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Adsorption of *Rhizobium meliloti* to alfalfa roots: Dependence on divalent cations and pH

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Abstract

Adsorption of *Rhizobium meliloti* L5-30 in low numbers to alfalfa (*Medicago sativa* L.) roots was dependent on the presence of divalent cations, and required neutral pH. Adsorption was proportional to Ca and/or Mg concentrations up to 1.5 m*M*. Ca was not substituted by Sr, Ba or Mn. Adsorption was abolished and viability decreased at pH $\leqslant$ 6. When lowering pH, higher Ca concentrations were required to attain similar adsorption levels, indicating a marked interactive effect between Ca and H ions. Pretreatment of the roots with Ca and low pH did not affect subsequent adsorption of the bacteria. However, Ca pretreatment of *R. meliloti* sustained further adsorption at low Ca levels and low pH substantially affected their ability to adsorb. Low pH appears to affect the stability of binding causing desorption of the previously bound bacteria. The presence of saturating concentrations of heterologous *R. leguminosarum* bv. *trifolii* A118, did not prevent the expression of divalent cations and pH requirements, as well as their interaction. Our results suggest that rhizobial binding to the root surface already shows the Ca and pH dependence of alfalfa nodulation, which was generally associated to some event prior to rhizobial penetration of root hairs.

Introduction

Soil bacteria of the genus Rhizobium infect leguminous plants to produce nitrogen-fixing nodules. Initial adsorption of rhizobia to the legume root surface is one very early interaction between symbionts in the complex host-specific infection process. Many reports have suggested that host specificity for the rhizobial partner is already expressed during adsorption (Caetano-Anollés and Favelukes, 1986b; Dazzo *et al.*, 1976; Dazzo *et al.*, 1984; Kato *et al.*, 1980; Stacey *et al.*, 1980), while many others pointed out a non-host-specific adsorption mechanism (Badenoch-Jones *et al.*, 1985; Caetano-Anollés and Favelukes, 1986a; Mills and Bauer, 1985; Pueppke, 1984; Smit *et al.*, 1986; Vesper and Bauer, 1985). In alfalfa, we have found that homologous *R. meliloti* binds to the root surface in a specific mode different from a non-specific mode of adsorption also exercised by these rhizobia and other heterologous bacteria (Caetano-Anollés and Favelukes, 1986b). In these studies, the addition of heterologous strains of rhizobia decreased adsorption by *R. meliloti* only partially, while other homologous *R. meliloti* caused almost complete inhibition (Caetano-Anollés and Favelukes, 1986b).

In many plant-bacteria interactions, adsorption to root surfaces is dependent on divalent cations. Thus, binding of *Pseudomonas fluorescens* to radish roots (James *et al.*, 1985) and *Pseudomonas tolaasii* to barley roots (Nissen, 1973) is stimulated by Ca and Mg. Similar cation effects occur in bacterial adherence to inert surfaces (Fletcher, 1988; Marshall *et al.*, 1971; Stanley, 1983). In contrast Ca and Mg have been shown to inhibit the binding of

Agrobacterium tumefaciens to plant cells from suspension culture (Ohyama *et al.*, 1979) and of *Azospirillum brasilense* to corn roots (Gafny *et al.*, 1986). Recently, Smit *et al.* (1986, 1987) found that adsorption of *R. leguminosarum* bv. *viciae* to pea root hairs requires neutral pH and bacteria grown in the presence of Ca. In this association, a Ca-dependent adhesin was proposed to be involved in rhizobial adsorption to root hairs (Smit *et al.*, 1987).

Nodulation of alfalfa is pH and Ca dependent (Munns, 1968; 1970). These phenomena have been linked to some event prior to rhizobial penetration of root hairs which probably coincides with root hair curling. As part of a physiological study of adsorption of *R. meliloti* to alfalfa roots, we report here on the influence of pH, divalent cations and their interaction upon this process. The possible role of these factors during preinfection will be addressed (A preliminary account of these studies was presented to the 4th North American Rhizobium Conference, Wailea, Hawaii, August 1985).

Materials and methods

Root adsorption of *R. meliloti* L5-30 (streptomycin-resistant strain obtained from G. Martinez-Drets, Montevideo, Uruguay; originally from J. Dénarié) and its nonmotile nonflagellated derivative LP101 (Caetano-Anollés *et al.*, 1988) was quantitatively determined as previously described (Caetano-Anollés and Favelukes, 1986a). In brief, late exponential cultures of rhizobia (OD_{500} = 0.4) in yeast extract-mannitol (YEM) medium (Caetano-Anollés and Favelukes, 1986a) were diluted to $2 \cdot 10^3$ bacteria/mL with nitrogen-free Fåhraeus solution (0.84 m*M* Na_2HPO_4, 0.74 m*M* KH_2PO_4, 0.015 m*M* ferric citrate, 0.49 m*M* $MgSO_4$, 0.90 m*M* $CaCl_2$, pH 7.0) (Fåhraeus, 1957) where sometimes the class and concentration of divalent cations and the initial pH were modified. Seeds of alfalfa var. Dawson (supplied by Alexander and Co., Buenos Aires, Argentina) were surface sterilized with ethanol and mercuric chloride and germinated on water agar plates (Caetano-Anollés and Favelukes, 1986a). Fifteen 5-day old seedlings were incubated in 22.5 mL bacterial suspension with rotary agitation at 50 rpm and 28°C for 4 h, followed by 4 consecutive standardized washings with fresh mineral solution (Caetano-Anollés and Favelukes, 1986a). Bacteria adsorbed to root surfaces were individually detected as microcolonies which developed upon culture of the washed seedlings in embedding YEM agar supplemented with 100 $\mu g\,mL^{-1}$ streptomycin plus 25 $\mu g\,mL^{-1}$ cycloheximide. The number of root bound rhizobia was obtained by direct counting of the microcolonies closely apposed to the root surface, and the percentage of inoculated rhizobia that become adsorbed to roots in the chosen experimental conditions ('adhesiveness', A) was calculated (Caetano-Anollés and Favelukes, 1986a). Since the number of adsorbed bacteria increases linearly with the size of the inoculum within a range between 10 and 10^4 added bacteria/root (Caetano-Anollés and Favelukes, 1986a), reliable estimates of considerably low bacterial adsorption values can be obtained just by increasing the number of inoculant cells in the assays. pH and cell numbers were determined before and after the incubation of the bacteria with the roots for each experiment. The actual concentration of viable bacteria was obtained by plate counts in YEM agar using the overlay procedure.

In competition experiments, late exponential-phase cultures of the heterologous bacteria *R. leguminosarum* bv. *trifolii* A118 (obtained from INTA Castelar, Argentina; original denomination, TA1, from CSIRO, Australia) were centrifuged (12,000 *g*, 10 minutes), resuspended in mineral solution, and then added to the incubation medium to give 10^6–10^7 bacteria/mL. This strain did not develop colonies in the embedding YEM-streptomycin medium.

When biological buffers were used, the incubation medium was a modified Fåhraeus mineral solution: 0.11 m*M* Na_2HPO_4, 0.09 m*M* KH_2PO_4, 0.015 m*M* ferric citrate, supplemented with different levels of calcium (as $CaCl_2$) or magnesium (as $MgSO_4$) and 10 m*M* of PIPES [piperazine-N,N′-bis(ethanesulfonic acid)], HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) MOPS [3-(N-morpholino)propanesulfonic acid] or imidazole as their potassium salts.

All values are given with 95% confidence intervals. Averages and their confidence limits were obtained by proper weighting of the individual data (Caetano-Anollés and Favelukes, 1986a) in at least two independent experiments.

Results

pH requirement

Adsorption of *R. meliloti* L5-30 to alfalfa roots was highly sensitive to pH changes (Fig. 1). Plants and bacteria were incubated in Fåhraeus solution (low buffer capacity) adjusted to different pH. Sodium acetate was added to the medium to reduce pH variations during incubation since it had no measurable effects on adsorption. Adsorption decreased when pH was lowered from neutrality, and was abolished (A = 0.007 ± 0.013%) at pH 6.0 or below. This was not caused by a proportional loss of viability. However, bacterial numbers obtained by plate counts were affected below pH 6.0, and were zero at pH 4.5.

Decreasing pH from 7.0 to 5.0 did not cause agglutination of the bacteria but abolished bacterial motility, when cells were examined under the light microscope. Since bacterial motility contributes importantly to the rate and extent of contact of the bacteria with the roots (Caetano-Anollés *et al.*, 1988), pH effects on motility could account for the observed effects on adsorption. However this seems not to be the case. Adsorption of the non-flagellated mutant derivative LP101 was comparably affected by low pH. The percentage of LP101 bacteria bound to the roots after a 4 h incubation at pH 7.0 (0.5 ± 0.06%) decreased 5 times at pH 6.5 and more than 10 times at pH 6.0, suggesting that motility is not the only factor involved.

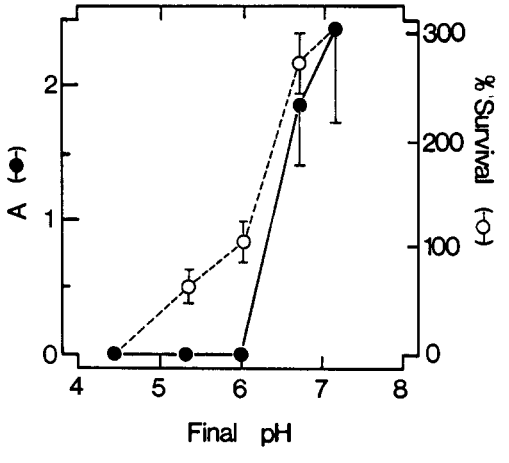

Fig. 1. Influence of pH on the adsorption of *R. meliloti* to alfalfa roots. Sets of 15 5-day-old seedlings were incubated during 4 h with $2 \cdot 10^3$ bacteria mL^{-1} in Fåhraeus mineral solution plus 1 m*M* sodium acetate at one of several defined pH within a range between pH 4.0 and pH 7.5, washed 4 times, and embedded in YEM agar supplemented with streptomycin (100 $\mu g\, mL^{-1}$) and cycloheximide (25 $\mu g\, mL^{-1}$). Microcolonies developed along the root surface were counted for each seedling after a 2-d incubation at 28°C. Bacterial adsorption was determined as the percentage of bacteria in the initial suspension that adhered to the roots after washing (adhesiveness, A). Bacterial survival estimates during incubation were obtained by comparing initial and final plate counts of the bacterial suspension in the presence of the plants. Values above 100% imply growth. *Bars* represent 95% confidence intervals and are not shown where smaller than the symbol.

Divalent cation requirement and interaction with pH

Adsorption was dependent on the presence of Ca and/or Mg but was independent of the counter anion nature (Table 1). Strontium, barium or manganese could not replace Ca or Mg. This selective requirement was not related to effects on bacterial motility. While adsorption of LP101 required divalent cations (Table 1), motility and chemotaxis of the wild type L5-30 was active in divalent-cation free solution (Caetano-Anollés *et al.*, 1988). Divalent cation levels had no effect on rhizobial viability and growth during the assays as expected from other studies (Loneragan and Dowling, 1958; Norris, 1958; Vincent, 1962). The adsorption response was strictly proportional to Ca and/or Mg over the concentration range up to 1.5 m*M* (Fig. 2). The pH changes during incubation caused by metabolic activities of plant and bacteria were low (0.1–0.2 units). Higher concentrations of divalent cations reduced initial pH considerably and caused marked pH decreases (up to 0.6 units) after incubation. The need for an efficient buffer without side effects was met by the use of PIPES of HEPES. MOPS and imidazole were markedly inhibitory to adsorption (62% and 54% respectively).

In the presence of PIPES and pH 7.0, maximal adsorption was obtained with 1.5 m*M* Ca or Mg after which a plateau was reached (Fig. 3, A and C). At lower pH (6.5) higher Ca levels (10 m*M*) were required for optimal adsorption. Furthermore, at comparable Ca concentrations, adsorption values were lower than those obtained at pH 7.0. Interestingly, at pH 6.5 Mg was unable to replace Ca; low adsorption levels were only obtained at high Mg concentration. Adsorption at pH 6.0 was considerably lower at all Ca levels tested.

Table 1. Adsorption of *R. meliloti* as affected by the ionic environment. Bacteria were incubated with roots for 4 h in a modified Fåhraeus mineral solution (pH 7.0) without divalent cations, but supplemented with the indicated salts. When both Ca and Mg were added, their concentration ratio was 1.8:1. Adsorbed bacteria and bacterial viability were determined as described in 'Materials and methods'

Strain	Compounds added	Divalent cations (m*M*)	Counter anions (m*M*)	A[a]
L5-30	None	0	0	0.004 ± 0.007
	K_2SO_4, KCl	0	2.29	0.03 ± 0.02
	$CaCl_2$, K_2SO_4	0.90	2.29	2.18 ± 0.32
	$MgSO_4$, KCl	0.49	2.29	1.47 ± 0.23
	$CaCl_2$, $MgSO_4$	1.39	2.29	3.03 ± 0.42
	$CaCl_2$, $MgCl_2$	1.39	2.78	2.76 ± 0.36
	$CaSO_4$, $MgSO_4$	1.39	1.39	2.92 ± 0.41
	$CaCl_2$	1	2	2.33 ± 0.54
	$Sr(NO_3)_2$	1	2	0.19 ± 0.13
	$BaCl_2$	1	2	0.06 ± 0.07
	$MnSO_4$	1	1	0.07 ± 0.08
LP101	None	0	0	0.01 ± 0.004
	$CaCl_2$, $MgSO_4$	0.69	1.14	0.21 ± 0.03
	$CaCl_2$, $MgSO_4$	1.39	2.29	0.44 ± 0.05

[a] Adhesiveness (A) values are weighted averages with 95% confidence intervals. Bacterial survival after 4 h of incubation in the presence of Ca, Sr, Ba or Mg salts was 130, 94, 109 and 78% respectively (values higher than 100% imply growth).

To study the influence of divalent cations and pH on the host-specific component of adsorption, adsorption of L5-30 was determined in the presence of high concentrations (10^6–10^7 bacteria/ml) of an heterologous strain, in an attempt to saturate the nonspecific mode of root adsorption common to both heterologous and homologous bacteria. *R. leguminosarum* bv. *trifolii* A 118 was chosen because it was the most inhibitory of 19 heterologous strains of rhizobia and agrobacteria tested (Caetano-Anollés and Favelukes, 1986b). Results resembled those obtained without heterologous bacteria (Fig. 3, B and D). Thus, the specific and nonspecific modes of adsorption are modulated by the ionic environment generated by Ca, Mg and H ions.

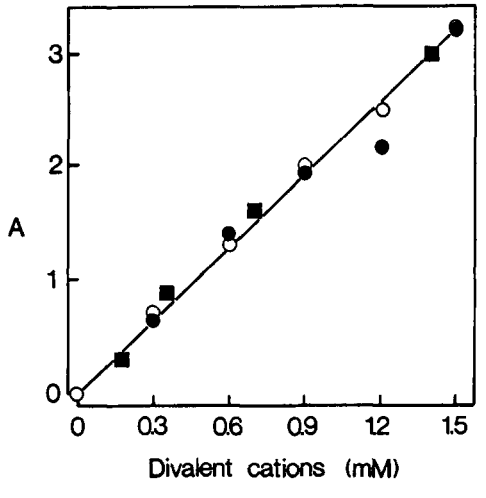

Fig. 2. The effect of divalent cations on adsorption of *R. meliloti* to alfalfa roots. The incubation medium was a Fåhraeus mineral solution (pH 7.0) containing different concentrations of $CaCl_2$ (○), $MgSO_4$ (●) or $CaCl_2$ plus $MgSO_4$ (Ca/Mg concentration ratio = 1.8) (■). Adsorption was determined after a 4 h incubation as in Fig. 1. For simplicity, 95% confidence intervals for this representative experiment are not shown but are less than 10% of the actual averages.

Preincubation experiments

Plants and bacteria were preincubated in the presence or absence of Ca and bacterial adsorption subsequently determined (Table 2). A decrease in adsorption was always observed when roots were preincubated in mineral solution. This effect may result from the loss of root secretion products important for the adsorption process, since it was abolished when adsorption was determined in the presence of the preincubating liquid (A. Lagares, unpublished). The requirement for Ca during adsorption was not substituted for by Ca pretreatment of the roots. In contrast, preincubation of the bacteria in Ca sustained bacterial adsorption at Ca

levels otherwise too low for it to occur. These results suggest that the Ca requirement was associated with bacterial components involved in the adsorption process rather than with the root surface. Since no Ca was provided during bacterial growth and the probable traces of the cation after dilution of the bacterial culture did not sustain bacterial adsorption (Table 2), Ca supplied during preincubation was either stored by the bacteria and used later during adsorption, or was directly used to activate relevant surface components.

In separate experiments, plants or bacteria were preincubated in acid or neutral pH (Table 3). Again, pretreatment of the roots had no effect on subsequent adsorption levels. However if the bacteria were preincubated at pH 6.0, only a slight recovery of adsorption was observed. Thus, acidity appears to affect irreversibly the competence of the bacteria but not of the roots during adsorption.

The time course of adsorption of *R. meliloti* L5-30 to alfalfa roots in Fåhraeus solution at pH 7.0 is shown in Fig. 4. Adsorption increased linearly with time during the first 5 h of incubation. In parallel, an acid pulse was added to the system after a 3 h incubation period (Fig. 4). Adsorption levels not only ceased to increase but decreased 2 h after the change in pH, indicating that acidity altered drastically the stability of binding, causing desorption of already adsorbed cells.

Discussion

The pH and calcium dependence of legume nodulation has been well documented (Albretch and Davis, 1929; Lie, 1969; Loneragan and Dowling, 1958; Lowther and Loneragan, 1968; Munns, 1968; 1970; Wood *et al.*, 1984). In the *R. meliloti*-alfalfa association, Munns (1968; 1970) found that the initial infection process is sensitive to acidity and Ca concentration. Nodulation was considerably reduced at pH 5.5 and was virtually abolished at pH 4.5 (Munns, 1968). Munns (1970) also found that Ca concentrations below 0.2 m*M* inhibit nodulation. This effect was not caused by alterations in root extension or root hair production. Since, infection thread initiation and following stages require Ca, but at very low levels (10 μM) (Munns, 1968) and as shown for subterranean clover, once the nodule is initiated development proceeds unhindered even at concentrations of Ca too low for plant growth (Lowther and Loneragan, 1968), the Ca-demanding step should occur early in the symbiotic process. It appears from these studies that the primary target of Ca and H ions effects is the rhizosphere or the surface of the root itself (rhizoplane), and that Ca and pH requirements for nodulation arise early during preinfection probably prior to the curling of root hairs.

Our results indicate that bacterial adsorption of

Table 2. Effect of preincubation with calcium of roots and bacteria on the adsorption of *R. meliloti* L5-30 to alfalfa. Plants (in groups of 15) and bacteria (diluted to $0.8–1.0 \cdot 10^5$ cells/mL) were preincubated separately in a modified Fåhraeus mineral solution without Mg (pH 7.0) in the presence or absence of calcium (as $CaCl_2$) at 50 rpm and 28°C and for 2 h and 1 h respectively. The plants were then transferred to a freshly diluted bacterial suspension containing $2 \cdot 10^4$ bacteria/mL and the bacteria (diluted to $2 \cdot 10^3$ cells/mL) incubated with untreated plants. Adsorption was determined 1 h later in the presence and absence of Ca as described in 'Materials and methods'. Adhesiveness (A) values are weighted averages from two independent experiments with 95% confidence intervals

Calcium concentration (m*M*) during:			Relative adsorption (% of control)[a]
Preincubation		Incubation	
Plants	Bacteria		
–	–	1.4	100
–	–	0.14	7 ± 3
–	–	0	0
1.4	–	1.4	54 ± 8
0	–	1.4	76 ± 18
1.4	–	0	2 ± 2
–	1.4	1.4	73 ± 9
–	0	1.4	77 ± 12
–	2.8	1.4	108 ± 26
–	4	1.4	63 ± 12
–	1.4	0.14	40 ± 5
–	1.4[b]	0.014	53 ± 13

[a] The average A value ± 95% confidence intervals for the control was 0.30 ± 0.04%.

[b] Bacteria were diluted to 10^6 cells/mL during preincubation.

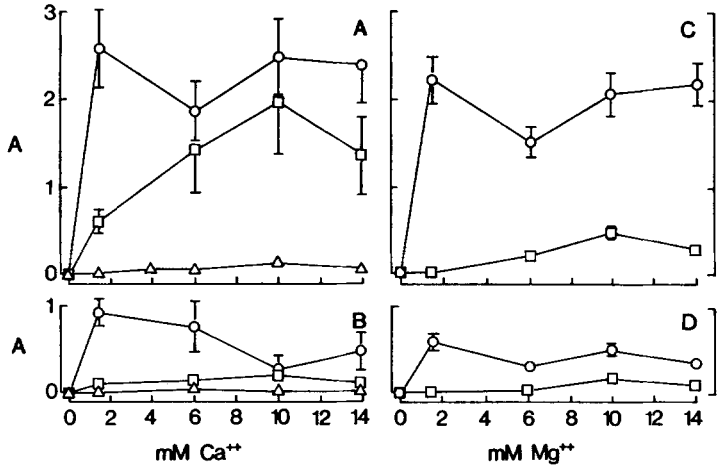

Fig. 3. The effect of the interaction between divalent cations and pH on adsorption of *R. meliloti* to alfalfa roots in the absence (**A** and **C**) or presence (B and D) of 10^6 heterologous *R. leguminosarum* bv. *trifolii* A118/mL. The incubation medium was buffered with 10 m*M* PIPES (see 'Materials and methods'), contained varying amounts of $CaCl_2$ or $MgSO_4$ and was adjusted to pH 7.0 (○), pH 6.5 (□) and pH 6.0 (△). Adsorption was determined after 4 h as in Fig. 1. The maximum pH variation during incubation in this experiment was 0.02 units. *Bars* show 95% confidence intervals and are not shown where smaller than the symbol.

roots shows a strict requirement for Ca and neutral pH, which correlates with the above mentioned dependence of alfalfa nodulation for both factors. These requirements cannot be explained simply by deficiencies in the growth of the bacteria or by decreased bacterial motility. We have also observed a marked interaction between Ca and pH effects on bacterial adsorption, which also parallels results obtained in nodulation studies with alfalfa (Munns, 1968). The sensitivity of the nodulation process to low pH is strongly influenced by Ca and though poorly understood it has been documented for a range of legumes (Andrew, 1976; Loneragan and Dowling, 1958; Munns, 1968; 1970).

Both pH and Ca requirements affect only the microsymbiont. Neither variable had much effect as a preinoculation treatment of the roots (Table 2 and 3). However, pre-exposure of the bacteria to

Table 3. Effect of preincubation of roots and bacteria at different pH on the adsorption of *R. meliloti* L5-30 to alfalfa. Plants (in groups of 15) and bacteria (diluted to 0.4–1.7·10^5 cells/mL) were preincubated in a modified Fahraeus mineral solution without Mg but containing 1.4 m*M* $CaCl_2$, at two different initial pH levels, pH 6.0 and 7.0, at 50 rpm and 28°C and for 2 h and 1 h respectively. The plants were then transferred to a freshly diluted bacterial suspension containing 2·10^4 bacteria/mL and the bacteria (diluted to 2·10^4 cells/mL) incubated with untreated plants. Adsorption was determined 1 h later at the two indicated initial pH, as described in 'Materials and methods'. Adhesiveness (A) values are weighted averages from two independent experiments with 95% confidence intervals. Note that absorption at pH 6.0 was not 0, since pH increased during incubation to 6.1 and 6.2 in the two experiments pooled in this Table

Initial pH during:			Relative adsorption (% of control)[a]
Preincubation		Incubation	
Plants	Bacteria		
–	–	7	100
–	–	6	36 ± 3
7	–	7	73 ± 6
7	–	6	37 ± 3
6	–	7	68 ± 6
–	7	7	83 ± 6
–	7	6	36 ± 3
–	6	7	45 ± 5

[a] The average A value ± 95% confidence intervals for the control was 0.46 ± 0.05%.

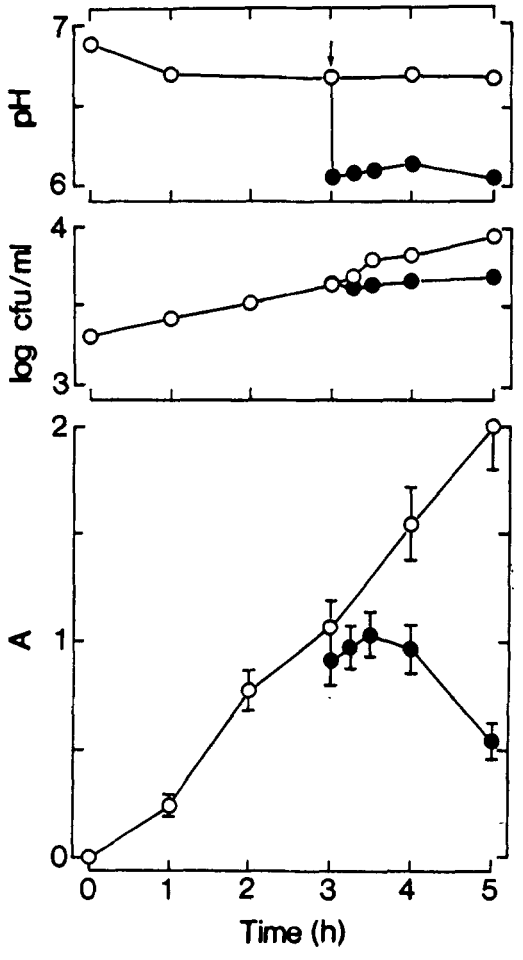

Fig. 4. Kinetics of adsorption of *R. meliloti* to alfalfa roots. Groups of 15 5-day-old seedlings were incubated with $2 \cdot 10^3$ bacteria mL^{-1} in Fåhraeus mineral solution (initial pH, 6.9) during different time intervals (○). In some cases, HCl was added (*see arrow* in upper panel) to decrease pH during incubation to around pH 6 (●), and the adsorption time course obtained was compared to that obtained at neutral pH. Bacterial adsorption was determined as described in 'Materials and methods' and expressed as adhesiveness (A). The rates of bacterial growth during incubation were also studied. As a control, an equivalent amount of acid was added at time 0. A, determined 5 h later was 0.25 ± 0.05 and the final number of bacterial colony forming units (cfu) in the incubation liquid, $5 \cdot 10^3$ cells/mL. *Bars* represent 95% confidence intervals in this representative experiment. Two other independent experiments showed the same trends. No bars are shown where the confidence interval is smaller than the symbol.

low pH suppressed adsorption at neutral pH while Ca sustained it at low Ca levels. In particular, acidity caused desorption of already bound rhizobia, suggesting that sustained neutral pH during incubation is required for the stability of binding. Still uncertain remains the localization of the effect on Ca during adsorption. Calcium could either bridge negatively charged groups on plant and bacterial surfaces after its stable association with bacterial components, and/or indirectly activate the bacteria for suitable adhesins. This still remains to be established.

Calcium requirements were quite specific, being only substituted for by Mg and not by other monovalent or divalent cations. These results contrast with the recent observation that previous growth of rhizobia in the presence of Ca — but not Mg — was required for adsorption to pea root hair tips, at the optimal pH of 7.5 (Smit *et al.*, 1987). However, we have found that during the adsorption process Mg replaces Ca *only* at neutral pH. At lower pH, Mg was unable to sustain adsorption, in agreement with the observation that Ca — but not Mg — was required for nodulation at the acid pH studied in alfalfa (Munns, 1970). Calcium and pH affected adsorption of *R. meliloti* in a similar manner regardless of whether or not heterologous bacteria were present during the assay. It is apparent from this result that Ca and pH requirements concurrently affect the nonspecific and the specific modes of adsorption described for alfalfa (Caetano-Anollés and Favelukes, 1986b).

Low pH and Ca concentration were shown to affect the ability of clover root exudates to induce the expression of nodulation genes in *R. leguminosarum* bv. *trifolii* (Richardson *et al.*, 1988a; 1988b). Low pH restricted the expression of *nod A::lacZ* translational fusion while the presence of Ca in the plant growth medium increased its induction at a range of pH. The presence of *nod* inducing flavones in root exudates was proposed to contribute to the acid sensitive and Ca dependent step in nodule formation (Richardson *et al.*, 1988a). It appears from our studies that the 'acid sensitive' step in the infection process coincides with the adsorption of the rhizobia to the root surface. The observation that Ca is required for the adsorption process, and that the presence of Ca lowers the apparent critical pH necessary for both nodulation and adsorption to occur, further supports this view. At present we can only speculate that both *nod* gene expression and adsorption jointly limit nodulation under low Ca and acidic conditions until a causal relationship between these two symbiotic events is found.

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