The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots

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*Azospirillum brasilense* in a motile Gram-negative bacterium that can adapt its flagellation to different environments. Cells growing in a liquid culture possess only a single polar flagellum; growth on a solid surface additionally induces multiple lateral flagella. The polar flagellum is primarily used for swimming, i.e. locomotion of the bacterium in a liquid environment, whereas the lateral flagella allow the bacteria to swarm over a solid surface. We have previously described a completely non-motile *A. brasilense* mutant (Sp7 p90D084), and shown that this mutant has a drastically reduced adsorption capacity to wheat roots. In the present work, we present several lines of evidence demonstrating that adsorption to wheat roots is mediated by the polar flagellum of *A. brasilense*. First, the non-adsorbing mutant Sp7 p90D084 forms no polar and no lateral flagella, but is otherwise undistinguishable from wild-type *A. brasilense*. Second, disintegration of the flagella by heat or acid eliminates adsorption. Third, using a polyclonal antiserum against the polar flagellum filament protein (Fla1), we have isolated out of a collection of 3000 Tn5-B30-induced mutants, three additional and genetically different non-flagellate mutants. Like Sp7 p90D084, these mutants show a severely reduced adsorption capacity to wheat roots. Finally, purified polar flagella bind to wheat roots in *vitro*.

Introduction

*Azospirillum brasilense* belongs to a group of soil bacteria often referred to as Plant Growth Promoting Rhizobacteria or PGPR, because they can stimulate plant growth either directly, or indirectly by suppression of plant pathogens. In view of their potential use as biofertilization or biocontrol agents, these bacteria have recently become the focus of intensive research efforts (Davison, 1988; Lugtenberg et al., 1991; Michiels et al., 1989). Regardless of their mode of action, PGPR depend on efficient plant root colonization to stimulate plant growth. Several bacterial properties are believed to contribute to this colonization capacity, such as chemotaxis towards root exudates, metabolism of root exudate components, suppression of competing micro-organisms, and, most importantly, the ability to bind to the plant root surface.

Attachment of *A. brasilense* to a large variety of plant roots, to suspended plant cells, and to inert surfaces has been reported (e.g. Umali-Garcia *et al.*, 1980; Jain & Patriquin, 1984; Murty & Ladha, 1987; Bashan & Levanony, 1988; Eyers *et al.*, 1988; Gafny *et al.*, 1986). However, only a few studies give information on the attachment mechanism and on the nature of the bacterial cell surface structures involved. A 100 kDa protein that agglutinates red blood cells was suggested to be involved in adsorption, because upon EDTA extraction of the protein from the bacteria, these lost their adsorption capacity (Madi & Henis, 1989). This protein was not further characterized however, and was not shown directly to bind to wheat roots.

In a previous study, we have shown that attachment of *A. brasilense* to wheat roots proceeds in two steps (Michiels *et al.*, 1991). First, bacteria adsorb rapidly and reversibly on the root surface. Several hours later, they become irreversibly anchored on the adsorption site. Anchoring is probably mediated by a bacterial polysaccharide that binds calcofluor, a dye specific for β-1,3 and β-1,4 linked polysaccharides, since mutants that did not react with the dye (Michiels *et al.*, 1990) were deficient in anchoring (Michiels *et al.*, 1991). The adsorption step, on the other hand, could be eliminated by protease treatment of the bacteria, suggesting the involvement of a cell-surface component of a protein nature (Bashan & Levanony, 1988; Michiels *et al.*, 1991). We have described an *A. brasilense* mutant that is...
completely non-motile (non-swimming and non-swarming) and defective in adsorption (Croes et al., 1991). This suggested a role for the *A. brasilense* polar and/or lateral flagella in wheat root adsorption. Two additional observations support the involvement of the polar flagellum. First, *A. brasilense* adsorbs to various surfaces in a polar fashion, and adsorbed bacteria can often be seen rotating around their longitudinal axis (Patriquin, 1981; Umali-Garcia et al., 1980; Patriquin et al., 1983; our laboratory, unpublished observations). Second, we found no difference in adsorption capacity between bacteria grown on a solid (having both a polar and lateral flagella) or in a liquid medium (having only the polar flagellum) (Tarrand et al., 1978), and have always used the latter in our adsorption experiments (Michiels et al., 1991). In the present work, we present several independent lines of evidence for the role of the *A. brasilense* polar flagellum as a wheat root adhesin.

**Methods**

**Bacterial strains and culture conditions.** The strains used in this study are listed in Table 1. They were grown at 30 °C on Luria-Bertani medium (LB) (Sambrook et al., 1989) supplemented with 2.5 mM-CaCl₂ and 2.5 mM-MgSO₄. Defined medium for growth tests of *Azospirillum brasilense* was AB minimal medium (MMAB) (Vanstockem et al., 1987). Antibiotics were added at the following concentrations: kanamycin, 25 μg ml⁻¹; tetracycline, 10 μg ml⁻¹.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of bacterial proteins. Bacterial outer membrane proteins were isolated by a modification of the method of Hurlbert & Gross (1983) as follows. Cells from a 250 ml overnight culture in supplemented LB were collected by centrifugation (6000 r.p.m., 20 min), and resuspended in 5 ml water per g of wet cells. RNAase [100 U (g cells)⁻¹] and DNAase (100 U (g cells)⁻¹) were added, the suspension was chilled on ice for 15 min, passed twice through a French Press (2 x 10⁶ p.s.i.; about 138 MPa) with preheated cylinder, and kept on ice. Residual cells were removed by centrifugation (12000 r.p.m., 10 min), and the supernatant was centrifuged (20000 r.p.m., 30 min, 4 °C). The pellets were resuspended in 0.5 % Sarkosyl, incubated for 30 min at 37 °C, and collected again by centrifugation (20000 r.p.m., 30 min, 4 °C). The membranes were then resuspended in phosphate-buffered saline (PBS) (Harlow & Lane, 1988), collected again (30000 r.p.m., 30 min, 5 °C), and finally resuspended in 5 ml PBS. Proteins were extracted from the outer membranes with phenol, and prepared for and subjected to 2-D PAGE as described previously (De Mot & Vanderleyden, 1989). Total bacterial protein preparations were made as described (De Mot & Vanderleyden, 1989). Electrophoretic separations were by isoelectric focusing in the first dimension, and SDS-PAGE in the second dimension. Gels were stained with Coomassie blue.

DNA hybridization. Total bacterial DNA was extracted, digested with the restriction endonucleases EcoRI and HindIII, electrophoresed, and blotted on Hybond N membranes (Amersham), using standard techniques (Sambrook et al., 1989). DNA from the Tn5-B30 delivery plasmid (Simon et al., 1989) was prepared by alkaline lysis (Sambrook et al., 1989), and labelled with digoxigenin-dUTP using a random primed labelling kit (Boehringer Mannheim). Hybridization was carried out at 65 °C (Sambrook et al., 1989), and signals were detected using a chemiluminescent detection kit (Boehringer Mannheim).

**Isolation of polar and lateral flagella.** We adopted the procedure described by De Pamphilis & Adler (1971) with some modifications. Portions (1 ml) of a fresh *A. brasilense* culture were spread on fifty 10 x 10-cm-square Petri dishes containing supplemented LB solidified with 1.5 % (w/v) agar. After 48 h incubation at 30 °C in a humid atmosphere, cells were collected in 5 ml 10 mM-Tris/HC1 (pH 8.0) per plate. The bacteria were pelleted by centrifugation (4000 r.p.m., 15 min) and resuspended in 100 ml of the same buffer. This suspension was mixed for 40 s at maximum speed in a Waring Blender to break the flagella. The bacteria and bacterial cell debris were removed from the mixed cell suspension by centrifugation for 15 min at 12000 r.p.m. From the supernatant liquid, flagella were pelleted by ultracentrifugation for 90 min at 22000 r.p.m, and resuspended in 1 ml of sterile water. This suspension was called the crude flagellar suspension. For further purification and separation of polar and lateral flagella, 134 g dried CaCl₂ was added and the volume of the suspension was adjusted to 30 ml with 10 mM-Tris/HC1 (pH 8.0). A density gradient was then established by spinning the samples for 60 h at 22000 r.p.m. in a Beckmann SW28 rotor. Two opaque bands were obtained and carefully removed from the gradient with a Pasteur pipette, dialysed against water, lyophilized, and resuspended in 500 μl water. The material from each band and the crude flagellar suspension were inspected by transillumination electron microscopy and subjected to SDS-PAGE. The crude flagellar suspension contained a mixture of thick and thin flagella, being the polar and lateral flagella respectively (Tarrand et al., 1978), that was composed of two major proteins of 100 kDa and 45 kDa. The opaque band with the highest density contained only thick flagella and yielded the 100 kDa protein upon SDS-PAGE. The other band corresponded to the lateral flagella and the 45 kDa protein. These results will be reported elsewhere. As judged by SDS-PAGE with Coomassie blue staining, the CaCl₂ purified flagella were > 95 % pure (see Fig. 4a).

**Preparation of flagella-specific antisera.** Rabbits were immunized with purified preparations of polar and lateral flagella (Harlow & Lane, 1988). Optimal working concentration and specificity of each antisera were determined by Western immunoblotting using crude flagellar extracts. The antisera against the polar flagellum (AS-Fla1), used at a 1:150000 dilution, reacted specifically with the 100 kDa polar flagellum flagellin (Fla1). AS-Fla1 did not react with flagellar or total protein extracts from the mutants lacking polar flagella. The antisera induced with the lateral flagella (AS-Fla2) was used at the same concentration, and reacted with the 45 kDa lateral flagella flagellin (Fla2), but also with two other components present in the extract which do not stain with Coomassie blue (probably lipopolysaccharides, see Results). However, since it did not cross-react with Fla1 (see Fig. 4), AS-Fla2 was considered to be useful for this study.

<table>
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<tr>
<th>Strain</th>
<th>Relevant properties*</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Sp7</td>
<td>Wild-type strain; ATCC 29145</td>
<td>Tarrand et al. (1978)</td>
</tr>
<tr>
<td>Sp7 p90D084</td>
<td>Sp7 derivative carrying ~ 13 kb deletion in 90 MDa plasmid; Ads Fla1*</td>
<td>Croes et al. (1991)</td>
</tr>
<tr>
<td>Sp7 XB7</td>
<td>Tn5-B30 mutant; Fla1*</td>
<td>This work</td>
</tr>
<tr>
<td>Sp7 XIIIIE8</td>
<td>Tn5-B30 mutant; Fla1*</td>
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* Ads, adsorption-deficient; Fla1*, lacking the polar flagellum filament protein.
Quantification of bacterial adsorption to wheat roots. The adsorption assay used in this work has been described in detail elsewhere (Michiels et al., 1991). Briefly, live excised wheat roots are shaken for 2 h at 30 °C in five separate 5 ml suspensions each containing about 10⁶ radio-labelled A. brasilense cells. The radioactivity remaining on each root after washing is expressed as a percentage of the total amount of radioactivity added to the root, and the mean of the five values is called the percentage adsorption.

Isolation of A. brasilense Tn5-B30 mutants defective in the production of the polar flagellum flagellin (Fla1). Random mutagenesis in A. brasilense Sp7 was performed with transposon Tn5-B30 (Simon et al., 1988) as described earlier for Tn5 (Van der Lee et al., 1987). Approximately 3000 mutants were grown overnight individually in PVC multiwell plates, and the optical density (OD₅₅₀) was recorded with an automatic reader. Plates were emptied, washed twice with PBS (Harlow & Lane, 1988), and incubated consecutively for 2-h periods at 37 °C with bovine serum albumin (BSA, 3% (w/v) in PBS), AS-Fla1 antiserum (1:50000 in PBS), and alkaline phosphatase-conjugated goat-anti-rabbit antiserum (1:3000, Bio-Rad). After each incubation, plates were washed twice with PBS. After the last wash, plates were washed three times with Tris-buffered saline (TBS) (Harlow & Lane, 1988), and twice with 10 mM-diethanolamine, 0.5 mM-MgCl₂, p-Nitrophenyl phosphate (0.1% in 10 mM-diethanolamine, 0.5 mM-MgCl₂) was added as a substrate for alkaline phosphatase. When the colour had developed well, reactions were stopped with 0.1 M-EDTA, and the OD₅₅₀ was measured with an automatic reader. For each mutant, this value was expressed relative to the culture cell density as OD₅₅₀/OD₅₅₀ (A) and compared with the values (mean of five replicates) similarly obtained for the wild-type Sp7 (B), and the non-flagellate mutant Sp7 p90D084 (C). When A – C < 0.3(B – C), the absence of a polar flagellum in these bacteria was further tested by three criteria: (1) the inability to swim was examined by phase contrast light microscopy of a fresh liquid culture; (2) the inability of bacteria from a fresh culture to be agglutinated by the AS-Fla1 antiserum (1:100) was also verified by phase contrast light microscopy; (3) direct visualization of flagella by transmission electron microscopy of bacteria, negatively stained with 2% (w/v) phosphotungstic acid (pH 7.0).

Wheat root-binding assay for flagella. Five equally sized wheat roots, obtained as described by Michiels et al. (1991) were gently shaken at 30 °C in 5 ml MPCL. (Lavigne, 1987) containing 10 μg purified polar flagella ml⁻¹ and the same concentration of purified lateral flagella. After 2 h incubation, roots were removed from the flagellar suspension, washed gently three times with water, and dried on absorbant paper. Root proteins were extracted in 2 ml extraction buffer as described by De Mot & Vanderleyden (1989). Two identical 8-25% (w/v) SDS gradient polyacrylamide gels were run with a 2 μl sample from each extract together with a sample obtained from untreated roots as a control, using the Phast System (Pharmacia). After electrophoresis, both gels were blotted onto Immobilon PVDF membranes (Millipore) with the Phast System electroblotting equipment. The presence of Fla1 and Fla2 on the blots was analysed by immunodetection with AS-Fla1 and AS-Fla2, respectively. A horseradish-peroxidase-conjugated goat anti-rabbit secondary antiserum was used in combination with 4-chloro-1-naphthol as a substrate to develop the blots (Harlow & Lane, 1988).

Results

The non-motile mutant Sp7 p90D084 lacks polar and lateral flagella

A. brasilense Sp7 p90D084, constructed by exchange of a 13 kb BamHI fragment from the p90 plasmid with a kanamycin resistance cartridge, is completely non-motile on solid and in liquid media, and is deficient in wheat root adsorption (Croes et al., 1991). Growth rates on different media and all other tested properties were as in the wild-type (Croes et al., 1991). We analysed the presence of flagella in this mutant by different methods. First, the presence of the polar flagellum was tested by adding AS-Fla1 antiserum to cells from a fresh broth culture or from a fresh plate. No agglutination could be observed by phase contrast light microscopy. AS-Fla2 was not used for this purpose because it was not sufficiently specific for the lateral flagella (see Methods). Second, the 100 kDa (Fla1) and 45 kDa (Fla2) flagellins from, respectively, the polar and the lateral flagella could not be detected by SDS-PAGE in a crude flagellar extract obtained from this mutant (data not shown). This extract further failed to produce opalescent bands upon CsCl gradient centrifugation. Finally, transmission electron microscopy of negatively stained mutant cells from a liquid culture and from a plate revealed the complete absence of flagella (Fig. 1).

2-D PAGE protein analysis of mutant Sp7 p90D084

Since A. brasilense adsorption to wheat roots involves a protease-sensitive component of the bacterial cell surface (Michiels et al., 1991), we compared the outer membrane proteins of wild-type strain Sp7 with those of the non-adsorbing mutant Sp7 p90D084 by 2-D PAGE (Fig. 2a). Both fingerprints were identical, and contained one major spot, corresponding to a 40 kDa protein with low pI value, and five other minor spots. Flagella were not extracted by this membrane protein isolation procedure. A comparison of the 2-D PAGE fingerprints of total proteins from both strains revealed that the mutant lacked an acidic protein with an approximate molecular mass of 100 kDa (circled spot in Fig. 2b). This protein was shown by immunoblot analysis to be the Fla1 flagellin (data not shown). The acidic protein (molecular mass, 28 kDa; marked by a square in Fig. 2b) appearing in the mutant was neomycin phosphotransferase (De Mot & Vanderleyden, 1989), expressed from the Tn5-derived kanamycin resistance cassette inserted in this mutant (Croes et al., 1991). Apart from these two proteins, there were no significant differences in the total protein patterns of both strains. The 45 kDa Fla2 flagellin was not present in the extracts because these were from cells grown in liquid cultures. In conclusion, we could not detect any defect other than the absence of flagella in mutant Sp7 p90D084. Next, we wanted to investigate whether this defect was responsible for the observed deficiency in adsorption of the mutant to wheat roots.
Disintegration of flagella by heat or acid eliminates adsorption

Bacterial flagellar filaments are supramolecular assemblies of thousands of flagellin subunits, that can be reversibly dissociated by heat or acid treatment (Joys, 1988). We subjected *A. brasilense* bacteria from a fresh liquid culture to a 5 min heat shock at different temperatures. After treatment, the bacteria were immediately centrifuged and resuspended in the original
After the final wash, they were resuspended in the pH 7.0
Motility and agglutination of the bacteria by AS-Fla1
root adsorption capacity at 65°C. The wheat roots was tested separately using a radiolabelled

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The initial screening for Fla1- mutants by ELISA yielded
and microscopy results, we concluded that these mutants produce no extracellular Fla1 protein. It is possible that
Fla1 is still produced intracellularly. In this location, however, it cannot contribute to wheat root adsorption of

Table 3 compares wheat root adsorption of the three newly isolated mutants, Sp7 p90D084, and the wild-type
strain Sp7. In all four Fla1- mutants, adsorption capacity was reduced to a background level, comparable to
Escherichia coli adsorption capacity (Michiels et al., 1991). These results strongly suggest that the presence of
the polar flagellum is required for wheat root adsorption. Since bacteria and roots were constantly agitated during
the adsorption assay, the chances of contact with the root surface for flagellated and non-flagellated cells were
the same. Thus, the role of motility was eliminated in our experiments, and our results show that the polar
flagellum was involved in adsorption in a direct way. This was further confirmed in a direct binding assay
between flagella and wheat roots.

Isolation of additional A. brasilense Fla1- mutants by immunosscreening

The initial screening for Fla1- mutants by ELISA yielded
30 mutants out of a collection of approximately 3000 isolates. Further evaluation by light and electron
microscopy reduced the number to 11 genuine Fla1- mutants lacking the polar flagellum. All the mutants had
normal growth rates in complex and in defined media with different carbon and nitrogen sources, and normal
 colony and cell morphology. On the basis of the ELISA

Table 2. Effect of heat and acid treatments on polar flagellum and wheat root adsorption of A. brasilense

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volume of fresh medium to prevent reassociation of the
flagella at room temperature. Removal of the polar
flagellum was evaluated microscopically by inspecting
motility and agglutination of the bacteria by AS-Fla1
antiserum. The effect of heat treatment on adsorption to
wheat roots was tested separately using a radiolabelled
bacterial culture. From the results, summarized in Table
2, it can be concluded that motility and agglutination
with AS-Fla1 are reduced at 55°C and are completely
lost at 65°C. Further, this apparent loss of the polar
flagellum coincides with a sixfold reduction of wheat
root adsorption capacity at 65°C.

A similar experiment was set up for the low pH
treatment. In this case, fresh bacteria were washed three
times in 50 mM-citrate/phosphate buffers at different pH.
After the final wash, they were resuspended in the pH 7.0
buffer and evaluated for motility and agglutination with
AS-Fla1, and for wheat root adsorption (radiolabelled
bacteria). The results indicated a loss of the polar
flagellum below pH 50 which is again concomitant with
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We performed a DNA hybridization using a Tn5-B30
specific probe on EcoRI- and HindIII-digested total
DNAs of these mutants. This allowed us to distinguish
three genetically different mutants, with Tn5-B30 inser-
tions in different EcoRI and HindIII restriction frag-
ments (Fig. 3). The size of the mutated EcoRI and
HindIII fragments for each mutant can be calculated
from the hybridization data. Since the smallest EcoRI
fragment is 10 kb (without Tn5-B30, which measures
6 kb), the positions of the three transposon insertions
must be separated by at least 10 kb. Hence, the insertions
must define more than one gene, and most likely also
more than a single gene cluster. Also, a comparison of
the present data with the restriction map of the A.
brasilense p90 plasmid, shows that none of the three new
mutations map on this plasmid, and thus that the new
mutants differ from the non-flagellate mutant Sp7
p90D084 described earlier (Croes et al., 1991).

Adsorption to wheat roots of A. brasilense Fla1-
mutants

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Binding of isolated polar flagella to wheat roots

A mixture of CsCl-purified polar and lateral A. brasilense
flagella was incubated with wheat roots, under the same
conditions as used in the bacterial adsorption assay.
After three gentle washes, a protein extract of the roots
was prepared, and the presence of Fla1 and Fla2
flagellins in this extract was analysed by SDS-PAGE and
Western immunoblotting with flagellin-specific antisera.
The immunoblot developed with AS-Fla1 showed no
Fig. 3. Autoradiogram of hybridization on blots of restricted total DNAs from *A. brasilense* Tn5-B30 Fla1 Ка mutants with a Tn5-B30-
specific probe. Estimated sizes of the fragments are indicated in kb. (a) *EcoR*I-digested total DNA. Since *EcoR*I does not cleave Tn5-B30,
single hybridization signals are found. (b) *HindIII*-digested total DNA.
Since *HindIII* cleaves Tn5-B30 once, two hybridization signals are
found. For mutant XVIII(B, both bands are the same size.

Table 3. Adsorption to wheat roots of *A. brasilense* Sp7
and Fla1 mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage adsorption ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp7</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>Sp7 p90D064</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>Sp7 XB7</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>Sp7 XVII(B</td>
<td>0.29 ± 0.12</td>
</tr>
<tr>
<td>Sp7 XVIII(B</td>
<td>0.28 ± 0.13</td>
</tr>
</tbody>
</table>

signal for the control extracts from wheat roots that had not been incubated with flagella, and an intense band
corresponding to Fla1 for the treated roots (Fig. 4b). An
identical immunoblot developed with AS-Fla2 showed a
faint background signal for the untreated roots and a
similar signal for the treated roots (Fig. 4c).

As already pointed out in Methods, AS-Fla2 is less
specific than AS-Fla1. It reacts rather poorly with Fla2,
and strongly with two components in the flagellar
preparation that do not stain with Coomassie blue.
Therefore, the presence of only a background signal on
the immunoblots of proteins from treated roots de-
veloped with AS-Fla2, demonstrates that the roots have
been sufficiently washed to remove any non-binding
material. Thus, it can be concluded that, under the
conditions of this experiment, the polar flagella bind to
wheat roots. It also seems that Fla2 did not bind to the
same degree as Fla1. However, small amounts of Fla2
may have been missed due to the lower sensitivity of Fla2
detection.

Wheat root adsorption is not inhibited by anti-Fla1
antiserum or by excess polar flagella

If the polar flagellum is responsible for adsorption to
wheat roots, one would expect adsorption to be inhibited
by excess polar flagella and eventually by AS-Fla1. We
observed only a slight and statistically non-significant
decrease of adsorption in the presence of high concen-
trations (1:10 dilution) of AS-Fla1 (data not shown),
suggesting that the antibodies do not bind to the domains
of Fla1 involved in wheat root binding. Further, the
addition of excess polar flagella (200 µg ml⁻¹) did not
affect adsorption. This result seems to be in conflict with
the root-adhesive properties of the *A. brasilense* flagella,
but can be explained by taking into account the
particular properties of bacterial flagella (see Dis-

discussion).

Discussion

Earlier studies have shown that the rapid and reversible
adsorption of *A. brasilense* to wheat roots depends on a
bacterial protease-sensitive cell surface component
(Michiels *et al.*, 1991). In the present work, we have
identified this wheat root adhesin as a component of the
bacterial polar flagellar filament. Three independent pieces of evidence are presented.

First, we used heat and acid treatments to dissociate the flagellar filaments of A. brasilense cells. Loss of the filaments was monitored by the loss of swimming motility, and by the inability to be agglutinated by AS-Fla1, a polyclonal antiserum against the polar flagella. It was found that the same conditions that dissociated the polar filament also eliminated the ability to adsorb to wheat roots. Evidently, since physico-chemical methods like heat and acid treatment are likely to affect several outer membrane constituents, these results do not rule out the possible involvement of components other than the flagella in adsorption. The involvement of the lateral flagella can be excluded in our adsorption assay, since we always used bacteria grown in liquid growth medium, and these do not have lateral flagella (Tarrand et al., 1978). Heat treatment was also shown to eliminate adsorption of A. brasilense to suspended plant cells (Eyers et al., 1988).

Second, in order to confirm the possible involvement of the polar flagellum in wheat root adsorption, we tested four different mutants lacking the polar flagellar filament protein Fla1. One of these is a plasmid deletion mutant, previously described as adsorption deficient and non-motile (Croes et al., 1991), and which is shown here to be Fla1⁻. The three others were isolated in this study by immunoscreening with AS-Fla1, and they were all shown to be adsorption deficient. Since the adsorption experiments were carried out under well-agitated conditions, the role of bacterial motility can be discounted, and the adsorption deficiency of the non-flagellated mutants probably reflects the loss of a bacterial root adhesin. The lack of flagella is not due to a severe pleiotropic surface defect, since all the mutants have normal growth rates on different media, and normal colony and cell morphology. Because these four mutants carry mutations that are not closely linked, it is very unlikely that they would all share a common defect in one of the outer membrane proteins, in addition to the absence of the polar flagellum. Therefore, these results provide strong indirect evidence that the polar flagellum functions as a wheat root adhesin.

Finally, we showed that, under the same conditions as used to measure adsorption of whole bacteria, Fla1 bound effectively to wheat roots. This confirmed our assumption that the adsorption deficiency of the Fla1⁻ mutants is not caused by the lack of motility but by the lack of the flagellar adhesin.

At first glance, the finding that wheat root adsorption is not reduced by the addition of excess polar flagella seems contradictory to the proposed adhesin function of this flagellum. However, bacterial flagella possess the particular capacity to disintegrate into monomers and to reassemble, and there was no control on this process in our experimental set-up. Possibly, the free flagellar fragments adsorbed on the roots reassemble with flagella attached to the bacteria.

The best studied example of bacterial adhesion is probably the colonization of human or animal host cells by pathogenic Escherichia coli. The adhesins in this bacterium are minor constituents of heteropolymeric fibres pointing outward from the cells, called pili (Lindberg et al., 1986). Usually, the adhesins are located at the tip of the pili, a construction that warrants the optimal access to the receptor molecules on the host cell surface (Lindberg et al., 1987; Kuehn et al., 1992). A similar attachment strategy may be very widespread among Gram-negative bacteria, since the involvement of pili or fimbriae has been shown in many diverse interactions, such as the attachment of Seratia marcescens to the urinary tract (Mizunoe et al., 1991), or the adhesion of the periodontal pathogen Porphyromonas gingivalis to Actinomyces viscosus (Goulbourne & Ellen, 1991). Fimbriae have also been implicated in attachment of bacteria to plants, as in nitrogen-fixing Klebsiella spp. (Korhonen et al., 1983) and Enterobacter spp. (Hahtela et al., 1985) associating with grass roots, in Bradyrhizobium japonicum attaching to soybean roots (Vesper & Bauer, 1986), or in Pseudomonas syringae pv. phaseolicola, attaching to bean leaf stomata (Romanschuk & Bamford, 1986).

Bacterial flagella, in contrast to pili or fimbriae, are cell appendages that can rotate, and that serve primarily for locomotion. In terms of access to the target surface, one would predict that the location of a bacterial adhesin on the flagella or on the pili or fimbriae offers the same advantage. However, bacterial flagella have rarely been implicated in attachment, and often their only role is to increase the number of encounters between the bacterium and the surface by providing motility to the bacterium (Stanley, 1983; De Weger et al., 1987; Piette & Idziak, 1991). In these cases, the higher adsorption capacity of flagellated cells was lost when the experiment was conducted with vigorous agitation (Stanley, 1983; Lillard, 1985). Therefore, surface-adhesive properties should not be ascribed to flagella solely on the basis of a reduced adsorption of deflagellated bacteria or non-flagellate bacterial mutants, when no well agitated conditions were used. This was the case for instance in adhesion studies of Pseudomonas fluorescens (De Flau et al., 1990), and of Vibrio parahaemolyticus (Belas & Colwell, 1982). The polar flagella of P. fluorescens were implicated in the adsorption of this bacterium to a soil amoeba on the basis of light and electron microscopical observations (Preston & King, 1984). However, non-flagellated bacteria were not used in this study. To the best of our knowledge, A. brasilense represents the first
case of a flagellar adhesin shown to be involved in a bacterium–plant interaction (Smit & Stacey, 1990).

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