RESEARCH ARTICLE

The rhizobial adhesion protein RapA1 is involved in adsorption of rhizobia to plant roots but not in nodulation

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Abstract

The effect of the rhizobium adhesion protein RapA1 on *Rhizobium leguminosarum* bv. *trifolii* adsorption to *Trifolium pratense* (red clover) roots was investigated. We altered RapA1 production by cloning its encoding gene under the p*lac* promoter into the stable vector pHC60. After introducing this plasmid in *R. leguminosarum* bv. *trifolii*, three to four times more RapA1 was produced, and two to five times higher adsorption to red clover roots was obtained, as compared with results for the empty vector. Enhanced adsorption was also observed on soybean and alfalfa roots, not related to *R. leguminosarum* cross inoculation groups. Although the presence of 1 mM Ca^{2+} during rhizobial growth enhanced adsorption, it was unrelated to RapA1 level. Similar effects were obtained when the same plasmid was introduced in *Rhizobium etli* for its adsorption to bean roots. Although root colonization by the RapA1-overproducing strain was also higher, nodulation was not enhanced. In addition, *in vitro* biofilm formation was similar to the wild-type both on polar and on hydrophobic surfaces. These results suggest that RapA1 receptors are present in root but not on inert surfaces, and that the function of this protein is related to rhizosphere colonization.

Introduction

The formation of N_2-fixing nodules on legume roots by the action of rhizobia is a dynamic and complex process that consists of several related steps. Among the earliest of such steps is rhizobial adsorption to roots, which precedes a characteristic root hair deformation called root hair curling, and root hair penetration through infection threads (Gage, 2004). It has been suggested that rhizobia attached to root hair tips are able to respond to flavonoids released by the plants, inducing their *nod* genes for the synthesis and release of lipochitooligosaccharides (Brencic & Winans, 2005). Localized actions of lipochitooligosaccharides redirect root hair growth and create the multifaceted root hair pocket for rhizobia at the curled root hair, which is required for initiation of infection threads (Esseling *et al.*, 2004). In addition to their attachment to root hair tips, rhizobia are able to attach to other plant surfaces, including epidermal cells of symbiotic and nonsymbiotic plant species (Smit *et al.*, 1992; Reddy *et al.*, 1997; Fujishige *et al.*, 2006), nonplant cells including those of bacteria, fungi and animals (Ausmees *et al.*, 2001; Seneviratne & Jayasinghearachchi, 2003; Horiuchi *et al.*, 2005), and inert surfaces such as soil particles or glass (Fujishige *et al.*, 2006; Russo *et al.*, 2006). Thus, in addition to being required for the multifaceted root hair pocket, rhizobial adsorption to surfaces other than root hair tips could fulfil diverse requirements for rhizosphere colonization or establishment as biofilms in soil (Ramey *et al.*, 2004).

The ability of rhizobia to adhere to plant and soil surfaces enables them to colonize these niches in competition with multiple microbial species. The rhizosphere is a particularly rich and complex niche (Watt *et al.*, 2006). It is continuously modified and shaped by plant root exudates (Walker *et al.*, 2003), and diverse plant–microorganism and microorganism–microorganism interactions take place there (Barea *et al.*, 2005). As the susceptibility of root hairs to infection is transient (Bhuvaneswari *et al.*, 1980), and rhizobial cells normally constitute 0.001–0.1% of all soil bacterial biomass, rhizobial adsorption to legume root surfaces must be highly

accurate for the organisms to gain access to the site of infection at the right time and place in competition with other soil microbiota. Hence, different modes of rhizobial adsorption to root surfaces coexist, as demonstrated by experiments in which adsorption of an antibiotic-resistant 'indicator' strain was completely inhibited by competition against an excess concentration of sensitive strains from the same species but not from different species (Caetano-Anollés & Favelukes, 1986b; Lodeiro *et al.,* 1995; Lodeiro & Favelukes, 1999).

Much work has been done to characterize rhizobial adsorption to roots, but few specific molecules have been recognized as adhesins having such a role. Most of these adhesins are agglutinins of plant or bacterial origin, and are considered to act by binding to more or less specific ligands either on the surface of cells of their partner or on cells of their own species (Ho *et al.*, 1990; Wisniewski *et al.,* 1994; Dazzo *et al.,* 1984). Given the complex nature of this rhizobial adsorption, certain adhesins could mediate a given class of adsorption, for instance to root surfaces, but not a different class, for instance to inert surfaces.

Among those rhizobial adhesins already characterized, a series of rhizobial adhesion proteins (Rap) were isolated from *Rhizobium leguminosarum* bv. *trifolii* using the phage display technology (Ausmees *et al.*, 2001). These proteins have a domain in common, the rap domain, and are known as RapA, RapB and RapC. RapA has two rap domains, and two isoforms of this protein, termed RapA1 and RapA2, are expressed in *R. leguminosarum* bv. *trifolii*. The RapA1 protein is an extracellular, Ca^{2+}-binding protein that recognizes a polysaccharide from the bacterial surface, and promotes rhizobial autoaggregation through cell poles (Ausmees *et al.*, 2001). Sera screening of diverse rhizobial species with an anti-RapA1 antibody gave cross-reaction only with the closely related *R. leguminosarum* bv. *viceae*, *R. leguminosarum* bv. *phaseoli* and *Rhizobium etli* (Ausmees *et al.*, 2001). In agreement with this result, homologous sequences to Rap proteins can be found only in the *R. leguminosarum* bv. *viceae* and *R. etli* genomes (González *et al.*, 2006; Young *et al.*, 2006). This restricted distribution of Rap together with the considerable gene redundancy observed suggest a specific and important role for these extracellular proteins.

A possible role for Rap proteins in the interaction between rhizobia and plant roots has not yet been found. The existence of a specific mode of adsorption of *R. etli* to common bean roots was described by Lodeiro *et al.* (1995). In contrast to other rhizobia, *R. etli* required the presence of Ca^{2+} and Mg^{2+} ions for specific adsorption in the presence of an excess concentration of competitor bacteria from a different species, but not for total adsorption in the absence of competitors. This specificity and the requirement for divalent cations parallel the above-mentioned characteristics of RapA1, suggesting that this protein could be involved in this Ca^{2+}-dependent mode of specific adsorption. Therefore, we wished to study whether RapA1 could mediate rhizobial adsorption to plant roots, as well as root colonization and rhizobial infection for nodule formation in the related system *R. leguminosarum* bv. *trifolii* – clover.

Given the high gene redundancy described above we did not attempt to obtain Rap mutants. Instead, we investigated the role of RapA1 via an alternative approach of obtaining strains able to overproduce this protein. By introducing *rapA1* in the stable plasmid pHC60 (Cheng & Walker, 1998), we were able to overproduce RapA1 in rhizobia while at the same time tagging the strain with the green fluorescent protein (GFP). This approach allowed us to characterize and quantify rhizobial adsorption and root colonization in diverse rhizobial backgrounds.

Materials and methods

Bacteria, plasmids and culture conditions

Rhizobium leguminosarum bv. *trifolii* R200 is a wild-type clover-nodulating isolate; *R. leguminosarum* bv. *trifolii* R201 is a cellulose-overproducing derivative from R200 (Ausmees *et al.*, 1999), and *R. etli* LP 1003S is a wild-type Streptomycin-resistant bean-nodulating isolate (Lodeiro *et al.*, 1995). These strains were grown in tryptone-yeast extract (TY) medium (Beringer, 1974) for DNA manipulation and conjugation, or in yeast-extract mannitol (YM) medium (Vincent, 1970) for adsorption, biofilm and plant experiments. *Escherichia coli* DH5α and S17-1 were grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989). Antibiotics were used at the following concentrations: streptomycin, 200 $\mu g\,mL^{-1}$; tetracycline, 2–6 $\mu g\,mL^{-1}$ (rhizobia) or 10 $\mu g\,mL^{-1}$ (*E. coli*); and cycloheximide, 100 $\mu g\,mL^{-1}$.

Standard DNA manipulations were performed according to Sambrook *et al.* (1989).

To obtain the plasmid pRKrapAS, a 1.0-kpb HindIII fragment containing *rapA1* was obtained from *R. leguminosarum* bv. *trifolii* R200 total DNA, and cloned in the HindIII site of pRK404A (Ditta *et al.*, 1985; Scott *et al.*, 2003) downstream of the vector p*lac* promoter and in the same orientation (Ausmees *et al.*, 2001).

To obtain the plasmid pHCrapAS for *rapA1* overproduction and GFP labelling, the *rapA1* gene was cut from pRKrapAS with HindIII, and subcloned into pBluescript SK(−). The insert orientation was checked by digestion with EcoRV. Furthermore, the fragment containing *rapA1* was cut with ClaI and BamHI and cloned into pHC60 (Cheng & Walker, 1998) downstream of the vector p*lac* promoter and in the same orientation to create pHC60::*rapA1*. With this procedure, the *gfp* gene was removed from pHC60. To reintroduce *gfp* downstream of *rapA1*, it was cut from pGreenTir (Miller & Lindow, 1997) with SacI, and

pHC60::*rapA1* was digested with the same enzyme. The two fragments were then ligated to create pHCrapAS. This plasmid was transferred from *E. coli* DH5α to *E. coli* S17-1 and mobilized to *R. leguminosarum* bv. *trifolii* R200 or *R. etli* LP 1003S by conjugation.

Protein extraction and analysis

Bacteria were grown in 150 mL liquid YM medium and then removed from the broth with two consecutive centrifugations at 15 000 ***g*** for 30 min. Extracellular proteins were precipitated from the supernatants with 5% trichloroacetic acid (w/v). The pellets were washed three times with acetone and resuspended in polyacrylamide gel electrophoresis (PAGE) loading buffer (Laemmli, 1970). Samples were quantified by the Bradford (1976) method using bovine serum albumin as standard, and equal amounts of total proteins were loaded in polyacrylamide gels. After sodium dodecyl sulphate (SDS)-PAGE separation, gels were either silver-stained or electroblotted to polyvinylidene fluoride membranes (immobilon^{TM}-P Millipore) for Western blot analysis. The RapA1 protein was detected on the membranes with a specific antibody developed on rabbit (Ausmees *et al.*, 2001) and anti-rabbit alkaline phosphatase conjugate. The intensity of bands was assessed with a Kodak 1D v.3.5.0 image analysis software (Kodak Scientific Imaging Systems).

Adsorption of rhizobia to legume roots or inert surfaces

To measure adsorption of rhizobia to legume roots, a method previously described by Caetano-Anollés & Favelukes (1986a) and Lodeiro *et al.* (1995) was used. In brief, 15 (clover) or 10 (bean) seedlings per treatment were incubated for 4 h in a rhizobial suspension of *c.*10^3 cells mL^{-1} in N-free modified Fåhraeus solution (MFS) (Lodeiro *et al.*, 2000) at 28 °C with rotary shaking at 50 r.p.m. CFU plate counting at the beginning and at the end of these incubations showed that no loss of viability occurred during incubation. Rootlets with adsorbed rhizobia were washed four times, each by shaking with fresh MFS for 1 min at 120 r.p.m. After washing, two different methods were employed to quantify rhizobial adsorption.

In method I, the rootlets were distributed on the bottom of Petri dishes, and overlaid with molten (45 °C) YM agar supplemented with cycloheximide and the appropriate antibiotic concentration for selection of the assayed indicator strain. After plate incubation at 28 °C, rhizobia remaining adsorbed on the embedded root surfaces developed microcolonies, which were counted along the visible surface of each primary root under a stereomicroscope equipped with a Leica fluorescence module at × 25 magnification. We then estimated the total number of rhizobial CFU on the whole root surface as described by Caetano-Anollés & Favelukes (1986a). Total counts of microcolonies on all primary roots, expressed as the percentage of the total number of CFU present in the original inoculum, represented the adsorption index, %*A*. Thus, the adsorption index represents the number of CFU adsorbed per 100 CFU inoculated. Confidence intervals ($P < 0.05$) were obtained by employing the relationship given by Caetano-Anollés & Favelukes (1986a), which takes into account the binomial distribution of the adsorption index.

In method II, roots were vortexed for 2 min in the presence of 0.5-mm-diameter glass beads and the supernatants were diluted and plated in YM agar with the corresponding antibiotic for the rhizobial strain used. After colony development, CFU were counted, corrected for the dilution factor, and %*A* as well as confidence intervals were calculated as indicated for method I.

To measure rhizobial adsorption to inert surfaces, 2.5 g sand, or 5 g soil or vermiculite, or 10 g polypropylene beads per flask were used. All these quantities refer to dry weight. Rhizobia were incubated with these materials for 4 h at 28 °C and 50 r.p.m. and washed as described above. After washing, adsorbed rhizobia were counted according to method II, except that glass beads were not included at the vortexing step.

For microscopic observations, rhizobia were inoculated at a cell density of *c.* 10^7 rhizobia mL^{-1}, incubated at low agitation at 28 °C for 4 h and the roots were then washed and the rhizobia on them were observed as described (Smit *et al.*, 1986). For visualization of infection threads, roots were observed under a fluorescent microscope 5 days after inoculation in Fåhraeus (1957) slide assemblies.

Biofilm quantification

Rhizobia were grown in YM medium at 28 °C on a rotary shaker, 180 r.p.m., to an $OD_{500\,nm}$ of 1.0. Then, rhizobia were diluted in MFS to an $OD_{500\,nm}$ of 0.1. The microtitre plate assay for biofilm quantification was used as described by Fujishige *et al.* (2006). Briefly, 150 μL of cells or Fåhraeus solution was added to individual wells of 96-well polystyrene plates. The plates were sealed with sterile parafilm 'M' and incubated at 28 °C. At different times the medium was removed and the $OD_{500\,nm}$ was measured to verify that there was no difference in growth rate among the wells. The biofilms were then stained with 0.1% crystal violet for 20 min. In different experiments, dye excess was washed according to O'Toole & Kolter (1998), Fujishige *et al.* (2006) or Rinaudi *et al.* (2006). To test biofilm formation on a polar surface, the polystyrene plates were replaced by glass tubes.

Plant nodulation assays

Nodulation rate was evaluated by inoculating 100 clover plants or 35 bean plants germinated as described by Lodeiro

et al. (1995) with the indicated concentrations of log-phase YM-grown rhizobia per plant. Plants were grown in square dishes with solid MFS (1.5% agar) for clover or in plastic growth pouches watered with MFS for bean. Nodules were counted at fixed time intervals on the same plants during 20 days of growth in the greenhouse at 26/18 °C day/night temperature. To confirm that nodules were indeed occupied by GFP-labelled rhizobial strains, at the end of the experiments nodules were observed with a stereomicroscope equipped with a Leica fluorescence module at × 25 magnification. In addition, uninoculated controls were included in all experiments both with clover and with bean.

Results and discussion

Expression of RapA1 and plasmid stability

Rhizobium leguminosarum bv. *trifolii* R200 and *R. etli* LP 1003S overproduced RapA1 when carrying either pHCrapAS or pRKrapAS, as confirmed by Western blot analysis in six independent experiments. A typical result with *R. leguminosarum* bv. *trifolii* R200 is shown in Fig. 1, where RapA1 production was analysed at two bacterial culture ages: early and late logarithmic phase ($OD_{500\,nm}$ 0.20 and 0.95, respectively). Both in the control and the RapA1-overproducing strain, five times more RapA1 was accumulated in the supernatants at late logarithmic phase in comparison with early logarithmic phase, as expected from the longer secretion time. By contrast, R200 pHCrapAS cells had three to four times more RapA1 than control R200 pHC60 cells at both culture ages (coefficient of variation among experiments being within 20%). Although rhizobia containing these plasmids had a small reduction in total biomass production as compared with the R200 wild-type, RapA1 overproduction did not affect growth kinetics as compared with bacteria carrying empty vector as controls. These results indicated higher RapA1 production and secretion rates for R200 pHCrapAS cells at the growth interval analysed.

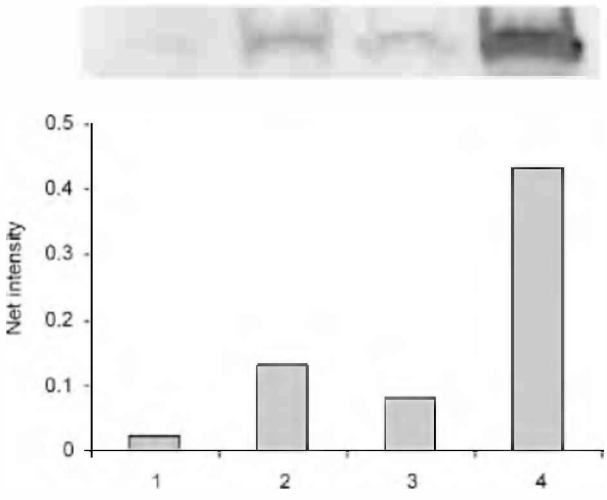

Fig. 1. Production of RapA1 at different culture ages. Upper panel: Western blot of *Rhizobium leguminosarum* bv. *trifolii* R200 extracellular proteins visualized with an anti-RapA1 antibody developed in rabbit and anti-rabbit antibody conjugated with alkaline phosphatase. Lower panel: net intensity of bands from upper panel. Lane 1: R200 pHC60 early log phase; lane 2: R200 pHCrapAS early log phase; lane 3: R200 pHC60 late log phase; lane 4: R200 pHCrapAS late log phase.

As pRK404A or pRKrapAS tended to be lost by rhizobia *in planta* (data not shown) we studied the stability of pHC60 and pHCrapAS for further use in plant inoculation experiments. To this end, we inoculated red clover or bean plants with *R. leguminosarum* bv. *trifolii* R200 or *R. etli* LP 1003S, respectively, each rhizobial strain carrying either pHC60 or pHCrapAS, and after 25 days we observed at least 100 nodules from each inoculation treatment under a stereomicroscope equipped with UV lamp. In both plant species we corroborated that 100% of nodules contained green fluorescent rhizobia, in agreement with previous reports with *Sinorhizobium meliloti* – alfalfa (Cheng & Walker, 1998). In addition, total numbers of nodules obtained were similar between the plasmid-carrying and wild-type strains without plasmid (data not shown).

Adsorption of rhizobia to clover roots

Adsorption of *R. leguminosarum* bv. *trifolii* to red clover roots and *R. etli* to common bean roots was studied by incubating rhizobia and plants according to Caetano-Anollés & Favelukes (1986a). Subsequently, two different methods were applied in parallel for quantification.

In method I, the microcolonies from adsorbed CFU were counted directly upon the roots with a stereomicroscope. With this method we observed that RapA1-overproducing R200 pRKrapAS cells adsorbed five times more than the wild-type R200 cells (%*A* values being 0.92 ± 0.29 and 0.18 ± 0.04, respectively), although microcolony visualization upon the roots was difficult owing to the translucent character of *R. leguminosarum* bv. *trifolii* colonies, which made it difficult to distinguish them from the roots beneath. Therefore, in the following we worked with the green fluorescent cells carrying pHC60 vector or the derived plasmid pHCrapAS, microcolonies of which were easily observed with a stereomicroscope equipped with UV lamp. This method allowed us to count individual adsorption events, independently of the number of bacteria adhering to each adsorption site (see Caetano-Anollés & Favelukes, 1986a). Thus, either a single bacterium or a bacterial clump adsorbed to a single root site developed one microcolony and, therefore, each one was counted once irrespective of the initial number of bacterial cells that gave rise to the microcolony.

Table 1. Adhesiveness (%*A*) of *R. leguminosarum* bv. *trifolii* R200 to red clover roots in relation to RapA1 overproduction

		Strain		
Incubation time (h)	CFU count method*	R200 pHC60 (1)	R200 pHCrapAS (2)	Ratio (2/1)
4	I	0.26 ± 0.07	1.22 ± 0.33	4.67
4	II	0.44 ± 0.17	1.64 ± 0.50	3.73
24	II	2.69 ± 0.44	9.50 ± 2.15	3.53

*Method I: roots with adsorbed rhizobia were laid on the bottom of Petri dishes and covered with molten YM agar; CFU developed from adsorbed rhizobia were counted directly upon the roots. Method II: roots with adsorbed rhizobia were vortexed for 2 min in the presence of glass beads and the supernatants were plated on YM agar; CFU developed from released rhizobia were then counted.

In method II, adsorbed rhizobia were removed from the root surfaces by vortexing, and counted in YM agar plates. Control plants incubated in YM agar as in method I following vortexing had no bacterial colonies, thus confirming that this treatment resulted in the release of all adsorbed rhizobia from the roots. Therefore, the total number of bacteria firmly associated with the roots was counted, irrespective of whether each bacterial cell was in direct contact with the root, or associated with another bacterial cell(s) into a clump attached to the root. Individual adsorption events were not discerned and, thus, single rhizobial cells give different results than clumps of bacteria adsorbed to a single site.

Therefore, both methods are complementary in detecting individual adsorption events and in estimating the mean size of bacterial clumps adsorbing to each site on the root (here a 'site' is considered as the minimal root surface that can be discerned under the stereomicroscope; as an estimate, this area is around 20 μm^2).

Overproduction of RapA1 increased adsorption of *R. leguminosarum* bv. *trifolii* to red clover roots both in short incubations of 4 h or in a longer incubation of 24 h (Table 1). This effect was observed in short incubations either comparing individual adsorption events by counting microcolonies developed on the roots, or comparing total numbers of bacteria released by vortexing (in the longer incubation, counting method I could not be used because the high number of microcolonies overlapped and could not be discerned). Therefore, the effects of RapA1 were not due to bacterial autoaggregation only, but this protein also promoted rhizobial adsorption to root surfaces. By comparing total cell counts on the roots with total cell counts from supernatants after vortexing of the roots, we were able to estimate that microcolonies on the roots were originated, as a mean, in single cells or associations of two cells, similar to the extent of cell association observed by light microscopy in bacterial cultures (data not shown).

Table 2. Adhesiveness (%*A*) of *R. leguminosarum* bv. *trifolii* R200 to red clover roots in relation to RapA1 overproduction, with or without the addition of 1 mM $CaCl_2$ to the YM culture medium during rhizobial growth. In all cases, Method I was used for CFU count

	Strain		
$CaCl_2$ addition	R200 pHC60 (1)	R200 pHCrapAS (2)	Ratio (2/1)
Yes (a)	0.94 ± 0.34	1.83 ± 0.26	1.95
No (b)	0.56 ± 0.22	1.09 ± 0.18	1.93
Ratio (a/b)	1.67	1.68	

To assess the effect of Ca^{2+}, we grew the rhizobia in YM broth supplemented with 1 mM $CaCl_2$. Under these conditions, total biomass did not increase as compared with regular YM broth (where Ca^{2+} is provided in the yeast extract; see Vincent, 1970), thus indicating that Ca^{2+} was not limiting for growth in YM. In two independent experiments, adsorption levels increased by 50% when rhizobia grew in the presence of 1 mM $CaCl_2$, irrespective of the production level of RapA1. By contrast, the mean relative increase in adsorption observed in the same experiments as due to RapA1 overproduction was around 85%. Thus, these results indicated that RapA1 activity on rhizobial adsorption to clover roots did not require Ca^{2+} addition to the growth medium, although there was an effect of this cation on general adsorption (results from a typical experiment is shown in Table 2).

We also studied adsorption to nonsymbiotic roots such as common bean, alfalfa or soybean. *Rhizobium leguminosarum* bv. *trifolii* R200 adsorbed to all root species tested, and in all cases RapA1 overproduction increased adsorption levels either measured on the roots or by vortexing and counting released bacteria. In this regard, the function of RapA1 was carried out without host symbiotic specificity (Table 3). This may be interpreted in the sense that RapA1 recognizes, in addition to the bacterial surface polysaccharide (Ausmees *et al.*, 2001), some plant surface polysaccharide that is present in all four legumes tested. Reciprocally, other adhesins from rhizobia belonging to alfalfa or soybean cross inoculation groups might be recognized also at the red clover root surface. Examples of such agglutinins are the α-L-fucoside-binding lectin in *Rhizobium lupini* (Wisniewski *et al.*, 1994) or the BJ38 protein in *Bradyrhizobium japonicum* (Ho *et al.*, 1990), which is nonhomologous to RapA1 (E. J. Mongiardini *et al.*, unpublished data). Therefore, different modes of adsorption could exist for bacteria using different adhesin sets. This could explain earlier results of rhizobial adsorption to roots under competition with strains from the same or different rhizobial species. Consistently, it was observed in *S. meliloti* (Caetano-Anollés & Favelukes, 1986b), *R. etli* (Lodeiro *et al.*, 1995) and *B. japonicum* (Lodeiro & Favelukes, 1999) that adsorption of an antibiotic-resistant strain to its symbiotic host root was inhibited at

Table 3. Adhesiveness (%*A*) of *R. leguminosarum* bv. *trifolii* R200 to roots of different plants species in relation to RapA1 overproduction

Plant species	CFU count method*	Strain		
		R200 pHC60 (1)	R200 pHCrapAS (2)	Ratio (2/1)
Red clover	I	0.31 ± 0.03	0.96 ± 0.08	3.15
	II	0.61 ± 0.07	2.67 ± 0.22	4.37
Bean	I	0.06 ± 0.02	0.41 ± 0.10	6.40
	II	0.80 ± 0.22	1.88 ± 0.39	2.34
Alfalfa	I	0.28 ± 0.03	0.55 ± 0.05	1.96
	II	0.42 ± 0.05	2.63 ± 0.22	6.25
Soybean	I	0.20 ± 0.03	1.07 ± 0.17	5.27
	II	1.53 ± 0.32	11.15 ± 1.61	7.28

*Method I: roots with adsorbed rhizobia were laid on the bottom of Petri dishes and covered with molten YM agar; CFU developed from adsorbed rhizobia were counted directly upon the roots. Method II: roots with adsorbed rhizobia were vortexed for 2 min in the presence of glass beads and the supernatants were plated on YM agar; CFU developed from released rhizobia were then counted.

Table 4. Adhesiveness (%*A*) of *R. leguminosarum* bv. *trifolii* R200 to different inert substrates in relation to RapA1 overproduction. In all cases, Method II was used for CFU count, except that addition of glass beads during vortexing was omitted.

Substrate	Strain		
	R200 pHC60 (1)	R200 pHCrapAS (2)	Ratio (2/1)
Sand	0.92 ± 0.18	1.11 ± 0.26	1.21
Vermiculite	1.58 ± 0.31	2.04 ± 0.47	1.29
Soil	0.24 ± 0.06	0.41 ± 0.08	1.71
Polypropylene	0.020 ± 0.004	0.023 ± 0.006	1.15

90–99% by a 10 000-fold excess concentration of sensitive rhizobial cells when these competitors belonged to the same species, but only at 50–60% when competitors were from a different, nonsymbiotic species. However, the present results raise the possibility that similar observations could be obtained also if rhizobia and host roots belong to different cross-inoculation groups.

We also measured adsorption on inert surfaces, such as polypropylene beads, sand, vermiculite and soil. Very low levels of adsorption were obtained on polypropylene beads, indicating poor rhizobial adhesiveness to this hydrophobic surface. By contrast, adsorption to sand, vermiculite and soil could be measured, although there was a high variability in the results (Table 4). RapA1 overproduction had no influence in vermiculite or sand and induced only a small increase in adsorption to soil, thus suggesting that the presence of RapA1 receptors in these surfaces is unlikely, except in soil, which might contain remnants of plant macromolecules in its organic matter.

In another experiment, we introduced pRK404A, pRKrapAS, pHC60 or pHCrapAS into *R. etli* and measured its adsorption to common bean roots. The effects observed were very similar to those described above with *R. leguminosarum* bv. *trifolii* – red clover, thus indicating that the action of RapA1 on nonsymbiont-specific adsorption can be observed also in a different bacterial species genomic background. *Rhizobium etli* possesses two homologous genes with 79% and 83% identity to *R. leguminosarum* bv. *trifolii rapA1*, but cross infection of common bean by *R. leguminosarum* bv. *trifolii* or red clover by *R. etli* was not observed either with or without pHCrapAS (data not shown).

Root colonization and biofilm formation

Direct microscopic observation of red clover roots with attached R200 pHC60 or R200 pHCrapAS was possible using inocula of higher concentration than in the adsorption experiments documented above. Roots incubated for 4 h with *c.* 10^7 rhizobia mL^{-1} and washed as described by Smit *et al.* (1986) are shown in Fig. 2a–d. Although this comparison is not quantitative, the more abundant adsorption events obtained with R200 pHCrapAS are readily apparent, especially on root hair tips. Bacterial clumps seem denser than calculated from adsorption experiments, but this could be explained by the three- to fourfold higher inoculum density used here.

Longer-term colonization was observed 24 h after inoculation. As shown in Fig. 2e–h, roots were overlaid with bacteria with some accumulations around root hairs, with larger bacterial clouds formed by the RapA1-overproducing cells. Rhizobial clouds appeared to be embedded in an extracellular matrix, synthesis of which could be stimulated in the rhizosphere conditions given that, as indicated by *in vitro* biofilm experiments, they could not be formed in the absence of the plant. It is unknown whether the RapA1 exopolysaccharide receptor (Ausmees *et al.*, 2001) is part of this extracellular material. This result, together with the continued increase in adsorption 24 h after inoculation (Table 1), may suggest that RapA1 overproduction could also lead to denser biofilm formation.

To assess this aspect quantitatively, we measured biofilms formed on transparent surfaces by employing a method already described with different staining procedures (O'Toole & Kolter, 1998; Fujishige *et al.*, 2006; Rinaudi *et al.*, 2006). We quantified biofilm formation under different conditions, namely: with rhizobia suspended in YM broth or MFS, or rhizobia at different growth states, or with the addition of different amounts of sucrose ranging from 0.1% to 2 % (w/v), or with different incubation times and washing procedures, or incubating in polystyrene cuvettes or glass tubes. With all these variants we observed that, despite the higher root colonization observed in Fig. 2, the amounts of biofilm obtained were similar for the parental strain R200 without plasmids, or R200 pHC60, or R200 pHCrapAS, as compared with the cellulose-overproducer

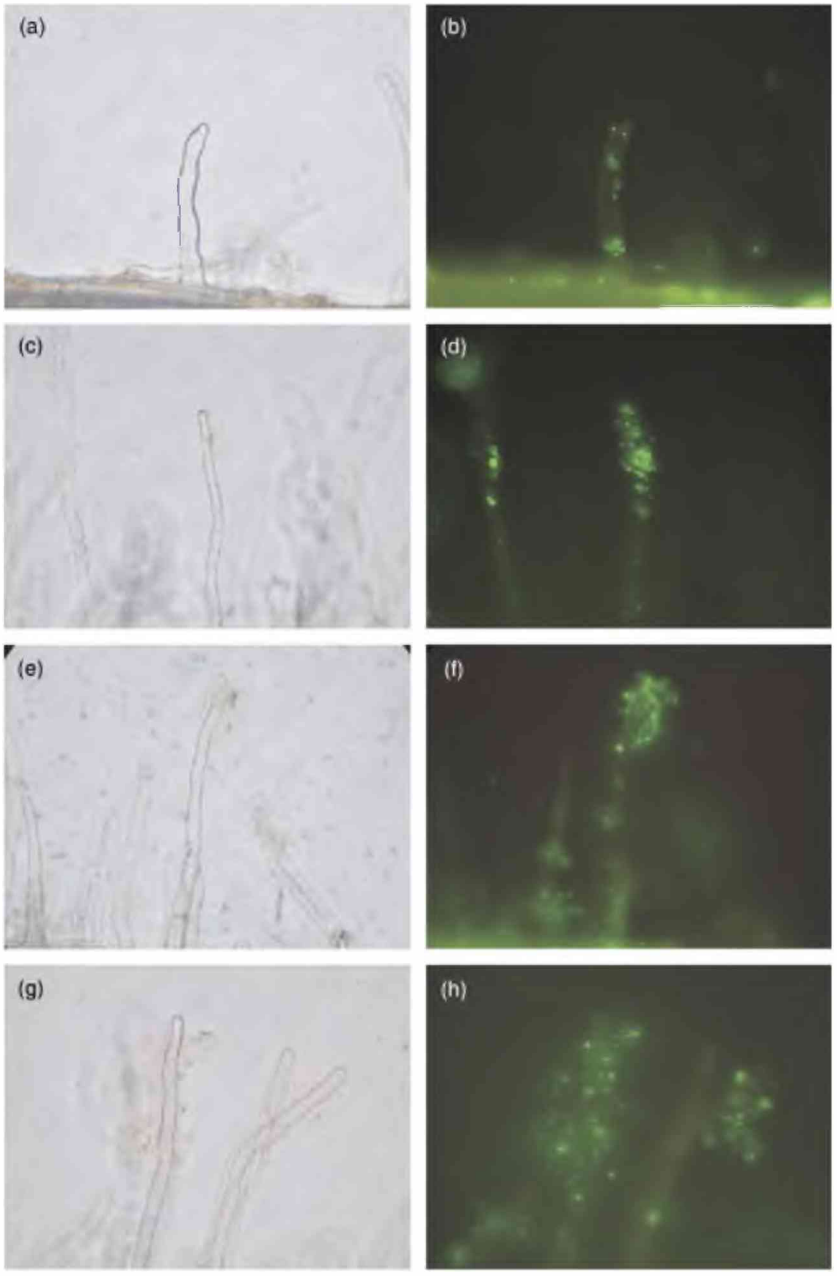

Fig. 2. Root hairs colonization by *Rhizobium leguminosarum* bv. *trifolii* R200 pHC60 (a, b, e, f) or R200 pHCrapAS (c, d, g, h) after 4 h (a–d) or 24 h (e–h) of rhizobia–plant incubation. Images shown in (a), (c), (e) and (g) were obtained with light microscopy, while those in (b), (d), (f) and (h) were obtained with fluorescence microscopy. Magnification: × 400.

R201 strain used as positive control. In Fig. 3 a typical result is shown. In this experiment, the biofilms were stained with 0.1% crystal violet for 20 min, excess dye was removed with three changes of sterile water and the dye that stained the biofilms was solubilized with 95% ethanol (Fujishige *et al.*, 2006). The amount of dye remaining bound to the cells was quantified by measuring the absorbance at 570 nm.

Although RapA1 was involved in root adsorption and colonization, its overproduction did not enhance either rhizobia autoagglutination *in vitro* (Ausmees, 2001) or biofilm formation on inert surfaces (Fig. 3). Our results indicated that RapA1 overproduction also did not increase rhizobial initial adsorption to polypropylene beads or sand (Table 4), suggesting that the lack of an increase in biofilm size on similar materials could be a related phenomenon. However, for adsorption quantification the rhizobia adsorbed on the beads were subjected to a washing procedure that in principle should destroy the biofilm structures (Stoodley *et al.*, 2002). Thus, even if the initial adsorption to polystyrene cuvettes were the same for the wild-type and the RapA1-overproducing strains, any possible influence of RapA1 on later biofilm development should be observed in

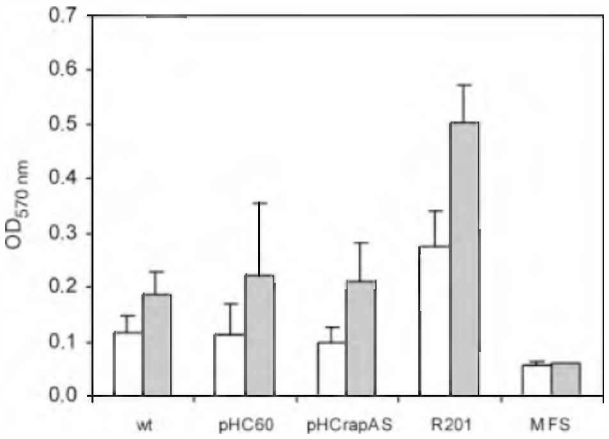

Fig. 3. Biofilm formation (mean absorbance at 570 nm ± SD) by *Rhizobium leguminosarum* bv. *trifolii* R200 wild-type, R200 carrying pHC60 or R200 carrying pHCrapAS. The cellulose-overproducing strain R201 was included as a positive control, and MFS without bacteria as a negative control. Bacteria were suspended in MFS with 1% sucrose, and the microtitre plate assay was used according to Fujishige *et al.* (2006). Incubations were performed over 24 h (white) or 48 h (grey). Error bars represent SD.

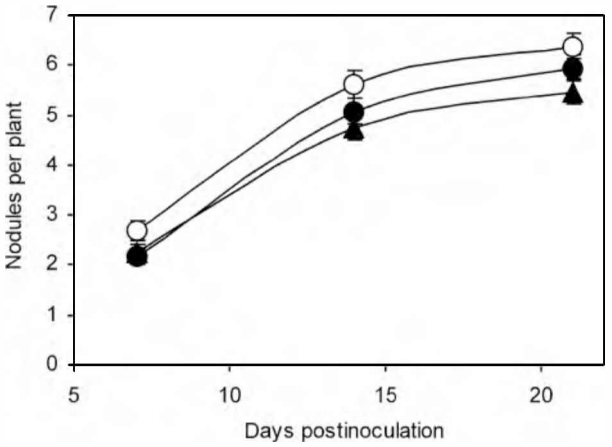

Fig. 4. Red clover nodulation at different times by *Rhizobium leguminosarum* bv. *trifolii* R200 wild-type (open circles), R200 pHC60 (filled circles) or R200 pHCrapAS (triangles). Inoculum concentrations: $3.58 \times 10^3 \pm 1.59 \times 10^3$ CFU mL^{-1} (R200 wild-type); $4.45 \times 10^3 \pm 2.52 \times 10^3$ CFU mL^{-1} (R200 pHC60); $5.77 \times 10^3 \pm 2.08 \times 10^3$ CFU mL^{-1} (R200 pHCrapAS). Bars represent mean SE ($n = 100$). Where not shown, the mean SE was smaller than the symbol.

the microtitre plate assay. If we assume that RapA1 overproduction continues in the *in vitro* biofilm development conditions, these results might indicate that either RapA1 receptors are limiting, or that this agglutinin does not play a role in this process.

Nodulation kinetics

We also assessed the ability of these strains to form infection threads and to nodulate red clover. No significant differences were observed under the fluorescence microscope in the structure of infection threads produced by each strain. In agreement with this, red clover nodulation kinetics measured with nonsaturating inocula concentrations followed similar trends with *R. leguminosarum* bv. *trifolii* R200 wild-type, R200 pHC60 and R200 pHCrapAS (Fig. 4). Similarly, there was no difference in common bean nodulation kinetics between *R. etli* 1003S wild-type, *R. etli* 1003S pHC60 and 1003S pHCrapAS (data not shown). In both experiments, uninoculated control plants produced no nodules. Therefore, neither the higher number of adsorption events nor the denser bacterial clouds formed by the RapA1-overproducing strains at the root hair tips (Fig. 2) increased nodulation rate. Earlier reports indicated that there exists a saturating rhizobial concentration for early nodulation, of the order of 10^6 rhizobia mL^{-1}, above which further increases of rhizobial concentration at the rhizosphere do not have an effect on nodulation rate (Bhuvaneswari *et al.*, 1980). The rates of adsorption increase observed in the RapA1-overproducing strain with both quantification methods were two to five times over that of the control strain (see above). This is equivalent to inoculating plants with two- to fivefold concentrated rhizobial suspensions, which given the suboptimal rhizobial concentration used for nodulation assays is not sufficient for saturation, which requires rhizobial concentrations two to three orders of magnitude higher. This indicates that other factors, not related to saturating effects, were responsible for the lack of nodulation response to RapA1.

Concluding remarks

In this work the function of RapA1 on rhizobial adsorption to root surfaces was established. The results suggest that RapA1 might fulfil functions related to early root adsorption and colonization for establishment at the rhizosphere. This environmental niche is complex, with root exudates varying in chemical composition at different root portions, and with diverse cell surface structures at root hairs, root epidermis and root tip. In addition, microbial biodiversity is particularly high in the rhizosphere and therefore adsorption and colonization of the root surface at the infection points might be subjected to harsh competition. Thus, differently adapted modes of rhizobial adsorption and colonization should coexist for overall rhizosphere occupation. Hence, RapA1-mediated adsorption seems to be one particular mode of adsorption, without influence on nodulation kinetics in the absence of competitors. Further work is required to assess the effects of RapA1 on competition for root colonization and nodulation both in controlled and in field conditions.

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