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Lectin-Enhanced Accumulation of Manganese-Limited *Rhizobium leguminosarum* Cells on Pea Root Hair Tips

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The ability of *Rhizobium leguminosarum* 248 to attach to developing *Pisum sativum* root hairs was investigated during various phases of bacterial growth in yeast extract-mannitol medium. Direct cell counting revealed that growth of the rhizobia transiently stopped three successive times during batch culture in yeast extract-mannitol medium. These interruptions of growth, as well as the simultaneous autoagglutination of the bacteria, appeared to be caused by manganese limitation. Rhizobia harvested during the transient phases of growth inhibition appeared to have a better attachment ability than did exponentially growing rhizobia. The attachment characteristics of these manganese-limited rhizobia were compared with those of carbon-limited rhizobia (G. Smit, J. W. Kijne, and B. J. J. Lugtenberg, J. Bacteriol. 168:821–827, 1986, and J. Bacteriol. 169:4294–4301, 1987). In contrast to the attachment of carbon-limited cells, accumulation of manganese-limited rhizobia (cap formation) was already in full progress after 10 min of incubation; significantly delayed by 3-*O*-methyl-D-glucose, a pea lectin haptenic monosaccharide; partially resistant to sodium chloride; and partially resistant to pretreatment of the bacteria with cellulase. Binding of single bacteria to the root hair tips was not inhibited by 3-*O*-methyl-D-glucose. Whereas attachment of single *R. leguminosarum* cells to the surface of pea root hair tips seemed to be similar for both carbon- and manganese-limited cells, the subsequent accumulation of manganese-limited rhizobia at the root hair tips is apparently accelerated by pea lectin molecules. Moreover, spot inoculation tests with rhizobia grown under various culture conditions indicated that differences in attachment between manganese- and carbon-limited *R. leguminosarum* cells are correlated with a significant difference in infectivity in that manganese-limited rhizobia, in contrast to carbon-limited rhizobia, are infective. This growth-medium-dependent behavior offers an explanation for the seemingly conflicting data on the involvement of host plant lectins in attachment of rhizobia to root hairs of leguminous plants. Sym plasmid-borne genes do not play a role in manganese-limitation-induced attachment of *R. leguminosarum*.

Attachment of rhizobia to tips of developing root hairs of leguminous plants is thought to be an early and necessary step in the infection process leading to the development of nitrogen-fixing root nodules. The physical interaction between rhizobia and the growing root hair tips precedes the following infection stages in the nodulation process: marked root hair curling, infection thread formation, development of a root cortical meristem, and root nodule morphogenesis (7). Nodulation is a host-plant-specific process; e.g., *Rhizobium leguminosarum* (*R. leguminosarum* biovar *viciae*) exclusively nodulates pea, vetch, lentil, and sweet pea, whereas *Rhizobium trifolii* (*R. leguminosarum* biovar *trifolii*) nodulates only clover. Host specificity is determined by one or more events preceding the outgrowth of the infection thread in the root hair (e.g., see references 22 and 32). In fast-growing rhizobia, many essential nodulation genes (*nod* genes), including the genes determining host specificity, are located on a large Sym (symbiosis) plasmid. Flavones and flavanones secreted by the roots of leguminous plants induce most of the rhizobial *nod* genes (25, 28, 32, 36, 38).

Neither the molecular mechanism of root hair attachment nor its relation to *nod* genes and *nod* gene induction is fully understood. Available data from several laboratories are difficult to compare in view of the widely various experimental conditions used (e.g., see references 2, 5, 6, 12, and 35). In particular, the role of host plant lectin as a determinant of host-specific attachment has been a matter of debate (2, 8, 15, 20, 29).

In our laboratory, it has recently been observed that the conditions under which the rhizobia are grown are of prime importance in their ability to attach to root hairs. Nutrient limitation always coincides with optimal attachment, and the type of limitation determines whether host lectins are involved in the attachment process (19, 30, 31). Carbon limitation induces a non-host-specific attachment mechanism in *R. leguminosarum* in which neither host plant lectins nor *nod* genes are involved (30). The results of attachment assays with carbon-limited rhizobia could be explained by assuming a two-step attachment mechanism: (i) attachment of the bacteria to the root hair surface per se, via a rhizobial Ca^{2+}-dependent adhesin, followed by (ii) aggregation of the bacteria at the root hair tip mediated by cellulose fibrils (cap formation) (31).

In the present study, the ability of *R. leguminosarum* cells, grown in yeast extract-mannitol (YM) medium, to attach to pea root hair tips was investigated during various growth phases. YM medium has been used as a standard growth medium for rhizobial cells in many laboratories. Consistent with earlier results (30), it was found that growth inhibition of *R. leguminosarum* coincides with good attachment ability, and the availability of manganese ions was identified as the growth-limiting factor in YM medium. Comparison of the attachment characteristics of manganese- and carbon-limited rhizobia revealed a number of striking

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differences, especially with respect to the role of pea lectin in the attachment process.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *R. leguminosarum* 248 harboring Sym plasmid pRL1JI (16) is able to nodulate and fix N_2 on peas (*Pisum sativum* L.). Its Sym plasmid-cured derivative 248^c (RBL1387 [27]) is Nod$^-$ Fix$^-$. YM medium (40) contains the following components per liter of deionized water: K_2HPO_4, 1.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; yeast extract (Difco Laboratories, Detroit, Mich.), 1.0 g; and mannitol, 10.0 g (pH 6.8). TY medium (3) contains the following components per liter of deionized water: tryptone (Difco), 5.0 g; yeast extract (Difco), 3.0 g; and $CaCl_2 \cdot 2H_2O$, 1.0 g. A^+ medium (33) contains the following components per liter of deionized water: yeast extract (Difco), 0.8 g; glucose, 10.0 g; mannitol, 3.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; KH_2PO_4, 0.993 g; and K_2HPO_4, 0.318 g (pH 6.8).

Synthetic medium used for the Mn^{2+}-limited chemostat culture (B^- glutamate medium) contained the following components per liter of deionized water: K_2HPO_4, 0.318 g; KH_2PO_4, 0.993 g; $MgSO_4 \cdot 7H_2O$, 0.54 g; $CaCl_2 \cdot 2H_2O$, 0.04 g; sodium glutamate, 0.5 g; KNO_3, 0.5 g; biotin, 0.1 mg; thiamine, 0.1 mg; FeNa-EDTA, 33.5 mg; KCl, 40 mg; H_3BO_3, 3.175 mg; Na_2MoO_4, 2.5 mg; $ZnSO_4 \cdot 7H_2O$, 0.25 mg; $CuSO_4$, 0.1 mg; and $MnSO_4 \cdot 4H_2O$, 0.305 μg (pH 6.5). The bacteria were grown in a laboratory chemostat (1-liter volume; BioLafitte SA, St. Germain en Laye, France) at an O_2 concentration of 200 μM at 28°C with a dilution rate (*D*) of 0.1 h^{-1}. The A_{620} value of the culture at steady state was 0.16.

Bacteria were maintained on slants consisting of solidified A^+ medium at 4°C. Bacteria for attachment assays were grown at 28°C in 100-ml Erlenmeyer flasks containing 50 ml of YM medium with vigorous aeration (180 rpm [incubator shaker; New Brunswick Scientific Co., Edison, N.J.]). Growth was monitored by measuring the A_{620} with a Vitatron colorimeter and by direct cell counting with a hemacytometer.

Plants. Pea seeds (*P. sativum* L. cv. Rondo) were obtained from Cebeco, Rotterdam, The Netherlands. Seeds were surface sterilized, and seedlings were cultured by the method described by Smit et al. (30).

Attachment assay. The attachment assay used was described previously by Smit et al. (30). Briefly, lateral pea roots were immersed for up to 2 h in a suspension of 1.5×10^8 to 2.0×10^8 rhizobial cells ml^{-1} in 25 mM phosphate buffer, pH 7.5, at room temperature with gentle agitation (2 rpm [rotary table; TAAB Laboratories, Reading, United Kingdom]). After incubation, the roots were washed and attachment was quantified by randomly screening at least 100 developing root hairs with a phase-contrast microscope (×400 magnification). Attachment was ranked from class 1 (no attached bacteria) to class 4 (many attached bacteria forming a caplike aggregate on top of the root hair). The percentage of root hairs of each class was calculated. The variability of this test is about 5% and depends largely on the condition of the roots.

Inhibition of attachment by monosaccharides or NaCl was tested by immersion of the seedlings in the bacterial suspension supplemented with one of these compounds.

Cellulase treatment. To test the effect of pretreatment with cellulase, bacteria to be used in the attachment assay were pretreated with purified cellulase by the method of Smit et al. (31).

Induction of *nod* genes. Sym plasmid-borne *nod* genes were induced by batch cultivation of the bacteria in YM medium supplemented with 0.7 μM naringenin (H. P. Spaink, unpublished results; see also reference 38).

Spot inoculation test. The low-inoculum-concentration spot inoculation test used has been described in detail by Díaz et al. (14). Briefly, a 5-μl drop of inoculum was placed 0.5 cm above the root tips of 4-day-old pea seedlings (on the trichoblast zone). After 7 days of culture, the seedlings were carefully removed from the gravel and the roots were fixed in 4% paraformaldehyde–phosphate-buffered saline (each 10 ml of fixative contained the following: paraformaldehyde [20%], 2 ml; phosphate-buffered saline [pH 7.2], 5 ml; deionized water, 3 ml). Phosphate-buffered saline contained the following per liter of deionized water: NaCl, 16 g; KCl, 0.4 g; $Na_2HPO_4 \cdot 2H_2O$, 3.0 g; and KH_2PO_4, 0.4 g. The inoculation zone (1 cm) was excised and sectioned on a freeze-microtome (section thickness, 150 μm). The sections were stained with 1% methylene blue and examined by phase-contrast microscopy. Progression of infection thread growth in the zone of inoculation was taken as a measure of infectivity.

The susceptibility of different pea roots to infection by rhizobia shows a large variability. The low-inoculum-concentration spot inoculation test described above may result in 0 to about 60 infection threads by inoculation zone per root. Approximately one of five primary pea roots appears to be resistant to infection. However, the ratio between the number of infection threads protruding into the outer, middle, or inner cortical cell layers, respectively, is constant, regardless of the total number of infection threads. Therefore, differences in infectivity of *R. leguminosarum* cells can be illustrated by comparing the mean number of infection threads in each category (see Table 4).

RESULTS

Transient growth interruptions in YM medium are caused by Mn^{2+} limitation. Batch-cultured cells of *R. leguminosarum* 248 and its Sym plasmid-cured derivative 248^c grew exponentially in YM medium to an A_{620} of 0.25. At that growth stage, autoagglutination of cells begins. Direct cell counting showed that, between the A_{620}s of 0.25 and 0.30, the cell number did not increase. Two other interruptions of growth were detected at A_{620}s of 0.40 to 0.55 and 0.70 to 0.90. These three growth interruptions can be visualized clearly by plotting the number of cells during culture growth against the optical density in a so-called *n/A* curve (Fig. 1). The stationary growth phase started at an A_{620} of 1.3. Each successive growth interruption was characterized by autoagglutination of the rhizobia, leading to the apparent decrease in cell number as measured by direct cell counting (Fig. 1). The autoagglutination of the bacterial cells suggested that a nutritional factor had become limiting (21). The growth-limiting factor appeared to be Mn^{2+}, since addition of at least 0.1 μM $MnSO_4$ per liter of medium resulted in a complete disappearance of both the growth interruptions and the autoagglutination (Fig. 1). Addition of an extra supply of the other medium constituents had no effect (data not shown). Therefore, it was concluded that the transient growth interruptions of *R. leguminosarum* 248 and 248^c and the simultaneous autoagglutination of the bacterial cells in YM medium are caused by Mn^{2+} limitation.

Ability to attach to root hair tips as a function of growth stage. The ability of rhizobia to attach to pea root hair tips

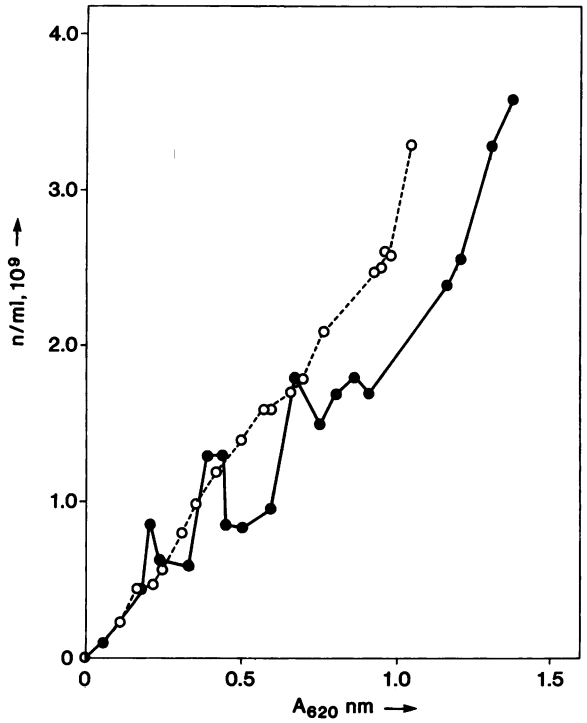

FIG. 1. Relationship between cell number and optical density at 620 nm (an *n/A* curve) of *R. leguminosarum* 248 cells grown in YM medium (●) or in YM medium supplemented with 0.1 μM $MnSO_4$ (○). Autoagglutination of the bacteria during the growth interruptions causes an apparent decrease in the number per milliliter.

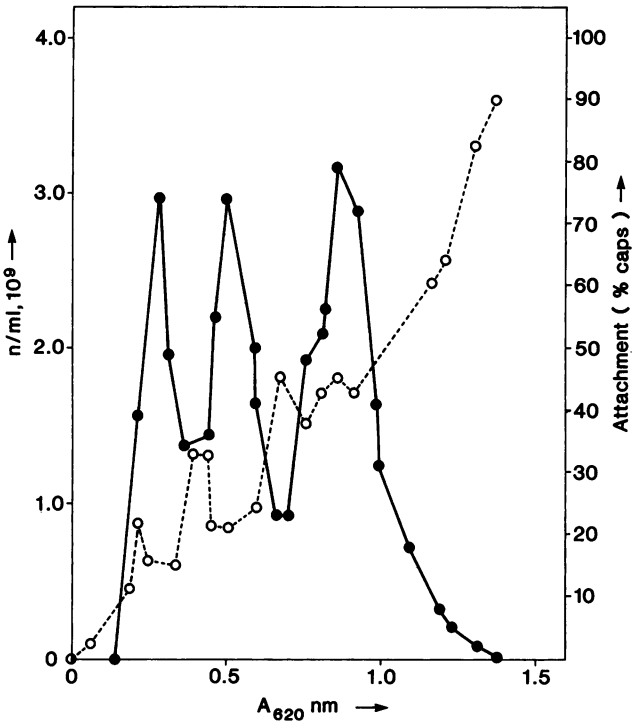

FIG. 2. Relation between the *n/A* curve of *R. leguminosarum* 248 cells growing in YM medium and the ability of the cells to form caps on pea root hair tips (class 4 attachment). The number of bacteria (○) and the percentage of class 4 attachment (●) are both plotted against the A_{620} of the culture.

was investigated as a function of growth stage. Rhizobia harvested during the transient growth interruptions appeared to have a better attachment ability than did exponentially growing rhizobia. After the percentage of developing root hairs with class 4 attachment was plotted in the *n/A* curve, a positive correlation between the growth interruptions and cap formation was observed (Fig. 2), which again illustrates that growth inhibition of rhizobia coincides with good attachment ability (for a description of the situation during carbon limitation, see reference 30).

The effect of Mn^{2+} limitation on attachment ability was investigated over time by using cells harvested at an A_{620} of 0.85 (Table 1). Growth in YM medium supplemented with 0.1 μM Mn^{2+} appeared to result in delayed attachment. In conclusion, growth of *R. leguminosarum* under Mn^{2+} limitation increases the rate of attachment to pea root hair tips.

Comparison of the effects of manganese and carbon limitation on rhizobial attachment to pea root hair tips. Comparison of the attachment characteristics of Mn^{2+}- and C-limited *R. leguminosarum* cells revealed a number of remarkable differences. (i) Cap formation by Mn^{2+}-limited bacteria could already be observed after 10 min of incubation with the pea roots, whereas in the case of C-limited bacteria, cap formation started after approximately 30 min (Fig. 3). Similar data were obtained after filtration of bacterial aggregates prior to the attachment assay; this finding shows that enhanced cap formation does not result from binding of bacterial clumps to the root hair tips. (ii) Similar to the case with C-limited rhizobia, optimal attachment of Mn^{2+}-limited cells occurred at pH 7.5, and attachment was completely inhibited at a pH ≤5.0. However, attachment by Mn^{2+}-limited cells appeared to be less H^+ sensitive, and a small percentage of caps could still be observed after incubation at pH 6.0, in contrast to the situation for C-limited cells (data not shown). (iii) Attachment by Mn^{2+}-limited cells appeared to be less sensitive to inhibition by salts than was attachment by C-limited cells. Even 100 mM NaCl, which completely abolished cap formation by C-limited rhizobia, still allowed cap formation by Mn^{2+}-limited cells on 25% of the root hairs (Fig. 4). (iv) To examine the possible role of pea lectin in the attachment process, we tested the influence of the presence of 3-*O*-methyl-D-glucose, a strong pea lectin hapten (34), on the attachment of Mn^{2+}-limited cells. D-Galactose was used as a nonhapten control. Addition of 100 mM D-galactose yielded results similar to those for controls, which had no added sugar. However, the presence of 100 mM 3-*O*-methyl-D-glucose delayed the attachment of Mn^{2+}-limited bacteria to a rate comparable with the attachment rate of C-limited

TABLE 1. Time course of attachment of *R. leguminosarum* 248 to pea root hair tips after growth in YM medium or in YM medium supplemented with $MnSO_4$[a]

Presence of $MnSO_4$ (0.1 μM)	Incubation time (min)	Attachment (%) in class:			
		1	2	3	4
No (control)	30	7	31	27	35
	60	3	8	35	54
	120	1	6	14	79
Yes	30	25	58	13	4
	60	3	15	58	24
	120	0	1	9	90

[a] Bacteria were harvested at an A_{620} of 0.85.

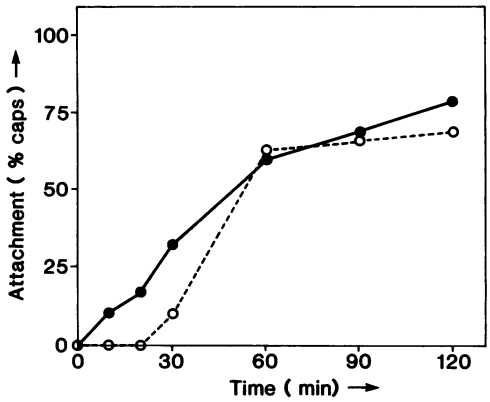

FIG. 3. Time course of cap formation (class 4 attachment) on pea root hair tips by *R. leguminosarum* 248 cells. The rhizobia were grown in either YM medium (●) or TY medium (○) and harvested at A_{620}s of 0.85 and 0.70, respectively.

rhizobia (Table 2). C-limited rhizobia do not show hapten-inhibited attachment (30). (v) The attachment properties of YM medium-grown, cellulase-pretreated rhizobia in comparison with those of cells incubated in buffer are shown in Table 3. Comparable data from C-limited cells (from Smit et al. [31]) are included. The dramatic shift from attachment classes 3 and 4 to class 2 attachment caused by cellulase pretreatment of C-limited cells is much less pronounced with Mn^{2+}-limited cells. This result suggests that, for Mn^{2+}-limited cells, cellulose fibrils are not the only adhesins involved in accumulation of rhizobia on pea root hair tips.

Role of the Sym plasmid in attachment. To determine the possible involvement of Sym plasmid-borne nodulation genes in Mn^{2+}-limitation-induced attachment, the attachment characteristics of *R. leguminosarum* 248 (wild type) were compared with those of its Sym plasmid-cured derivative, 248^c. The attachment characteristics of both strains were found to be indistinguishable in all respects, including the delay in attachment in the presence of 3-*O*-methyl-D-glucose. Induction of *nod* genes by the flavanone naringenin (0.7 μM) (32, 38) did not lead to any change in the attachment behavior of the wild-type strain (data not shown).

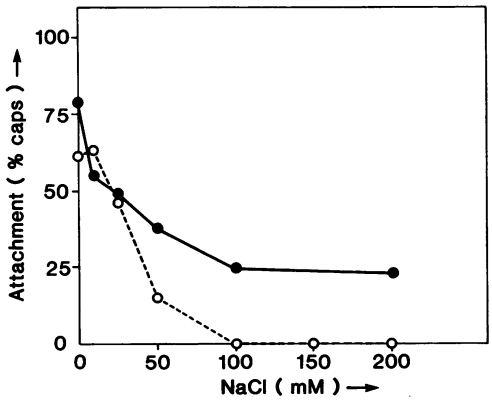

FIG. 4. Influence of sodium chloride on cap formation by *R. leguminosarum* 248 cells on pea root hair tips. The rhizobia were grown in either YM medium (●) or TY medium (○) and harvested at A_{620}s of 0.85 and 0.70, respectively. NaCl was added to the bacterial suspension just before the addition of the roots, and attachment was measured after 2 h of incubation.

TABLE 2. Influence of the addition of monosaccharides on the attachment of *R. leguminosarum* 248 cells to pea root hair tips[a]

Sugar added	Incubation time (min)	Attachment (%) in class:			
		1	2	3	4
None	30	7	32	27	34
	60	3	8	35	54
	120	1	6	14	79
D-Galactose (100 mM)	30	5	34	27	34
	60	3	8	31	58
	120	0	2	9	89
3-*O*-Methyl-D-glucose (100 mM)	30	14	48	26	12
	60	4	10	46	40
	120	0	2	11	87

[a] Bacteria were cultivated in YM medium and harvested at an A_{620} of 0.85.

These results exclude a role for Sym plasmid-borne genes in Mn^{2+}-limitation-induced attachment.

Manganese-limited continuous culture. To determine, by using an independent approach, whether Mn^{2+} limitation is indeed responsible for the described attachment behavior, the attachment characteristics of *R. leguminosarum* 248 cells grown in mineral medium in an Mn^{2+}-limited chemostat culture were investigated. Chemostat-cultured Mn^{2+}-limited cells attached to pea root hair tips in a manner similar to that of batch-cultured Mn^{2+}-limited cells, as characterized by quick attachment, only partial inhibition by 100 mM NaCl, and delayed attachment in the presence of 3-*O*-methyl-D-glucose (data not shown). These results confirm that Mn^{2+} limitation is responsible for the described attachment characteristics and that Mn^{2+}-limited cells differ in their attachment characteristics from C-limited cells.

Influence of attachment behavior on infection thread formation. To determine whether differences in attachment behavior were reflected in subsequent root infection steps, infection thread formation in pea roots was studied after spot inoculation with Mn^{2+}-limited, Mn^{2+}-supplied, and C-limited *R. leguminosarum* 248 cells. The number of infection threads in the inoculation zone that ended in the outer, middle, and inner cortex, respectively, was judged by phase-contrast microscopy after staining the root sections with methylene blue. Table 4 shows that spot inoculation with Mn^{2+}-limited cells harvested during the three successive growth interruptions results in an increasing number of infection threads protruding into the inner cortical layers. However, spot inoculation with exponentially growing cells from YM medium (A_{620}, 0.12) or with cells cultured in YM medium supplemented with Mn^{2+} (A_{620}, 0.85) resulted in an

TABLE 3. Influence of pretreatment with cellulase on attachment of *R. leguminosarum* 248 cells to pea root hair tips[a]

Growth medium	Treatment	Attachment (%) in class:			
		1	2	3	4
YM	Buffer	2	23	15	60
	Cellulase	4	55	9	32
TY	Buffer	7	14	11	68
	Cellulase	21	77	2	0

[a] Bacteria were cultivated in YM medium or TY medium and harvested at A_{620}s of 0.85 and 0.70, respectively. Attachment was measured after an incubation time of 120 min. For further details, see reference 30.

TABLE 4. Number of infection threads per root protruding into the cortex of pea roots after low-inoculum-concentration spot inoculation with *R. leguminosarum* 248 cells grown under various conditions

Growth medium	A_{620}	No. of threads which ended in the following cortical zone[a]:		
		Outer	Middle	Inner
YM	0.12 (exponentially growing cells)	8.8	5.0	0
	0.28 (Mn^{2+}-limited cells)	4.4	11.5	6.4
	0.49 (Mn^{2+}-limited cells)	1.0	4.3	7.7
	0.85 (Mn^{2+}-limited cells)	0	2.7	13.7
YM + 0.1 μM $MnSO_4$	0.85	10.2	4.4	0.8
TY	0.70 (C-limited cells)	0	0	0

[a] Mean number from four inoculation experiments.

infection pattern in which most infection threads reached only the outer cortical layers. Interestingly, C-limited rhizobia did not induce infection thread formation in the inoculation zone at all (Table 4). However, prolonged presence in the pea rhizosphere apparently restores the infectivity of C-limited rhizobia, resulting in infection and nodulation of root tissue, particularly lateral roots, formed after inoculation. Taken together, the results show that a delay in infection thread formation is correlated with a delay in the attachment process and that differences in attachment between Mn^{2+}- and C-limited *R. leguminosarum* 248 cells are correlated with a significant difference in infectivity.

DISCUSSION

Manganese limitation leads to transient growth interruptions and an increased rate of attachment of *R. leguminosarum*. Mangenese appeared to be the first growth-limiting nutritional factor for *R. leguminosarum* 248 and 248[c] in YM medium (Fig. 1). However, the phenomenon of three successive growth interruptions as a result of limitation of one nutrient is hard to imagine. Our results clearly show (Fig. 1) that the first interruption at an A_{620} of 0.25 is directly caused by Mn^{2+} limitation. Our current hypothesis is that the next phenomenon is adaptation to the limited availability of the factor. Growth limitation followed by elevation of the level of the limiting factor is commonly used as a method for synchronization of cell divisions. The approximately twofold increase in cell number after each growth interruption is indicative of cell division synchronization in this case. This explanation raises the possibility that the limitation of the nutrient itself does not increase the attachment rate of *R. leguminosarum* but that the nondividing condition of the rhizobial cell determines the increased ability to attach to other rhizobia (autoagglutination) and to accumulate on pea root hair tips. Attachment studies with differently synchronized *Rhizobium* cell cultures in various mineral media are necessary to examine this possibility in more detail. The primary limiting factors for growth of rhizobia in the soil and in the rhizosphere of leguminous plants are unknown and remain to be determined.

Role of pea lectin in attachment of Mn^{2+}-limited *R. leguminosarum* cells. Taken together, the experiments relating to the influence of 3-*O*-methyl-D-glucose, NaCl, and pretreatment with cellulase on attachment of Mn^{2+}-limited rhizobia to pea root hair tips provided evidence that, unlike the situation for C-limited rhizobia, cellulose fibrils are not the only adhesins involved in cap formation (31). In view of the observed inhibition by hapten, pea lectin is probably involved as an enhancer of the accumulation of Mn^{2+}-limited rhizobia at the root hair tip. Carbon limitation (TY medium) apparently eliminates the possibility of lectin-mediated attachment (30). This might be explained by restricted synthesis of surface polysaccharides under C-limiting conditions, since rhizobial lectin receptors are found in various surface polysaccharide fractions: extracellular polysaccharides (EPS), capsular polysaccharides, and/or lipopolysaccharides (LPS) (1, 9, 23, 24, 26, 37). Late-logarithmic-phase TY medium-grown *R. leguminosarum* cells are not encapsulated and produce greatly reduced amounts of high-molecular-weight EPS (G. J. Medema and J. W. Kijne, unpublished results). Manganese limitation in the presence of excess carbon apparently allows synthesis and exposure of lectin receptor molecules in rhizobia. The results of the attachment experiments with YM medium-grown rhizobia have been corroborated by the attachment experiments with rhizobia grown in a chemostat under Mn^{2+}-limiting conditions. Interestingly, direct binding of single cells to the root hair tip surface (class 2 attachment) is not inhibited in the presence of 3-*O*-methyl-D-glucose. Preliminary results indicate that a Ca^{2+}-dependent adhesin is involved in class 2 attachment of Mn^{2+}-limited rhizobia (G. Smit, unpublished results) as it appears to be the case for C-limited rhizobia (31). The results described can be incorporated into our two-step model for rhizobial attachment to pea root hair tips (31) by assuming that the first attachment step (class 2 attachment) is similar for both Mn^{2+}- and C-limited cells, and that the second step (cap formation) is accelerated by lectin molecules under appropriate culture conditions. Mutants of *R. leguminosarum* unable to produce or expose the Ca^{2+}-dependent adhesin and/or lectin receptor molecules will be used to test this model.

Lectin-recognition hypothesis. The lectin-recognition hypothesis was proposed by Dazzo and Hubbell (11) to explain host-specific attachment in the *R. trifolii*-clover symbiosis. According to this hypothesis, clover lectin (trifoliin A) recognizes similar saccharide sequences both on the surface of the bacterium and on the root hair and cross-bridges these sequences in a complementary fashion. The clover lectin hapten 2-deoxyglucose specifically inhibits the attachment of *R. trifolii* to clover root hairs (12), "reducing the high level of bacterial adhesion to that characteristic of background" (8). Dazzo's results have been corroborated by Zurkowski (39). On the other hand, several investigators, e.g., Badenoch-Jones et al. (2), did not observe hapten-inhibitable attachment of *R. trifolii* to clover root hairs.

Our work (30, 31, and this study) shows that the use of different growth media for rhizobia results in the expression of different attachment mechanisms. In addition to the well-known effect of culture age on expression of rhizobial lectin receptors (13, 23, 33), this work offers an explanation for the seemingly conflicting data on the involvement of lectins in rhizobial attachment to root hairs of leguminous plants. We assume that the "background" attachment observed by Dazzo et al. (12) is similar to class 2 attachment as defined in our experiments. It should be noted that the lectin-mediated, "high level of bacterial adhesion" in Dazzo's experiments is much lower than the level of attachment observed in root hair caps. This difference might be explained by the different conditions during the attachment

assay, most notably by the different pH of the incubation medium.

We propose that, until the growth conditions in the rhizosphere of leguminous plants have been exactly defined, statements about the attachment characteristics of rhizobia should be based on experiments which use various growth media.

Is enhanced cap formation related to infection thread formation? We found a positive correlation between lectin-enhanced accumulation of *R. leguminosarum* on pea root hair tips and the rate of infection thread formation in the inoculation zone of pea roots (Table 4). This result corroborates our earlier finding of a correlation between the location of lectin on the surface of pea roots and infection by *R. leguminosarum* (14). However, the relation between cap formation and infection thread formation is unclear. Cap formation per se does not necessarily coincide with successful nodulation. (i) C-limited rhizobia form caps but no infection threads (this study). (ii) Cellulose-negative mutants of *R. leguminosarum* do not form caps but nodulate pea roots successfully (31). It has been suggested that *Rhizobium* lectin receptor molecules are involved in infection thread formation (1). If so, enhancement of attachment under the conditions of the attachment assay might be a coinciding secondary effect indicative of a compatible interaction between the bacteria and the host plant target cells. Our observation that TY medium-grown rhizobia are noninfective at the time of inoculation might be explained by the lack of lectin receptor molecules and/or by the lack of EPS in general (4) on the rhizobial surface.

Role of the Sym plasmid in attachment. From studies of the *R. trifolii*-clover symbiosis, evidence is accumulating that lectin receptor synthesis and/or exposure is under Sym plasmid control (10, 39). For *R. leguminosarum*, we could not demonstrate any involvement of Sym plasmid-coded gene products in the attachment process. These data correspond with our earlier observation that several *Rhizobium* species are able to bind pea lectin (33). However, it has been reported (17, 18) that *R. leguminosarum* produces at least two different pea lectin receptors, one in the EPS fraction and one in the LPS fraction. Pea lectin binding to rhizobial EPS appeared to be non-species specific (18). This extracellular pea lectin receptor might be similar to the receptor involved in enhanced accumulation of *R. leguminosarum* on pea root hair tips.

Interestingly, Kamberger (18) showed that in Ouchterlony cross-precipitation tests with LPS fractions from several *Rhizobium* species and with lectins from the homologous host plants, LPS from *R. leguminosarum* was precipitated exclusively by pea and lentil lectin. The presence of a species-specific pea lectin receptor in *R. leguminosarum* LPS was reported earlier by Wolpert and Albersheim (37). This receptor might be an analogous to the species-specific lectin receptor in the capsule of *R. trifolii* demonstrated by Dazzo and co-workers (10). Conclusive demonstration of its existence, taking into account our present knowledge of *nod* genes, *nod* gene induction, and growth medium effects, will be a part of our future research.

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