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Involvement of both Cellulose Fibrils and a Ca^{2+}-Dependent Adhesin in the Attachment of *Rhizobium leguminosarum* to Pea Root Hair Tips

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We have previously described an assay for the attachment of *Rhizobium* bacteria to pea root hair tips (cap formation) which was used as a model to study the attachment step in the nodulation process. Under all conditions tested, a positive correlation was observed between the percentage of fibrillated cells and the ability of these bacteria to form caps and to adhere to glass, suggesting that fibrils play a role in the attachment of *Rhizobium leguminosarum* to pea root hair tips and to glass (G. Smit, J. W. Kijne, and B. J. J. Lugtenberg, J. Bacteriol. 168:821–827, 1986). In the present paper the chemical and functional characterization of the fibrils of *R. leguminosarum* is described. Characterization of purified fibrils by infrared spectroscopy and cellulase treatment followed by thin-layer chromatography showed that the fibrils are composed of cellulose. Purified cellulose fibrils, as well as commercial cellulose, inhibited cap formation when present during the attachment assay. Incubation of the bacteria with purified cellulase just before the attachment assay strongly inhibited cap formation, indicating that the fibrils are directly involved in the attachment process. Tn*5*-induced fibril-overproducing mutants showed a greatly increased ability to form caps, whereas Tn*5*-induced fibril-negative mutants lost this ability. None of these Tn*5* insertions appeared to be located on the Sym plasmid. Both types of mutants showed normal nodulation properties, indicating that cellulose fibrils are not a prerequisite for successful nodulation under the conditions used. The ability of the fibril-negative mutants to attach to glass was not affected by the mutations, indicating that attachment to pea root hair tips and attachment to glass are (partly) based on different mechanisms. However, growth of the rhizobia under low Ca^{2+} conditions strongly reduced attachment to glass and also prevented cap formation, although it had no negative effect on fibril synthesis. This phenomenon was found for several *Rhizobium* spp. It was concluded that both cellulose fibrils and a Ca^{2+}-dependent adhesin(s) are involved in the attachment of *R. leguminosarum* to pea root hair tips. A model for cap formation as a two-step process is discussed.

Attachment of the soil bacterium *Rhizobium* species to the developing root hairs of leguminous plants is considered to be an early step in the host-specific infection process which leads to a nitrogen-fixing symbiosis. In fast-growing rhizobia many essential nodulation genes (*nod* genes), including the genes determining host specificity, are located on a large Sym(biosis) plasmid. Neither the molecular mechanism of attachment nor its relation to *nod* genes is understood. It has been proposed that host plant lectins are involved in the attachment process in a host-specific manner (3, 6, 7, 27, 31). However, a number of recent reports provided evidence for a non-lectin-mediated mechanism of attachment (1, 18, 26). It was observed recently in our laboratory that growth conditions of the bacteria are of prime importance for the results of attachment assays in that optimal attachment always coincided with limitation for a nutrient and that the kind of limitation determined whether lectins are involved in the attachment process. Stationary growth in tryptone-yeast (TY) medium is caused by carbon limitation which induces attachment of *Rhizobium leguminosarum* 248 cells to pea root hair tips as well as to glass in a Sym plasmid-independent process. Since pea lectin haptenic monosaccharides do not inhibit attachment it is unlikely that pea lectins play a role in carbon-limitation-induced attachment (26). Also, other rhizobia, e.g., *R. trifolii* and *R. phaseoli*, adhered to pea root hair tips, which also points to a non-host-specific attachment mechanism. The rhizobia produced extracellular fibrils 5 to 6 nm in diameter and up to 10 μm long. A positive correlation between the presence of extracellular fibrils and the ability to attach to pea root hair tips and to glass was found, suggesting a role of these fibrils in attachment (26).

In the present paper, we describe the chemical and functional characterization of these fibrils. They appear to be composed of cellulose. By using Tn*5* mutants that lack fibrils it was shown that these appendages are indeed required for attachment, especially for cap formation, but not for nodulation. A second, Ca^{2+}-dependent adhesin was identified which was found to be also involved in attachment. A model for rhizobial attachment to root hair tips is discussed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Rhizobium*, *Agrobacterium*, and *Escherichia coli* strains are listed in Table 1. The composition of the media A^+ and TY has been described previously (26). RMM medium contains (per liter of deionized water): K_2HPO_4, 2.05 g; KH_2PO_4, 1.45 g; $MgSO_4 \cdot H_2O$, 0.5 g; NaCl, 0.15 g; NH_4NO_3, 0.5 g; glucose, 2.0 g; $CaCl_2$, 0.147 g; biotin, 0.2 mg; thiamine, 0.2 mg; calcium pantothenate, 0.01 mg; $CuSO_4 \cdot 5H_2O$, 0.345 mg; $MnSO_4 \cdot 4H_2O$, 6.09 mg; $ZnSO_4 \cdot 7H_2O$, 0.947 mg; H_3BO_3, 12.69 mg; $Na_2MoO_4 \cdot 2H_2O$, 3.98 mg; and NaFe-EDTA, 0.13 g; final pH 6.5. YMB medium contains (per liter of deionized water): K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; mannitol, 10.0 g; and yeast extract (Difco Laboratories, Detroit, Mich.), 0.4 g; final pH 7.0. LC medium contains (per

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TABLE 1. Bacterial strains

Strain	Relevant characteristics[a]	Reference
Rhizobium leguminosarum 248	*R. leguminosarum* harboring Sym plasmid pRL1JI, Cam^r	12
RBL1465 RBL1466 RBL1467 RBL1468 RBL1469	Tn*5* fibril-overproducing mutants of strain 248[b]	This work
RBL5039	*R. trifolii* cured of its Sym plasmid, Str^r	10
RBL5506	RBL5039, Cam^r	21
RBL5515	RBL5039, Rif^r	21
RBL5523[c]	RBL5039 harboring the *R. leguminosarum* Sym plasmid pRL1JI::Tn*1831*[b]	21
RBL5760 RBL5761 RBL5762 RBL5763	Tn*5* fibril-negative mutants of strain RBL5523	This work
R. trifolii 0403		6
R. phaseoli 1233		11
Agrobacterium tumefaciens 1251	*A. tumefaciens* GMJ9017	24
Escherichia coli 1830	*E. coli* harboring suicide plasmid pJB4JI (Tn*5*)	2

[a] Str, Streptomycin; Rif, rifampin; Cam, chloramphenicol.
[b] Tn*5* codes for kanamycin resistance (Km^r); Tn*1831* codes for spectinomycin resistance (Sp^r).
[c] RBL5523 is called *R. leguminosarum* since the pRL1JI Sym plasmid harbors the host-specificity-determining genes. In a previous paper this strain was called *R. trifolii* (26).

liter of deionized water): tryptone (Difco), 10.0 g; yeast extract (Difco), 5.0 g; NaCl, 8.0 g; Tris, 0.121 g; and $MgSO_4 \cdot 7H_2O$, 2.46 g; final pH 6.6. *Rhizobium* species and *E. coli* were maintained on solid A^+ medium and solid LC medium, respectively. For attachment assays, bacteria were cultivated at 28°C either in Erlenmeyer flasks or in a chemostat. In the former case, bacteria were grown in 50 ml of TY medium on a rotary shaker (180 rpm) and harvested at an A_{620} value of 0.70. Growth in a chemostat was in TY medium, and the A_{620} value was kept at 0.7; $D = 0.05\ h^{-1}$; and the dissolved oxygen concentration was kept at a level of >70% saturation by regulating the stirring rate. For fibril purification, bacteria were grown in 2-liter Erlenmeyer flasks containing 1.25 liter of TY medium and shaken at 160 rpm at 28°C. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following concentrations (milligrams per liter); kanamycin, 200; rifampin, 20; spectinomycin, 100; and chloramphenicol, 10.

Plant culture conditions. Seeds of pea (*Pisum sativum* cv. Rondo) and common vetch (*Vicia sativa* nigra) were surface sterilized and cultivated as described previously (26). For nodulation tests, pea seeds were inoculated with 0.1 ml of TY-grown bacteria (A_{620} value, 0.70) and placed separately in coarse gravel soaked in sterile nitrogen-free medium (22). After 3 weeks the plants were screened for nodule formation. Common vetch seeds were placed on slopes containing Jensen medium (29), inoculated as described above, and screened for nodulation after 2 weeks.

Transposon mutagenesis and mutant screening. Transposon mutagenesis was performed by the method of Beringer et al. (2). Briefly, *E. coli* 1830 containing the suicide plasmid pJB4JI was mated with *R. leguminosarum* 248 or *R. leguminosarum* RBL5523 for 16 h at 28°C. Transconjugants were selected on RMM plates supplemented with kanamycin. Tn*5* mutants of *R. leguminosarum* 248 and RBL5523 were screened under UV light for altered fluorescence after 4 days of growth on RMM plates supplemented with kanamycin and 0.02% calcofluor white (CFW) (Sigma). For fibril isolation, testing of attachment ability, and testing of nodulation ability, the mutants were grown in the absence of kanamycin, a condition under which they appeared to be stable.

To establish whether the Tn*5*-insertions were Sym plasmid localized, fibril-negative Tn*5* mutants of *R. leguminosarum* RBL5523 were mated with *R. leguminosarum* RBL5506 (Cam^r) as the acceptor strain by the method of Beringer et al. (2). Under these conditions, Sym plasmid-localized Tn*5* insertions (Km^r) are transferred at a frequency of 10^{-2} to 10^{-3}, while a low frequency ($<10^{-6}$) points to a non-Sym plasmid-localized Tn*5* insertion (11). The same procedure was followed for fibril-overproducing Tn*5* mutants of *R. leguminosarum* 248 with *R. leguminosarum* RBL5515 (Rif^r) as the acceptor strain.

DNA probes and hybridization. Plasmid pNP520 (20) was used as a Tn*5* probe. Extraction of *Rhizobium* DNA and digestion with *Bam*HI restriction endonuclease were done as described by Maniatis et al. (15). DNA fragments of Tn*5* fibril-negative and fibril-overproducing mutants and their parent strains were transferred from agarose gels to nitrocellulose filters by the methods of Southern (26a). The conditions for hybridization with the ^{32}P-labeled Tn*5* probe prepared by nick translation were as described by Maniatis et al. (15).

Attachment assay. The attachment assay of rhizobia to pea root hairs is described in detail in a previous paper (26). Briefly, bacterial cells were centrifuged, suspended in 25 mM phosphate buffer [pH 7.5] to a final A_{620} value of 0.07 (which corresponds to 1.5×10^8 to 2.0×10^8 bacteria per ml), and added to lateral pea roots. After incubation for 2 h, the roots were washed 10 times in phosphate buffer and attachment was quantified by randomly screening at least 100 developing root hairs by phase-contrast microscopy. Attachment to the root hairs was separated into four classes: class 1, no attached bacteria; class 2, a few bacteria directly attached to the tip of the root hair; class 3, the root hair covered with bacteria; class 4, many attached bacteria, forming a caplike aggregate at the tip of the root hair (cap formation). The percentage of each class present was calculated.

Purification of extracellular fibrils. Rhizobia were grown in 5 liters of TY medium and harvested at an A_{620} value at which agglutination to glass started (see also reference 26). After growth of the bacteria under low Ca^{2+} conditions (1/20th or less of the usual Ca^{2+} supply), the cells in cultures with identical A_{620} values were harvested by centrifugation for 15 min in a Sorvall RC5B centrifuge with a GS3 rotor at 7,000 rpm. Fibrils were isolated by the trifluoroacetic acid extraction procedure for cellulose of Romanovicz and Brown (23). Briefly, pelleted cells were extracted with 0.5 N trifluoroacetic acid for 3 h at 37°C and centrifuged at 10,000 rpm for 15 min with an SS34 rotor. The pellet was subse-

quently reextracted with 2 N trifluoroacetic acid for 3 h at 121°C. Fibrils were purified by sedimentation in an Eppendorf centrifuge for 15 s at maximum speed, followed by three washings with deionized water, which yielded a white pellet.

Electron microscopy. Electron microscopy of bacteria and purified fibrils was performed after negative staining with phosphotungstic acid with a Philips EM300 electron microscope operating at 60 kV. Isolated fibrils were stained with a 2% phosphotungstic acid solution (pH 7.2), whereas a phosphotungstic acid concentration of 1% was found to be the optimal concentration for staining bacteria.

Cellulase purification and treatment. Grade C cellulase (Sigma) was further purified by gel filtration with Sephacryl S-300 (Pharmacia, Uppsala, Sweden) as the matrix and 25 mM phosphate buffer (pH 7.5) as the eluent. Based on the sizes of the elution peaks, impurities were estimated to represent approximately 40% of the total crude preparation. The eluted fractions were dialyzed for 18 h against 10-fold-diluted phosphate buffer at 4°C, lyophilized, and stored at −20°C. During cellulase purification the temperature was kept at 4°C and the pH was kept at 7.5 to prevent the enzyme from degrading the gel filtration matrix nor the dialysis tubing.

Cellulase activity of the fractions was tested by incubating 1 mg of cellulose (Sigma) with 1 mg of enzyme fraction for 72 h at 37°C in 100 mM sodium citrate buffer, pH 5.0. The released sugars were visualized by thin-layer chromatography as described below. Cellulase eluted as the first peak and was found to be free of impurities, as judged by silver staining of sodium dodecyl sulfate-polyacrylamide gels (14, 30). Only purified cellulase was used in further experiments. Bacteria harvested at an A_{620} value of 0.70 by centrifugation for 30 s in an Eppendorf centrifuge at maximum speed were suspended to a final A_{620} value of 0.35 in 100 mM sodium citrate buffer (pH 5.0) and treated with cellulase at a final concentration of 1 mg/ml in sodium citrate buffer for 2 h at 28°C. Subsequently, the bacteria were harvested by centrifugation in an Eppendorf centrifuge and suspended to a final A_{620} value of 0.070 in 25 mM phosphate buffer (pH 7.5) for testing their attachment ability. Controls were incubated in sodium citrate buffer without cellulase and, if appropriate, supplemented with 10 mM glucose.

Characterization of fibrils. Purified fibrils were hydrolyzed with 6 N HCl for 24 h at 100°C or digested with cellulase at a final concentration of 1 mg/ml in 100 mM sodium citrate buffer (pH 5.0) for 72 h at 37°C. The released sugars were identified by comparison with standards by thin-layer chromatography on cellulose sheets (Sigma) developed with *n*-butanol–pyridine–water (6:4:3, vol/vol/vol). Spots were visualized by spraying with a solution of 1.27% *p*-anisidine and 0.166% phthalic acid in ethanol (17). The enzymatically treated fibrils were also investigated by electron microscopy. Isolated fibrils were further characterized by infrared spectroscopy. Spectra were recorded on a Philips Uncicin spectrophotometer with a KBr pellet.

RESULTS

Fibril purification and characterization. Initially, the fibrils were thought to be proteinaceous filamentous fimbriae, since fimbriae were found to be involved in a number of plant-bacterium associations (5, 9, 25, 28). However, fimbria isolation procedures, e.g., the method of Korhonen et al. (13), were unsuccessful (G. Smit, unpublished data).

Fibril purification was successful when procedures for the isolation of cellulose (fibrils) were used. Purified fibrils usually consisted of aggregated bundles (Fig. 1). The purified fibrils had the same characteristics as the fibrils observed on the cell surface of the bacteria (26). The yield of the fibril-overproducing strain RBL1465 (see below) was approximately sixfold higher than that of the wild-type *R. leguminosarum* 248, 2.4 and 0.4 mg, respectively. The yield of strain 5523 was 3.2 mg. Enzymatic digestion of purified fibrils of *R. leguminosarum* 248, RBL5523, and RBL1465 for 72 h with cellulase resulted in liberation of glucose as the only sugar as judged by thin-layer chromatography (data not shown). As a control experiment, purified fibrils of *R. leguminosarum* 248 digested with cellulase were screened for fibrils by electron microscopy. Fibrils could no longer be detected after this treatment. Infrared spectra of fibrils purified from *R. leguminosarum* 248, RBL5523, and the fibril-overproducing mutant RBL1465 were identical to the infrared spectrum of commercial cellulose (Fig. 2). These results show that the extracellular fibrils are composed of cellulose.

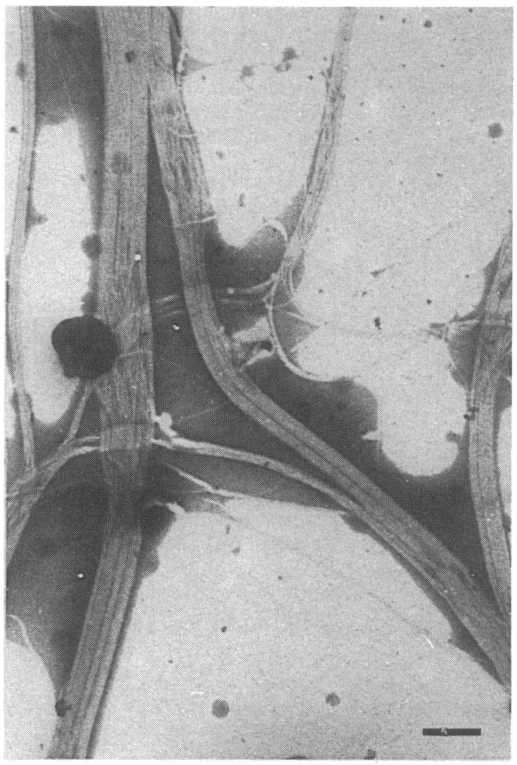

FIG. 1. Electron micrograph of purified fibrils of *R. leguminosarum* 248. Fibrils were negatively stained with phosphotungstic acid. Bar, 200 nm.

Effect of purified cellulose fibrils, commercial cellulose, and carboxymethyl cellulose on attachment. Isolated cellulose fibrils of *R. leguminosarum* 248 as well as commercial cellulose and water-soluble carboxymethyl cellulose inhibited the attachment of *R. leguminosarum* 248 when they were added to the bacteria just before incubation with pea roots (Table 2).

Isolation and characterization of fibril-overproducing and fibril-negative Tn*5* mutants. Since both *R. leguminosarum* 248 and RBL5523 produce extracellular fibrils, have a strong ability to attach to pea root hairs, and are able to nodulate peas, these strains were chosen as parent strains for Tn*5* mutagenesis. Of 10,000 tested Tn*5* mutants of *R. legumino-*

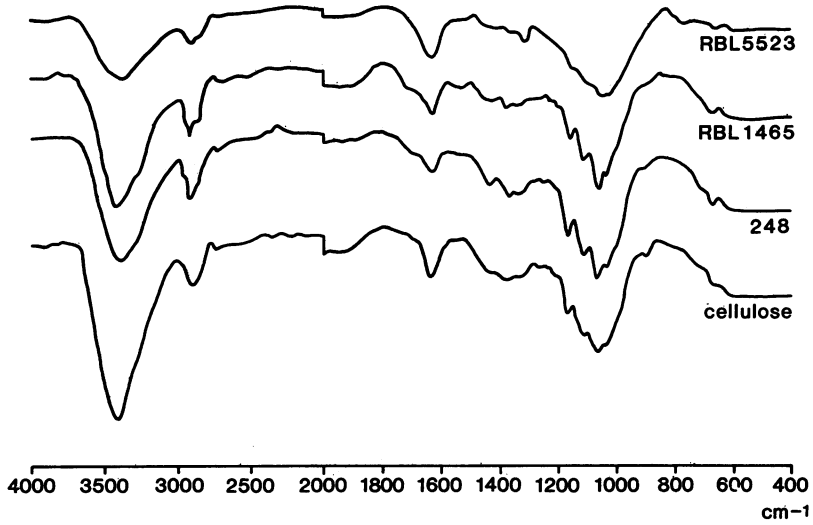

FIG. 2. Infrared absorption spectra of purified extracellular fibrils of *R. leguminosarum* 248, RBL5523, and the fibril-overproducing strain RBL1465 and of cellulose (Sigma). Spectra were measured with a KBr pellet. Infrared spectra of purified extracellular fibrils from these strains grown under low Ca^{2+} conditions were identical to the spectra shown in this figure.

sarum 248, 5 mutants showed a brighter fluorescence on agar in the presence of CFW (Fig. 3B). No fluorescence-negative mutants could be detected in the presence of CFW, presumably owing to the low fluorescence of the parent strain 248. The fibril-overproducing strains had an increased autoagglutinating ability, designated as flocculation (8), which was visible with the naked eye (Fig. 4B). Electron microscopic examination of these strains showed an overproduction of extracellular fibrils during all growth phases, particularly visible within autoagglutinated cell clumps.

From *R. leguminosarum* RBL5523, 4 of 1,000 tested Tn*5* mutants did not show fluorescence in the presence of CFW (Fig. 3D). No mutants with a brighter fluorescence could be isolated, which is presumably due to the high fluorescence of the parent strain RBL5523. In batch culture, the mutant strains did not show flocculation (Fig. 4D), and extracellular fibrils could not be detected by electron microscopy.

For both the fibril-overproducing strains of *R. leguminosarum* 248 and the fibril-negative strains of *R. leguminosarum* RBL5523 a low frequency of Kan^r transconjugants ($<10^{-6}$) was found in mating experiments with Sym plasmid-cured *R. leguminosarum* strains, which makes it very likely that none of the Tn*5* insertions is located on the Sym plasmid. Hybridization of *Bam*HI-digested total DNA with a ^{32}P-labeled Tn*5* probe showed two bands for every strain, owing to the presence of a unique *Bam*HI restriction site in the Tn*5* transposon. Hybridization resulted in fragments of different sizes for all mutants, indicating that the Tn*5* insertions are independent from each other. DNA from the wild-type strains did not show hybridization.

Attachment and nodulation ability of fibril-overproducing and fibril-negative mutants. Fibril-overproducing strains of *R. leguminosarum* 248 showed an increased attachment ability (Table 3). Although the percentage of class 4 attachment (cap formation) was only moderately increased, the size of the caps on the root hair tips was greatly increased

TABLE 2. Influence of purified cellulose fibrils of *R. leguminosarum* 248, commercial cellulose, and carboxymethyl cellulose on attachment of *R. leguminosarum* 248 cells to pea root hair tips[a]

Treatment	% Attachment in class[b]:			
	1	2	3	4
None	7	33	8	52
Purified cellulose fibrils	18	72	5	5
Commercial cellulose	32	61	1	6
Carboxymethyl cellulose	21	66	5	8

[a] Bacteria were harvested at an A_{620} value of 0.70. Purified cellulose, commercial cellulose, and carboxymethyl cellulose were added to the bacterial suspension just before the addition of the roots at a final concentration of 1 mg/ml.

[b] Class 1, No attached bacteria; class 2, few attached bacteria; class 3, apical portion of the root hair covered with bacteria; class 4, many attached bacteria forming a caplike structure on top of the root hair.

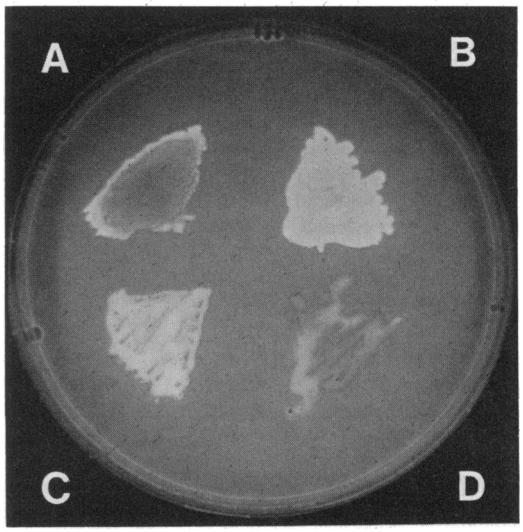

FIG. 3. Fluorescence under UV light of *R. leguminosarum* 248 (A), its fibril-overproducing mutant RBL1465 (B), strain RBL5523 (C), and its fibril-negative mutant RBL5760 (D). Rhizobia were cultivated on plates containing solid RMM medium supplemented with CF White at a final concentration of 0.02%.

TABLE 3. Attachment of wild-type rhizobia and their fibril-overproducing and fibril-negative mutants to pea root hair tips[a]

R. leguminosarum strain	Fibrillation[b]	% Attachment in class[c]:			
		1	2	3	4
248	+	0	25	17	58
RBL1465	++	4	15	4	77[d]
RBL1466	++	6	17	7	70[d]
RBL1467	++	3	17	9	71[d]
RBL1468	++	1	22	10	67[d]
RBL1469	++	2	17	7	74[d]
RBL5523	+	0	3	3	94
RBL5760	−	40	55	5	0
RBL5761	−	37	60	2	1
RBL5762	−	32	67	0	1
RBL5763	−	32	68	0	0

[a] Bacteria were harvested at A_{620} values at which agglutination of bacteria to glass started.
[b] +, Wild type; ++, fibril-overproducing mutant; −, fibril-negative mutant.
[c] See Table 2, footnote *b*.
[d] The size of caps was strongly increased in comparison with the size of the caps formed by the wild-type strain *R. leguminosarum* 248.

(data not shown). This indicates that the stronger attachment ability is mainly due to bacterial aggregation. Fibril-negative strains almost completely lost both class 3 and class 4 attachment (Table 3), which proves that the presence of fibrils is a prerequisite for cap formation. Neither fibril-overproducing mutants nor fibril-negative mutants were affected in their ability to agglutinate to glass. Also, neither of the mutants was significantly affected in their ability to nodulate peas and common vetch (data not shown).

Effect of cellulase treatment of whole cells on attachment properties. Incubation of *R. leguminosarum* 248 with purified cellulase just before the pea root attachment assay caused a strong decrease in both class 3 and class 4 (cap formation) attachment of the bacteria but did not decrease class 2 attachment. Instead, a shift from class 3 and 4 to class 2 attachment was observed. A control incubation with or without 10 mM glucose had no effect (Table 4).

Flocculation was observed during growth in batch culture of strain RBL5523 and fibril-overproducing mutants of strain 248, whereas fibril-negative mutants of strain RBL5523 did not flocculate at all (Fig. 4). Supplementation of TY medium with cellulase (1 mg/ml) resulted in a strong inhibition of flocculation of strain RBL5523 and the fibril-overproducing strain RBL1465, whereas the growth rate was not affected. Interestingly, agglutination of the bacteria to glass was not affected by the presence of cellulase in the growth medium.

TABLE 4. Influence of cellulase pretreatment of *R. leguminosarum* 248 cells on attachment to pea root hair tips[a]

Pretreatment	% Attachment in class[b]:			
	1	2	3	4
None	7	14	11	68
Cellulase	21	77	2	0
Glucose	4	21	6	69

[a] Bacteria were harvested at an A_{620} value of 0.70 and suspended in 100 mM sodium citrate buffer (pH 5.0) supplemented with either 1 mg of cellulase per ml or 10 mM glucose. After 2 h of incubation at 28°C, the bacteria were harvested by centrifugation, suspended in phosphate buffer, and used in the attachment assay. The control was treated under the same conditions except that cellulase and glucose were absent during the preincubation.
[b] See Table 2, footnote *b*.

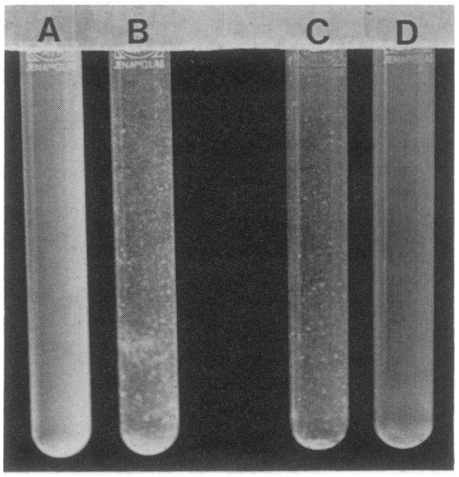

FIG. 4. Flocculation of *R. leguminosarum* 248 (A), its fibril-overproducing mutant RBL1462 (B), RBL5523 (C), and its fibril-negative mutant RBL5760 (D), grown in TY medium (with standard Ca^{2+} content), at A_{620} values at which agglutination of bacterial cells to glass started.

Growth with low Ca^{2+} reveals a second adhesin. Changing the Ca^{2+} concentration in TY medium strongly affected the ability of bacteria grown in TY medium to agglutinate to glass. Rhizobial strains grown with 50% or less of the standard Ca^{2+} content of 7 mM no longer formed a ring of agglutinated cells at the air-liquid interphase during early stationary growth (Fig. 5; Table 5). Rhizobial strains cultivated in TY medium supplemented with two to five times the standard Ca^{2+} content showed an increased ability to agglutinate to glass. Also the A_{620} value at which agglutination to

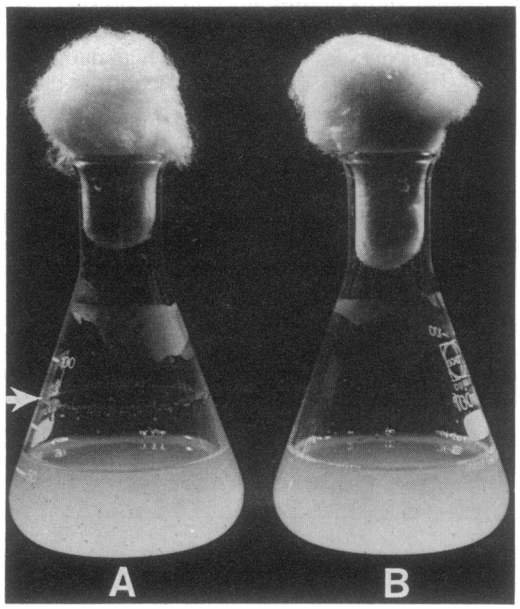

FIG. 5. Influence of Ca^{2+} concentration in the medium on the ability of *R. leguminosarum* 248 to agglutinate to glass at an A_{620} value of 0.70. (A) *R. leguminosarum* 248 grown in TY medium with the standard Ca^{2+} content of 7 mM; (B) *R. leguminosarum* 248 grown under low Ca^{2+} (0.35 mM) conditions. Agglutination to glass is visible as a ring of agglutinated bacteria at the air-liquid interface (arrow).

TABLE 5. Influence of Ca^{2+} concentration in TY medium on adhesion of *R. leguminosarum* 248 cells to glass and to pea root hairs[a]

Ca^{2+} conc (mM)[b]	Initiation of agglutination to glass (A_{620} value)	% Attachment in class[c]:			
		1	2	3	4
0.14	—[d]	64	32	0	4
0.35	—	54	28	4	14
0.70	—	40	48	2	10
1.4	—	39	49	6	6
3.5	—	31	43	9	17
7.0	0.65	6	26	13	55
14.0	0.40	6	26	17	51
28.0	0.18	17	13	18	52

[a] Bacteria were harvested at an A_{620} value of 0.70.
[b] The Ca^{2+} concentration in standard medium is 7 mM.
[c] See Table 2, footnote *b*.
[d] No agglutination to glass was observed.

glass started shifted to lower values in the latter cases (Table 5).

Low Ca^{2+} concentrations affected neither the flocculation of the bacteria nor the synthesis of extracellular fibrils, the latter result judged by electron microscopy, quantification, and infrared spectroscopy of purified fibrils (data not shown).

R. leguminosarum 248 grown in TY medium at 5% or less of the standard Ca^{2+} content showed a strong decrease in the ability to attach to pea root hair tips, as was illustrated by an increased percentage of root hairs without any rhizobia attached (class 1) and hardly any cap formation (class 4). In contrast, increasing the Ca^{2+} concentration in the growth medium did not affect attachment ability (Table 5). Also, for *R. leguminosarum* RBL5523, *R. trifolii* 0403, *R. phaseoli* 1233, *Agrobacterium tumefaciens* 1251, and the described fibril-overproducing and fibril-negative mutants of *R. leguminosarum* 248 and RBL5523, respectively, adherence properties to glass as well as to pea root hair tips were strongly reduced by decreasing the Ca^{2+} concentration in the medium (Table 6).

The addition of EDTA and ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) in concentrations up to 10 mM during the attachment assay did not affect the attachment ability of *R. leguminosarum* 248 (data not shown). After growth of *R. leguminosarum* 248 under low Ca^{2+} conditions but in the presence of 7 mM $SrCl_2$, ability to attach to glass and to pea root hair tips was similar to that of bacteria grown in the standard TY medium. In contrast, the presence of 7 mM $MgCl_2$ instead of $CaCl_2$ restored the ability to attach to pea root hair tips only weakly and the ability to agglutinate to glass not at all (Table 6). Autoagglutinating ability was not affected by either $SrCl_2$ or $MgCl_2$.

DISCUSSION

Role of cellulose fibrils in attachment of *Rhizobium* cells to pea root hair tips. The extracellular fibrils produced by *R. leguminosarum* 248 and RBL5523 were found to be composed of cellulose (Fig. 4). The fibrils were 5 to 6 nm in diameter (26), which is smaller than the diameter of cellulose microfibrils isolated in other laboratories from *Rhizobium* spp. (8) and *A. tumefaciens* (17). Interestingly, the diameter of the fibrils under study in our laboratory is similar to the diameter of cellulose microfibrils in plant cell walls (4).

Purified cellulose fibrils, commercial cellulose, and carboxymethyl cellulose added to the rhizobia just before the addition of pea roots caused a strong reduction in cap formation (Table 2). Fibril-negative mutants as well as

TABLE 6. Effect of divalent cations on adherence properties of a number of members of the family *Rhizobiaceae*

Strain	Divalent cations in growth medium (mM)	Ability to agglutinate to glass[a]	% Attachment to pea root hair tips[b] in class[c]:			
			1	2	3	4
R. leguminosarum 248	Ca^{2+} (7.0)[d]	+	6	26	13	55
R. leguminosarum 248	Ca^{2+} (0.35)	−	64	33	0	3
R. leguminosarum 248	Sr^{2+} (7.0)	+	2	30	5	63
R. leguminosarum 248	Mg^{2+} (7.0)	−	13	35	19	33
R. leguminosarum RBL1466	Ca^{2+} (7.0)[d]	+	6	17	7	70
R. leguminosarum RBL1466	Ca^{2+} (0.35)	−	38	28	7	27
R. leguminosarum RBL5523	Ca^{2+} (7.0)[d]	+	0	3	3	94
R. leguminosarum RBL5523	Ca^{2+} (0.35)	−	18	72	3	7
R. leguminosarum RBL5762	Ca^{2+} (7.0)[d]	+	32	67	0	1
R. leguminosarum RBL5762	Ca^{2+} (0.35)	−	67	31	1	1
R. trifolii 0403	Ca^{2+} (7.0)[d]	+	2	33	15	50
R. trifolii 0403	Ca^{2+} (0.35)	−	49	42	4	5
R. phaseoli 1233	Ca^{2+} (7.0)[d]	+	7	42	10	41
R. phaseoli 1233	Ca^{2+} (0.35)	−	56	35	5	4
A. tumefaciens 1251	Ca^{2+} (7.0)[d]	+	11	30	11	48
A. tumefaciens 1251	Ca^{2+} (0.0)	−	56	33	3	8

[a] Agglutination to glass was visible as a ring of agglutinated bacteria at the air-liquid interphase.
[b] Bacteria for attachment assays were harvested at A_{620} values at which agglutination to glass started. Bacteria grown at a low Ca^{2+} concentration were harvested at identical A_{620} values.
[c] See Table 2, footnote *b*.
[d] Represents the standard growth medium.

cellulase-treated wild-type strains were unable to form caps (Table 3). It may be argued, however, that cellulase treatment might reduce the attachment ability in an indirect way, namely, by liberating glucose, thereby abolishing carbon limitation, the condition which was found to result in optimal attachment ability (26). Since the presence of 10 mM glucose did not affect attachment (Table 2), abolishment of carbon limitation cannot be responsible for the reduced attachment ability of the rhizobia after cellulase treatment. We therefore conclude that cellulose fibrils are involved in the attachment of rhizobia to pea root hair tips.

Cellulose fibrils were also involved in the attachment of the closely related *A. tumefaciens* to carrot tissue culture cells (16). In *A. tumefaciens*, however, the fibrils are synthesized during the attachment process, possibly as a response to molecules of plant origin, whereas in *Rhizobium* species the fibrils are already present before incubation with the plant roots. Since an incubation of *R. leguminosarum* 248 with purified cellulase just before the attachment assay yielded only a low level of cap formation (Table 4), it seems reasonable to suppose that fibril production by *Rhizobium* species is not strongly induced by plant roots during the attachment assay, during which cellulase is not present.

Do cellulose fibrils play a role in nodulation? Fibril-negative mutants of *R. leguminosarum* RBL5523 as well as the fibril-overproducing mutants of *R. leguminosarum* 248 showed a normal nodulation behavior on pea and common vetch, indicating that cellulose fibrils, and thus the ability to form caps, are not essential for nodulation. Similarly, it has been reported that *A. tumefaciens* cellulose-negative mutants retain the ability to induce tumors, although indications were found that such mutants are less virulent under certain conditions (16). It is therefore clear that the possibility that fibrils are important under field conditions cannot be excluded; e.g., they might increase the competitiveness of the strain.

A second, Ca^{2+}-dependent adhesin. Ca^{2+} limitation is the only growth limitation found up to now which leads to poor attachment to root hair tips (Table 5). Nevertheless, fibril synthesis was not affected under low Ca^{2+} conditions as judged from fibril isolation, electron microscopic examination, and flocculation of bacteria in liquid medium. A low Ca^{2+} concentration in TY medium caused a decrease in the ability of the rhizobia to agglutinate to glass (Fig. 5), whereas previous experiments had shown that this ability was not affected by loss of fibrils. Therefore, low Ca^{2+} conditions lead to the simultaneous loss of the abilities to adhere to pea root hair tips and to glass. These results indicate the existence of a second, Ca^{2+}-dependent adhesin. The presence of EDTA or EGTA during the attachment assay did not inhibit the ability of *R. leguminosarum* 248 to attach to pea root hair tips, indicating that Ca^{2+} is not directly involved in the attachment process. Moreover, $SrCl_2$ and, to lesser extent, $MgCl_2$ were able to replace $CaCl_2$ in the medium without affecting the properties of *R. leguminosarum* 248 to adhere to pea root hairs, to autoagglutinate, and to agglutinate to glass (Table 6). These results indicate that the requirement for Ca^{2+} is not absolute but that a divalent cation is essential for synthesis, assemblage, or exposure of this adhesin.

The Ca^{2+}-dependent adhesin appears to be a common adhesin, since all rhizobial strains tested, including *A. tumefaciens*, lost the ability to agglutinate to glass as well as the ability to attach to pea root hair tips when the bacteria were cultured under low Ca^{2+} conditions (Table 6).

Location of genetic information for the two adhesins. Genetic examination of fibril mutants showed that all Tn*5* insertions were independent from each other and that they were not located on the Sym plasmid. The latter result is consistent with earlier observations (26) that the Sym plasmid-cured strains *R. leguminosarum* 248c and *R. trifolii* RBL5039 are indistinguishable in fibril formation and attachment behavior from their parental strains. It is likely that the Ca^{2+}-dependent adhesin is also not located on the Sym plasmid since Sym plasmid-cured strains of *R. leguminosarum* and *R. trifolii* as well as Ti plasmid-cured *A. tumefaciens* were not affected in their ability to agglutinate to glass and to adhere to pea root hair tips (26; unpublished data).

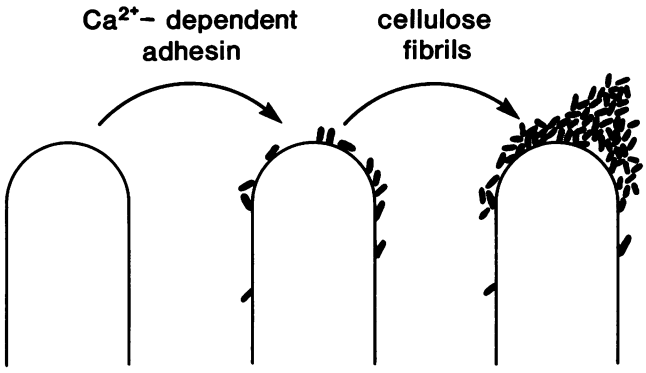

FIG. 6. Model for rhizobial attachment to pea root hair tips. Step 1 attachment is Ca^{2+} dependent and leads to the adhesion of single rhizobial cells to the tip of the root hair. Step 2 attachment is cellulose fibril dependent and results in formation of aggregates of bacteria on the tip of the root hair (caps).

Two-step model for root hair tip attachment. Since neither fibril-negative mutants nor wild-type rhizobia grown under Ca^{2+} limitation are able to form caps, both cellulose fibrils and the Ca^{2+}-dependent adhesin(s) must be involved in this attachment process. The results can be explained by a two-step attachment mechanism (Fig. 6). In the first step, in which the Ca^{2+}-dependent adhesin is involved, single rhizobial cells adhere to the surface of the root hairs (class 2 attachment). In the second step, other rhizobia adhere to the root hair-bound bacterial cells by interaction of cellulose fibrils in a process of autoagglutination, resulting in cap formation (class 4 attachment). If the first, Ca^{2+}-dependent step is affected, neither class 2 and 3 attachment nor cap formation will be observed. Flocculation of bacteria, however, will still occur since fibril synthesis is not affected under low Ca^{2+} conditions, and the few observed remaining caps are probably caused by aspecific adhesion of bacterial flocs to the root hair tip. Prevention of the second step, synthesis of cellulose fibrils, will also result in a strong inhibition of cap formation, but a single layer of rhizobia on the top of the root hair can still be formed corresponding with a relative high percentage of class 2 attachment, and the rhizobia retain the ability to infect the host plant. It has been suggested earlier (26) that cap formation is mainly due to bacterial autoagglutination. Consistent with this notion is the observation that autoagglutination of bacteria, flocculation, is positively correlated with fibrillation (Fig. 2) (8, 19). Proof that cellulose fibrils are involved in flocculation as well as in cap formation was obtained from the observations that fibril-overproducing strains strongly flocculate when grown in liquid TY medium and form caps on pea root hairs with a greatly increased size (Table 3; Fig. 4) and that fibril-negative mutants do not flocculate in liquid medium and do

not form caps on pea root hair tips (Table 3; Fig. 4). In conclusion, it seems most likely that cap formation is due to bacterial autoagglutination. Our results are consistent with this model and show that screening for class 4 attachment is useful for isolation of attachment-negative mutants affected in step 1 or 2, although cap formation itself is not essential for nodulation. This model has similarities with the model proposed by Matthysse et al. (17) for attachment of *A. tumefaciens* to carrot tissue culture cells in that both models propose two steps in which the second step is mediated by cellulose fibrils.

Our current research is focused on the isolation of mutants affected in the first step of the attachment process and on the characterization of the Ca^{2+}-dependent adhesin(s) to determine its (their) role in the nodulation process.

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