Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots

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Two *in vitro* assays are described that can distinguish between two modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots, and quantify the number of attached bacteria. The first assay measures *A. brasilense* adsorption (Ads), which reaches a maximal level within 2 h of incubation. Adsorption forces are weak, since adsorbed bacteria can be quantitatively removed by vortexing the root in water. In addition, adsorption is strongly reduced by pretreatment of *A. brasilense* cells with pronase E. The second assay measures *A. brasilense* anchoring (Anc), which begins only after 8 h of incubation and reaches a maximal level after 16 h. That adsorption and anchoring are different phenomena is further demonstrated by the properties of two classes of *A. brasilense* attachment mutants. The first class of mutants, deficient in the production of a particular surface polysaccharide, has completely lost anchoring capability, but maintains wild-type adsorption capacity (Ads⁺ Anc⁻). Mutants of the second class are defective in adsorption, but not in anchoring (Ads⁻ Anc⁺). On the basis of these data, a two-step attachment mechanism of *A. brasilense* to wheat roots is proposed. The first step consists of a rapid and weak adsorption and depends on bacterial surface protein; the second step is, at least *in vitro*, independent of the first and consists of firm anchoring of adsorbed and free bacteria by means of bacterial extracellular polysaccharide.

Introduction

Bacteria of the genus *Azospirillum* are known to colonize the rhizosphere of plants and to promote plant growth under certain conditions. They show only moderate host specificity in colonization, as is reflected by their isolation from a wide variety of plants all over the world. The capacity of azospirilla to colonize the rhizosphere, although not fully understood, is presumed to depend on several bacterial properties, like chemotaxis towards root exudates, a versatile metabolism including nitrogen fixation, antagonism towards competing micro-organisms, cyst formation allowing survival under adverse conditions, and, most importantly, the ability to bind to plant roots and soil particles (see reviews by Elmerich, 1984; Okon, 1985; Döbereiner & Pedrosa, 1987; Michiels *et al.*, 1989).

The mechanism of attachment to plant tissue has been well described in a number of other bacteria that interact with plants. For instance, in *Klebsiella pneumoniae* (Korhonen *et al.*, 1983), *Pseudomonas fluorescens* (Vesper, 1987) and *Bradyrhizobium japonicum* (Vesper & Bauer, 1986), attachment to grass, corn and soybean roots, respectively, could be attributed to the presence of

bacterial pili. A different *P. fluorescens* strain adsorbed to sand by means of a 34 kDa major outer-membrane protein, identified as flagellin (Deflaun *et al.*, 1990). Attachment of the symbiotic *Rhizobium* bacteria to their specific legume host plant is thought, at least in some cases, to be mediated by a root lectin that recognizes a specific receptor on the bacterial cell surface. Evidence for this lectin hypothesis was recently presented by Diaz *et al.* (1989), who made white clover susceptible to nodulation by *Rhizobium leguminosarum* bv. *viciae*, which normally nodulates pea, by expressing the pea lectin gene in white clover. Several bacterial cell-surface components have been implicated in the binding of the crown gall pathogen *Agrobacterium tumefaciens* to plant tissue: lipopolysaccharide, which in purified form inhibits binding of *A. tumefaciens* cells to wounded bean leaves (Whatley *et al.*, 1976); β -1,2-glucan, which is absent or not correctly transported to the periplasmic space in a number of non-attaching mutants (Puvanesarajah *et al.*, 1985; Cangelosi *et al.*, 1987, 1989); two outer-membrane proteins not related to flagella or pili (Matthysse, 1987); and cellulose fibrils, which are synthesized by *A. tumefaciens* after the initial adsorption, and which anchor the bacteria to the plant tissue (Matthysse *et al.*, 1981; Matthysse, 1983).

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Several studies have described attachment of *Azospirillum brasilense* to both plant roots and inert surfaces, but the mechanisms remain the subject of conflicting hypotheses. *A. brasilense* was shown to bind to grass (Umali-Garcia *et al.*, 1980), maize (Gafny *et al.*, 1986), wheat (Jain & Patriquin, 1984) and rice roots (Murty & Ladha, 1987), and to suspended *Zinnia* cells (Eyers *et al.*, 1988). *Azospirillum* cells often bound in higher numbers than other rhizosphere bacteria (Umali-Garcia *et al.*, 1980; Gafny *et al.*, 1986). Umali-Garcia *et al.* (1980) and Gafny *et al.* (1986) reported that attachment of *A. brasilense* to pearl millet and maize roots, respectively, was stimulated by lectin-like substances present in the root exudate. Involvement of a root lectin was also suggested by Del Gallo *et al.* (1989), who showed *in vitro* binding of wheat germ agglutinin by *A. brasilense* and *Azospirillum lipoferum* cells. Madi & Henis (1989), in contrast, isolated an *A. brasilense* cell-surface agglutinin that was necessary for adsorption of the bacteria to wheat roots. Bashan & Levanony (1988) reported that binding of *A. brasilense* to sand and to wheat roots could be eliminated by protease treatment of the bacteria, which supports the involvement of bacterial surface protein. They also studied the effect of cellulase on adsorption of *A. brasilense* to wheat roots. Treatment of the bacteria decreased adsorption moderately, whereas treatment of the wheat roots caused a significant increase in adsorption (Bashan & Levanony, 1989).

In the present paper, two classes of *A. brasilense* attachment mutants are distinguished using two different *in vitro* binding assays to wheat roots. This provides a genetic basis to postulate the involvement of adsorption and anchoring phenomena during attachment of *A. brasilense* to wheat roots.

Methods

Bacterial strains and culture conditions. The bacterial strains used are listed in Table 1. *Escherichia coli* and *Azospirillum brasilense* were grown at 37 °C and 30 °C, respectively. All strains were kept and restreaked every three weeks on fresh plates of Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) supplemented with 2.5 mM-CaCl₂ and 2.5 mM-MgSO₄. For adsorption and anchoring experiments, a single colony from a fresh stock plate was inoculated in 5 ml supplemented LB in a test tube and 20 µCi d[1',2',5'-³H]CTP (57 Ci mmol⁻¹, 2.11 TBq mmol⁻¹; Amersham) was added. Cultures were grown aerobically in a rotary shaker to an optical density (OD₆₀₀) of 1.0, corresponding to 3 × 10⁸ bacteria ml⁻¹, as determined by plating. When appropriate, 25 µg kanamycin sulphate ml⁻¹ was added to the plates or to the liquid cultures.

Cultivation of wheat seedlings and preparation of roots. Seeds of *Triticum aestivum* cv. Fidel (SES, Belgium) were surface-sterilized by immersion in 70% (v/v) ethanol for 3 min, washing three times in sterile distilled water, soaking three times for 30 min in a solution of sixfold diluted commercial bleach containing 0.5% sodium dodecyl sulphate, rinsing with sterile water for 60 min, soaking in fresh bleach/sodium dodecyl sulphate solution of the same composition for 60 min, and finally rinsing five times with sterile distilled water. Sterilized seeds were germinated on Nutrient Agar plates (Difco) for 3 d at 23 °C in the dark. Sterile seedlings were individually and aseptically transferred to cotton-plugged 200 × 22 mm glass tubes containing 30 g sterilized sand (0.4–0.8 mm). Per tube, 4 ml of MPCL nutrient solution (Lavigne, 1987) was added, and tubes were placed in a growth chamber with a 16 h day/8 h night cycle, a constant temperature of 23 °C, and a relative humidity of 80%. From 2–3-week-old seedlings with approximately equally-sized root systems, roots were cut, washed free of sand with sterile distilled water, and placed individually in sterile test tubes containing 4.5 ml MPCL medium.

Bacterial adsorption to wheat roots. Radioactively labelled bacteria grown as described above were collected by centrifugation, washed twice in sterile 0.85% NaCl to remove unincorporated label, and resuspended to the original volume in MPCL medium. The specific activity obtained in this way was in the range of 10⁻²–10⁻³ c.p.m. per bacterium. Portions (0.5 ml) of labelled and washed bacterial cells were

Table 1. *Bacterial strains*

Strain	Relevant properties*	Source or reference
<i>A. brasilense</i>		
Sp7	Wild-type strain (ATCC 29145); isolated from <i>Digitaria decumbens</i> ; Cal ⁺	Tarrand <i>et al.</i> (1978)
Sp7 p90Δ084	Sp7 derivative carrying approx. 13 kb deletion in 90 MDa plasmid; Cal ⁺ ; Km ^r	C. L. Croes and others, unpublished
7030	Nitrosoguanidine-induced derivative of Sp7; Cal ⁺ ; Sm ^r	C. Elmerich (Paris, France)
7030TN5-11	Tn5-induced 7030 mutant; Cal ^d ; Km ^r	Michiels <i>et al.</i> (1990)
7030TN5-12	Tn5-induced 7030 mutant; Cal ^d ; Km ^r	
7030TN5-21	Tn5-induced 7030 mutant; Cal ⁻ ; Km ^r	
7030TN5-22	Tn5-induced 7030 mutant; Cal ⁻ ; Km ^r	
7030TN5-23	Tn5-induced 7030 mutant; Cal ⁻ ; Km ^r	

* Cal refers to colony fluorescence on plates containing 200 µg Calcofluor ml⁻¹: Cal⁺, wild-type fluorescence; Cal⁻, no fluorescence; Cal^d, dimmed fluorescence. Sm^r, streptomycin resistant; Km^r, kanamycin resistant.

added to six test tubes containing a wheat root 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 distilled water for 1 min without agitation. The amount of radioactivity associated with the root was measured in a liquid scintillation counter, and for each root the percentage of the total added radioactivity (in 0.5 ml washed bacterial suspension) remaining on the root was calculated. Bacterial adsorption values in Tables 2 and 3 are the means of these percentages from three independent experiments with sixfold replications for each condition or each strain. Since the initial inoculum contained 3×10^8 bacteria ml^{-1} , 1% adsorption corresponds to 1.5×10^6 bacteria per root.

Bacterial anchoring to wheat roots. The same procedure and experimental design as for adsorption was used, except that NMF was used instead of MPCL and the incubation time was 16 h instead of 2 h. NMF is a nitrogen-free minimal salts medium containing 0.5% fructose, and induces high exopolysaccharide production (Michiels *et al.*, 1990).

Results and Discussion

Adsorption of *A. brasilense* Sp7 to wheat roots

Fig. 1(a) compares the adsorption kinetics of *A. brasilense* Sp7 and *E. coli* HB101. Adsorption of the *A. brasilense* strain starts immediately (15 min or less) after addition of the bacteria to the root, and after 1.5 h reaches a level of 2.6% of the initial inoculum, which corresponds to 3.9×10^6 bacteria per root. No substantial further increase is seen for a period up to 20 h. Adsorption of *E. coli* remains at least 20 times lower during a 20 h incubation time.

The strength of adsorption of *A. brasilense* to wheat roots was tested by comparing the efficiency in removing adsorbed bacteria of three wash procedures of increasing stringency. Vortexing three times for 5 s in water or in 2.5 mM-EDTA reduced adsorption about 25-fold (Table 2).

Next, we treated labelled *A. brasilense* cells with pronase E (2 mg ml^{-1} ; Sigma) for 2 h at 37 °C, removed pronase E by two washes in MPCL, and used the cells in our adhesion assay. Adhesion was reduced 20-fold, to a level comparable to *E. coli* HB101 adsorption. Viability of the cells was not affected by the pronase E treatment, as was shown by plating equal numbers of treated and untreated cells.

In conclusion, *A. brasilense* adheres to wheat roots in significantly higher numbers than *E. coli*, and adhesion is rapid, weak, and depends on bacterial surface protein.

Anchoring of *A. brasilense* Sp7 to wheat roots

In the anchoring assay, MPCL is replaced by NMF as incubation medium. It was previously shown that a high C/N ratio stimulates flocculation of *A. brasilense* in liquid culture, due to the production of a Calcofluor-binding fibrillar polysaccharide (Sadasivan & Neyra, 1985; Michiels *et al.*, 1990). With the present assay, we wanted

Fig. 1. Kinetics of adsorption (a) and anchoring (b) of *A. brasilense* Sp7 (●) and *E. coli* HB101 (▲) to wheat roots. The total number of bacteria added to the root (1.5×10^8) corresponds to 100%. Each point on the curves was determined as the mean of 12 values obtained from two independent experiments with sixfold replications.

to test whether this polysaccharide would promote binding of *A. brasilense* to wheat roots. The anchoring kinetics in Fig. 1(b) suggest that this is indeed the case. Two binding phases can be clearly distinguished: a rapid adsorption in the first 2 h of incubation, and an additional binding phase initiating after 8 h of incubation and reaching a constant level of 11.1% of the initial inoculum after 16 h, corresponding to 1.7×10^7 bacteria per root. Binding of *E. coli* HB101, by comparison, is not biphasic and is about 70 times lower after 16 h of incubation. Bacteria anchored to wheat roots in this assay could not be released by vortexing in water or in 2.5 mM-EDTA (Table 2).

It can be concluded that, in the conditions of the anchoring assay, *A. brasilense* binds to wheat roots in two clearly separated phases. The first phase is a rapid and weak adsorption, as described above. The second phase is the firm anchoring of adsorbed and not yet adsorbed bacteria. Anchoring begins only 8 h after transfer of the bacteria to the NMF incubation medium. This suggests that anchoring depends on the synthesis of bacterial extracellular polysaccharides, which is rapidly induced in NMF, but not in MPCL medium.

Table 2. Adsorption and anchoring of *A. brasilense* Sp7 to wheat roots as a function of washing stringency

Adsorption and anchoring are expressed as a percentage of the total number of bacteria incubated with the root (i.e. 1.5×10^8). Each value is the mean $\pm$ standard deviation of 18 values obtained from three independent experiments with sixfold replications for each wash procedure.

Binding mode	Wash procedure:			
	No wash; blot dry with absorbent paper	Immerse in H ₂ O 3 $\times$ 1 min	Vortex in H ₂ O 3 $\times$ 5 s	Vortex in 2.5 mM EDTA 3 $\times$ 5 s
Adsorption	3.7 $\pm$ 0.9	2.6 $\pm$ 0.8	0.11 $\pm$ 0.03	0.12 $\pm$ 0.04
Anchoring	12.6 $\pm$ 2.3	11.9 $\pm$ 2.1	11.1 $\pm$ 2.5	9.5 $\pm$ 1.7

Table 3. Adsorption and anchoring of *E. coli* and *A. brasilense* strains to wheat roots

Strain	Cal phenotype*	Adsorption†	Anchoring†	Attachment phenotype
<i>A. brasilense</i>				
Sp7	Cal ⁺	2.6 $\pm$ 0.8	11.1 $\pm$ 2.5	Ads ⁺ Anc ⁺
Sp7 p90Δ084	Cal ⁺	0.10 $\pm$ 0.05	9.9 $\pm$ 1.9	Ads ⁻ Anc ⁺
7030	Cal ⁺	3.5 $\pm$ 0.9	12.4 $\pm$ 2.4	Ads ⁺ Anc ⁺
7030TN5-11	Cal ^d	2.0 $\pm$ 0.5	2.1 $\pm$ 0.9	Ads ⁺ Anc [±]
7030TN5-12	Cal ^d	1.6 $\pm$ 0.3	1.4 $\pm$ 0.3	Ads ⁺ Anc [±]
7030TN5-21	Cal ⁻	4.2 $\pm$ 1.2	0.5 $\pm$ 0.2	Ads ⁺ Anc ⁻
7030TN5-22	Cal ⁻	4.5 $\pm$ 1.3	0.6 $\pm$ 0.2	Ads ⁺ Anc ⁻
7030TN5-23	Cal ⁻	4.0 $\pm$ 0.7	0.8 $\pm$ 0.3	Ads ⁺ Anc ⁻
<i>E. coli</i>				
HB101	Cal ⁻	0.11 $\pm$ 0.04	0.16 $\pm$ 0.09	Ads ⁻ Anc ⁻

* Cal refers to colony fluorescence on plates containing 200 µg Calcofluor ml⁻¹: Cal⁺, wild-type fluorescence; Cal⁻, no fluorescence; Cal^d, dimmed fluorescence.

† Adsorption and anchoring are expressed as a percentage of the total number of bacteria incubated with the root (i.e. 1.5×10^8). Each value is the mean $\pm$ standard deviation of 18 values obtained from three independent experiments with sixfold replications for each strain.

An A. brasilense mutant defective in adsorption, not in anchoring

As a part of our investigation on the functions encoded by the *A. brasilense* 90 MDa plasmid, we have constructed mutants carrying specific deletions in this plasmid (C. L. Croes and others, unpublished). In one of these mutants, Sp7 p90Δ084, adsorption to wheat roots was reduced 20-fold to a level comparable to *E. coli* HB101 (Ads⁻) (Table 3). Since this mutant still produces the Calcofluor-binding polysaccharide, and still flocculates in NMF medium (C. L. Croes and others, unpublished), we expected it to have wild-type anchoring capability (Anc⁺). This prediction was confirmed (Table 3). The existence of an Ads⁻ Anc⁺ mutant shows that anchoring of *A. brasilense* to wheat roots does not require high numbers of adsorbed bacteria.

A. brasilense mutants defective in anchoring, not in adsorption

Previously, we reported the isolation and characterization of *A. brasilense* Tn5 insertion mutants that are affected in the production of the Calcofluor-binding polysaccharide. These mutants were classified according to the level of fluorescence of the bacterial colonies on agar plates containing Calcofluor: Cal⁺ indicated wild-type fluorescence; Cal^d, dimmed fluorescence; Cal⁻, no fluorescence. We also showed a direct correlation between the production of this Calcofluor-binding polysaccharide and the flocculation ability of the mutants, since Cal⁻ mutants did not flocculate at all, and Cal^d mutants showed reduced flocculation compared to the Cal⁺ parent strain 7030 (Michiels *et al.*, 1990).

These mutants allowed us to test the hypothesis that

anchoring of *A. brasilense* to wheat roots would be mediated by this Calcofluor-binding polysaccharide. Indeed, it was found that the Cal^d and Cal⁻ mutants had at least 6-fold and 15-fold reduced anchoring capacity, respectively, compared to strain 7030, from which they were derived (Table 3). In addition, adsorption seems not to be significantly affected by the Cal mutations (Table 3). Thus the attachment phenotype of the Cal⁻ mutants is Ads⁺ Anc⁻.

Conclusions

We have provided evidence for the existence of two distinct modes of attachment of *A. brasilense* Sp7 to wheat roots. First, the binding kinetics in Fig. 1(b) clearly shown a biphasic pattern. Apart from the greater lag time, the second binding phase differs from the first in that binding is stronger than during the first phase. Also, first-phase binding can be eliminated by protease treatment of the bacteria prior to the assay. This suggests the involvement of bacterial surface components of a protein nature. For these reasons, we adopted the terminology adsorption (Ads) for first-phase binding, and anchoring (Anc) for second-phase binding. As opposed to adsorption, anchoring was shown to rely on a Calcofluor-binding surface polysaccharide, since mutants defective in the production of this polysaccharide are Anc⁻.

Adsorption and anchoring are two completely independent processes. This is already suggested by their dependence on two different bacterial surface components, but more rigorous evidence comes from the existence of both Ads⁻ Anc⁺ and Ads⁺ Anc⁻ mutants. These are the first *Azospirillum* attachment mutants reported. Both types of mutants also differ genetically, since the Cal⁻ mutants (Ads⁺ Anc⁻) are all chromosomal mutants (Michiels *et al.*, 1990), whereas Sp7 p90Δ084 (Ads⁻ Anc⁺) carries a deletion in the 90 MDa plasmid (C. L. Croes and others, unpublished).

In *Agrobacterium tumefaciens*, a similar two-step attachment process has been described (Matthysse *et al.*, 1981). In this plant pathogen, mutants defective in adsorption were all avirulent (Douglas *et al.*, 1982; Thomashow *et al.*, 1987; Matthysse, 1987), whereas anchoring mutants showed reduced virulence only when the plant inoculation site was washed with water (Matthysse, 1983). Thus, in natural conditions, anchoring can prevent *A. tumefaciens* from being washed from a wound site by rain. *In vivo* adsorption of *Azospirillum brasilense* to roots has been shown repeatedly (Umali-Garcia *et al.*, 1980; Bashan *et al.*, 1986; Gafny *et al.*, 1986), but it is not clear whether this bacterium can also anchor under natural conditions like *Agrobacterium tumefaciens*. Scanning electron micrographs of roots

inoculated with *Azospirillum* have revealed bacteria embedded on the root surface in a fibrillar matrix, but it has not yet been shown whether this matrix consists of bacterial polysaccharide or of root mucigel (Umali-Garcia *et al.*, 1980; our laboratory, unpublished). Nevertheless, it seems evident that the ability of *A. brasilense* to rapidly adsorb and firmly anchor in high numbers to plant roots, and possibly also to soil particles, contributes to its competitiveness in root colonization. The availability of the above-described mutants for various *in vivo* inoculation experiments will allow this hypothesis to be tested.

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