</em> promoter::<em>gusA</em> fusion</td>
<td>Vande Broek et al. (1993)</td>
</tr>
<tr>
<td>pFAJ31.13</td>
<td>Tc*; pLAFR3 derivative containing a constitutively expressed <em>A. brasilense</em> promoter::<em>gusA</em> fusion</td>
<td>Vande Broek et al. (1993)</td>
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</table>

* Ads* and Anc*, deficient adsorption or anchoring ability to wheat roots, respectively (Michiels et al., 1991); Cal* and Cal*, presence and absence, respectively, of colony fluorescence on plates containing calcofluor; Fla* and Fla*, presence and absence, respectively, of both the polar and lateral flagella; Mot* and Swarm*, absence of motility in liquid-rich medium as assayed by light microscopy (Moens et al., 1995) and on solid nutrient agar (Moens et al., 1995), respectively; Km, kanamycin; Sm, streptomycin; Tc, tetracycline.

inoculated plants. Bacterial colonization of a root was expressed as:

$$\frac{(A - B)}{C} \times 10^5$$

where A = A_{415} value of the tested root, B = mean A_{415} value of 10 non-inoculated seedlings and C = staining incubation time (min).

**Wheat root adsorption assay.** Bacterial adsorption to wheat roots was quantitatively measured as described previously (Croes et al., 1991). Briefly, bacteria were radioactively labelled by growing them to a cell density of $3 \times 10^9$ cells ml$^{-1}$ in 5 ml YEP medium supplemented with 20 μCi d[1',2',3'-$^3$H]CTP (57 Ci mmol$^{-1}$; 2.1 TBq mmol$^{-1}$); Amersham. Portions (0.5 ml) of the culture were then washed three times in 0.85% NaCl and transferred to test tubes containing equal-sized wheat roots in 4.5 ml MPCL. After 2 h incubation in a rotary shaker at 30°C, the bacterial suspensions were decanted and the roots were washed three times by immersion in 0.85% NaCl for 1 min without agitation. The amount of radioactivity retained on the root was measured in a liquid scintillation counter and bacterial adsorption was expressed as a percentage of the total added radioactivity. The experiment was carried out with six replications for each strain tested.

**RESULTS**

**Labelling of *A. brasilense* wild-type and mutant strains with the *gusA* expression plasmid pFAJ31.13**

The *A. brasilense* Sp245 promoter::gusA fusion plasmid pFAJ31.13 was conjugated into the *A. brasilense* strains Sp7 and 7030, the non-flagellated mutants Sp7p90A84 and NM304, the non-chemotactic mutant NM313, the exopolysaccharide mutants AB7001 and AB7002, the calcofluor-minus mutants 7030Tn5-23 and 7030Tn5-101 and the p115-cured mutant AB7200, as described in Methods. Phenotypic characteristics of these acceptor strains have been described elsewhere and are summarized in Table 1.

Before performing plant inoculation experiments, three preliminary *ex planta* experiments were carried out. Firstly, to avoid false conclusions due to interference with colonization caused by a reduced growth rate,
growth of the various wild-type and mutant strains harbouring pFAJ31.13 was compared when cultured in the complex YEP medium and in the minimal MMAB medium. All mutants were shown to have wild-type growth characteristics in both media. Secondly, to allow the correlation of GUS activity detected on inoculated wheat roots with the presence of cells of the Azospirillum strain tested, we examined whether the mutations in the various strains had any effect on the expression of the gusA gene of pFAJ31.13. GUS activity of the different A. brasilense strains carrying pFAJ31.13 was determined quantitatively when grown to late-exponential growth phase in rich YEP and minimal MMAB medium. For each strain, similar gusA expression levels were detected in both media. Strikingly, significant differences in pFAJ31.13 expression were found between the two A. brasilense wild-type strains Sp7 and 7030 (252 ± 26 Miller units and 129 ± 19 Miller units, respectively, when cultured in rich YEP medium). However, none of the mutants showed a gusA expression level significantly different from that of the corresponding parental strain. The reason for the reduction of expression of the gusA fusion plasmid in the 7030 derivatives as compared to the Sp7 derivatives is not known. Although 7030 was isolated as a streptomycin-resistant mutant of Sp7, it has previously been shown to differ in additional traits from its parent strain [e.g. the overproduction of red pigment, efficiency of Tn5 mutagenesis (Vanstockem et al., 1987) and electroporation (Vande Broek et al., 1989)]. In the third series of preliminary experiments, the stability of the gusA fusion plasmid pFAJ31.13 was assessed. Upon sub-culturing of Sp7(pFAJ31.13) and 7030(pFAJ31.13) in the absence of antibiotic selection for at least 10 generations, more than 90% of the bacterial populations (i.e. 93.5 ± 2.5% and 90.2 ± 7.5% for Sp7 and 7030, respectively) were found to have retained the recombinant plasmid.

**Fig. 1.** Histochemical analysis of initiation of wheat root colonization by *A. brasilense* wild-type and mutant strains carrying the gusA reporter plasmid pFAJ31.13. Wheat seedlings were inoculated with *Azospirillum*, grown in a hydroponic system containing nitrogen-free MPCL medium and stained with X-Glc 3–4 d after inoculation. Blue staining of the root surface indicates the presence of azospirilla. (a) Staining pattern of the root system of five wheat plants inoculated with Sp7(pFAJ31.13). (b) Initiation of root colonization by *A. brasilense* strains Sp7 (i), Sp7p90ΔA84 (ii), 7030 (iii), NM304 (iv) and NM313 (v) carrying pFAJ31.13.

**Histochemical analysis of primary colonization of the root hair zones of wheat by the various A. brasilense mutants**

In the first plant inoculation experiment, the association of each *A. brasilense* mutant with wheat roots was examined qualitatively by staining inoculated roots with the histochemical substrate X-Glc. Wheat seedlings were grown in a hydroponic system with nitrogen-free MPCL as plant nutrient solution and inoculated as described in Methods with the *A. brasilense* strains carrying
pFAJ31.13. Primary colonization sites were evaluated at 3 or 4 d post-inoculation. As has been found for inoculated wheat seedlings grown in sand (Vande Broek et al., 1993), a specific blue staining of the root hair zones during these first days of association was observed (Fig. 1a). The presence and absence of bacteria in blue-stained and non-stained zones, respectively, was confirmed by light microscopic analysis (data not shown). Colonization patterns were identical for both A. brasilense strains Sp7 and 7030.

Three mutants, namely the non-flagellated mutants Sp7p9OA84 and NM304, and the non-chemotactic mutant NM313, showed a strongly reduced colonization capacity. On seedlings inoculated with these mutants, no root staining or a faint blue staining restricted to the root hair zone was observed (Fig. 1b i, iii and v, respectively). For the other mutant strains, including the Cal- derivatives of A. brasilense 7030 (7030Tn5-23, 7030Tn5-101), staining patterns did not differ from those of the parental strains (data not shown).

Michiels et al. (1990) previously demonstrated that a high C/N ratio stimulates the biosynthesis of a calcofluor-binding PS in A. brasilense. Moreover, this PS promotes firm attachment of Azospirillum to wheat roots in short-term binding assays (Michiels et al., 1991). Therefore, to assess a role of the calcofluor-binding PS in root colonization, the colonization experiments were repeated in rooting medium containing 0.1% fructose. The results were consistent with the previous observations. In this medium, primary colonization of A. brasilense was again restricted to the root hair zones. No difference in the colonization capacity of the A. brasilense Cal-derivatives as compared to the parents could be detected. The only mutants that were affected in colonization were Sp7p9OA84, NM304 and NM313, indicating that under these conditions also chemotactic motility is important for the initiation of root colonization.

To rule out potential artifacts in the above described inoculation experiments caused by the use of only one constitutive A. brasilense promoter::gusA fusion, a second fusion plasmid, pFAJ31.2, was conjugated into Sp7, Sp7p9OA84, 7030, NM304 and NM313 and the qualitative colonization experiments were repeated with these transconjugants. Again, in both fructose-free and fructose-supplemented nutrient solution, the motility mutants Sp7p9OA84, NM304 and NM313 exhibited a strongly reduced colonization of the root hair zones, whilst the other mutants, including the Cal- mutants, appeared not to be affected (data not shown).

Quantitative analysis of GUS activity on wheat roots inoculated with the various A. brasilense mutants

To corroborate the histochemical observations described above we assayed GUS activity on the inoculated wheat roots quantitatively. We first tested whether there was a linear relationship between the number of cells and GUS activity. For this, samples containing different concentrations of A. brasilense Sp7 cells carrying pFAJ31.13 were tested for GUS activity at different times of incubation in the presence of PNPG (see Methods). A linear relationship between the number of cells and GUS activity was observed for cell densities from 10⁵ to 10⁹ cells ml⁻¹. Since additionally the expression level of the pFAJ31.13 fusion was shown to be the same in each mutant and in the corresponding wild-type strain (see above), the GUS activity detected on the root is a good measure of the number of bacteria present on the root. Results of the quantitative analysis are shown in Table 2 for the Sp7 derivatives and in Table 3 for the 7030 derivatives. The data are in agreement with the histochemical analysis, showing a strongly reduced colonization of wheat roots by Sp7p9OA84, NM304 and NM313. Again, no difference in colonization capacity of the calcofluor-minus 7030 derivatives, 7030Tn5-23 and 7030Tn5-101, as compared to 7030, and of the Sp7 derivatives AB7001, AB7002 and AB7200, as compared to Sp7, was detected, either in carbon-free rooting medium or in rooting medium supplemented with 0.1% of a metabolizable carbon source (fructose or malate).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plant nutrient solution</th>
<th>No C source</th>
<th>+ Fructose</th>
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<tbody>
<tr>
<td>Sp7</td>
<td>1.36*</td>
<td>6.95*</td>
<td></td>
</tr>
<tr>
<td>AB7001</td>
<td>0.65*</td>
<td>5.63*</td>
<td></td>
</tr>
<tr>
<td>AB7002</td>
<td>1.30*</td>
<td>6.95*</td>
<td></td>
</tr>
<tr>
<td>AB7200</td>
<td>0.55*</td>
<td>7.54*</td>
<td></td>
</tr>
<tr>
<td>Sp7p9OA84</td>
<td>0.35*</td>
<td>1.30*</td>
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</table>

The non-chemotactic mutant NM313 has a wild-type adsorption capacity to wheat roots

Croes et al. (1993) previously demonstrated that adsorption of A. brasilense to wheat roots is mediated by a proteinaceous component located at the polar flagellum. Transmission electron microscopy of cells of the non-chemotactic mutant NM313 revealed the presence of both lateral and polar flagella (data not shown). However, this method does not allow us to discern
Early colonization capacity was determined as described in the Methods section. Data presented are mean results from 10 seedlings. Within a given column, values followed by the same letter are not significantly different at \( P = 0.05 \). Negative values calculated according to the formula presented in Methods ( = non-colonized seedlings) were obtained with 6 out of 10 plants inoculated with NM304 or NM313 in the absence of C source and with 1 out of 10 plants with NM304 in the presence of fructose or malate. Plant nutrient solutions used were nitrogen-free MPCL or nitrogen-free MPCL supplemented with 0.1% fructose or 0.1% malate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No C source</th>
<th>+ Fructose</th>
<th>+ Malate</th>
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<tbody>
<tr>
<td>7030</td>
<td>0.55*</td>
<td>5.38*</td>
<td>1.07*</td>
</tr>
<tr>
<td>NM304</td>
<td>NGD*</td>
<td>0.61*</td>
<td>0.33*</td>
</tr>
<tr>
<td>NM313</td>
<td>NGD*</td>
<td>1.72*</td>
<td>ND</td>
</tr>
<tr>
<td>7030Tn5-23</td>
<td>0.69*</td>
<td>4.90*</td>
<td>ND</td>
</tr>
<tr>
<td>7030Tn5-101</td>
<td>0.42*</td>
<td>5.85*</td>
<td>ND</td>
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</table>

NGD, No GUS activity detected. The mean of the values obtained for the 10 seedlings according to the formula presented in Methods was negative (\(-0.15 \pm 0.13\) in the case of NM304, \(-0.01 \pm 0.23\) in the case of NM313).

ND, Not determined.

DISCUSSION

Previously, we described a root colonization assay using *Azospirillum* strains marked with the gusA fusion plasmids pFAJ31.13 and pFAJ31.2 (Vande Broek et al., 1993). The distribution of *Azospirillum* cells on the surface of monoxenically grown wheat roots was monitored during a period of 4 weeks after inoculation (Vande Broek et al., 1993). It was observed that during the first days of the association bacteria are not uniformly spread over the entire root surface but are specifically concentrated in the root hair zones and at the bases of lateral roots. By analysing the colonization capability of various previously isolated mutants of *Azospirillum* strains Sp7 and 7030, we here provide evidence that active bacterial motility towards the root hair zone is important for the initiation of root colonization at these sites.

Two non-motile *Azospirillum* mutants, Sp7p90ΔA84 and NM304, were found to exhibit a strongly reduced capacity to colonize the root hair zones of wheat. The two mutants carry a mutation in a different locus. Sp7p90ΔA84 was constructed by replacing a 13 kb BamHI restriction fragment of the p90 megaplasmid by a kanamycin resistance cartridge (Croes et al., 1991), whilst NM304 carries a Tn5-lacZ insertion into the chromosome (van Rhijn et al., 1990). However, since both mutants lack the polar flagellum, the affected primary root hair zone colonization by Sp7p90ΔA84 and NM304 does not strictly point to the requirement of bacterial motility for wheat root colonization. Croes et al. (1993) have previously demonstrated that the *Azospirillum* polar flagellum or a component located on the polar flagellum can function as a wheat root adhesin in short-term incubation studies. In these studies, non-flagellated mutants of *Azospirillum* Sp7, including the Sp7 derivative Sp7p90ΔA84, show a severely reduced adsorption capacity to wheat roots. Therefore, the reduced colonization of Sp7p90ΔA84 and NM304 may also result from the loss of the bacterial root adhesin located at the polar flagellum and mediating attachment of *Azospirillum* to possible specific receptor sites at the root hair zones. However, it is clearly demonstrated by the reduced colonization capacity of the non-chemotactic mutant NM313 that active movement of *Azospirillum* is important for the primary colonization of wheat roots at the root hair zones. This mutant contains a Tn5-lacZ insertion in the p90 megaplasmid at a position separated by at least 35 kb from the DNA region that is deleted in the p90 deletion derivative Sp7p90ΔA84 (Croes et al., 1991). NM313 still possesses both lateral and polar flagella and is motile in liquid rich medium as well as on nutrient agar plates. However it fails to exhibit chemotaxis towards some, but not all, of the amino acids, sugars and organic acids that have been tested (van Rhijn et al., 1990). Growth kinetics of NM313, as well as NM304 and Sp7p90ΔA84, under different physiological conditions were demonstrated to be identical to the wild-type. Since NM313 was shown in this study not to be affected in wheat root attachment, its reduced colonization capacity most probably results from the hampered motility of the bacterium towards the root hair zones.

No effect of the presence of the p115 megaplasmid and of the *A. brasilense* exoB1 and exoC genes in plant root colonization could be detected. In a previous study, Croes (1993) undertook a detailed phenotypical analysis of the 115 MDa plasmid but no function could be assigned to it. The *Azospirillum* exoB1 and exoC genes have been isolated by intergeneric complementation of *Sinorhizobium* (previously *Rhizobium*) *meliloti* exoB and exoC mutants for the production of
calcofluor-binding PS (Michiels et al., 1988) and have been shown to encode a UDP-glucose 4'-epimerase and a phosphomannomutase, respectively (De Troch et al., 1994, 1995). S. meliloti exo mutants are defective in the biosynthesis of the major exopolysaccharide, succinoglycan, and are symbiotically deficient, as shown by the production of amorphous, white, non-infected nodules on their host plants (Leigh et al., 1985; Long et al., 1988). In contrast to the S. meliloti exo mutants, A. brasilense strains carrying mutations in the exoB and exoC correcting loci are still fluorescent on agar medium containing calcofluor but produce a slightly modified exopolysaccharide with a lower molecular mass (Michiels et al., 1988). Apparently, as has been demonstrated in the present study, this slight modification of exopolysaccharide has no significant effect on the primary colonization of the root hair zones by Azospirillum.

In addition, the A. brasilense Cal- mutants, 7030Tn5-23 and 7030Tn5-101, appeared not to be affected in primary wheat root colonization. Studying the adhesion of A. brasilense to wheat roots in short-term in vitro binding assays, Michiels et al. (1991) demonstrated that Azospirillum–root attachment is a two-step process, similar to the attachment processes described for Agrobacterium and Rhizobium (Matthysse et al., 1981; Smit et al., 1987). In these three species, the first step involves a loose binding of single cells to the root surface. In the second step, bacteria become more firmly attached and additional free bacteria are entrapped to form large bacterial aggregates. PS have been found to mediate the second attachment step i.e. an as yet unidentified calcofluor-binding surface PS in the case of Azospirillum and cellulose fibrils in the case of Agrobacterium and Rhizobium. The fact that the Cal- mutants of A. brasilense are not affected in primary root colonization, at least not under the conditions tested here, is comparable with the wild-type nodulation and virulence phenotypes of cellulose-minus Rhizobium leguminosarum biovar viciae (Smit et al., 1987) and Agrobacterium mutants (Matthysse, 1983), respectively. Whether the calcofluor-binding PS of Azospirillum becomes more important for root colonization under other environmental conditions as tested in the present study remains to be elucidated. Matthysse (1983) demonstrated a reduced virulence of Agrobacterium cellulose-minus mutants when the inoculation site was washed with water. Similarly, for rhizobacteria such as Azospirillum where the plant-growth-promoting effect is most likely exerted while colonizing the plant root surface, the presence of fibrillar PS material might become of crucial importance under such conditions.

The ability of Azospirillum to attain significant populations on the host root system has been shown to be a prerequisite for their beneficial effects on plant growth (Bashan, 1986b). Nevertheless, despite the importance of root colonization, little is known about how these bacteria become distributed along the plant roots when present in or applied to the soil (for a review see Van de Broek & Vanderleyden, 1995b). In the present study, we demonstrated for the first time and directly by using different motility mutants that chemotactic movement is important for the initiation of wheat root colonization at the root hair zone. It can be hypothesized that compounds are specifically secreted by the root hair zones of wheat roots to which Azospirillum is attracted. Azospirillum strains have previously been shown in vitro to be attracted towards root exudates and root mucilage (Heinrich & Hess, 1985; Mandimba et al., 1986). NM313 fails to exhibit positive chemotaxis towards various amino acids, sugars and organic acids. However, whether the attractant of interest specifically released from the root hair zone is a common metabolite or a more specific compound remains to be elucidated. In any case, as evidenced in the present study, the presence of fructose or malate appears not to have an effect on the specific migration of Azospirillum towards the root hair zone.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of IMPACT BIO2-CT93-0053 and GOA 93/97-04 Vanderleyden. A.V.B. and M.L. are recipients of a postdoctoral and predoctoral fellowship of the ‘Fonds voor Wetenschappelijk Onderzoek’, respectively.

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Received 18 February 1998; revised 29 April 1998; accepted 22 May 1998.