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Roles of Flagella, Lipopolysaccharide, and a Ca^{2+}-Dependent Cell Surface Protein in Attachment of *Rhizobium leguminosarum* Biovar *viciae* to Pea Root Hair Tips

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The relationship between Ca^{2+}-dependent cell surface components of *Rhizobium leguminosarum* biovar *viciae*, motility, and ability to attach to pea root hair tips was investigated. In contrast to flagella and lipopolysaccharide, a small protein located on the cell surface was identified as the Ca^{2+}-dependent adhesin.

Attachment of rhizobia to developing root hairs is one of the first steps of the nitrogen-fixing root nodule symbiosis between rhizobia and the leguminous host plants. Recently, we reported that both cellulose fibrils and a Ca^{2+}-dependent adhesin of *Rhizobium leguminosarum* bv. *viciae* cells are involved in the two-step process of attachment of rhizobia to pea root hair tips (13). In the study reported here, the influence of Ca^{2+} limitation on motility and surface components of *R. leguminosarum* cells is described in relation to the ability of the cells to attach to pea root hair tips, and the Ca^{2+}-dependent adhesin is identified as a small cell surface protein.

Ca^{2+} is essential for motility of *R. leguminosarum*. Attachment ability (13) and motility of *R. leguminosarum* 248, harboring Sym plasmid pRL1JI (9), were found to decrease strongly under low-Ca^{2+} conditions. No motility was observed when the Ca^{2+} concentration in TY medium (12) was below 1.4 mM, whereas the growth rate was not affected. An electron microscopic study of rhizobia grown under Ca^{2+} limitation (13) showed that flagella were not present on the cell surface.

Purified flagella are not involved in attachment of *R. leguminosarum*. To determine the possible role of flagella as well as motility in attachment of rhizobia, the adhesin activity of purified flagella and the attachment ability of nonmotile mutants were determined. Flagella from *R. leguminosarum* 248, purified according to the method of Carsiotis et al. (5), appeared to be 12 to 13 nm in diameter and up to 4 μm long as judged by electron microscopy. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11) of purified flagella showed a dominant 32-kilodalton (kDa) protein (Fig. 1, lane I). Crude flagellum preparations from *R. leguminosarum* 248 grown at various Ca^{2+} concentrations, obtained as described above but without the density centrifugation step, showed that under low-Ca^{2+} conditions the 32 kDa band was missing, whereas the densities of several other bands had increased (data not shown). These data demonstrate that the 32-kDa protein represents the flagellar subunit of *R. leguminosarum*.

Thirty-three nonmotile Tn*5* mutants of *R. leguminosarum* 248 were isolated (6, 13) and examined for the presence of flagella by electron microscopy, and crude flagellum preparations isolated from these mutants were investigated by using SDS-PAGE. The nonmotile mutants could be divided into three classes. Class 1 contained mutants consisting of flagellumless cells that also lacked the major 32-kDa flagellin band in the crude flagellum preparation (strains RBL1484 through RBL1495); class 2 consisted of mutants that still possessed flagella but lacked an 18-kDa band present in the crude flagellum preparation (strains RBL1496 through RBL1507); and class 3 consisted of mutants possessing flagella and with a gel electrophoresis pattern of the crude flagellum preparations similar to that of the wild-type strain (RBL1508 through RBL1516) (Fig. 1). Mutants from classes 1 and 3 were indistinguishable from the wild-type strain with respect to attachment and nodulation ability on pea and common vetch, which indicated that motility and exposure of flagella are not essential for nodulation of *R. leguminosarum*.

An adhesin was experimentally defined as a surface component of rhizobia able to inhibit attachment of rhizobial cells to pea root hairs when supplied before or during an attachment assay. Attachment of *R. leguminosarum* was affected neither by incubation of the roots with purified flagella before incubation with the bacteria (Table 1) nor by addition of flagella during the attachment assay (data not shown). Taken together, these results demonstrate that flagella are not involved in attachment of *R. leguminosarum* and that reduced attachment ability as a result of Ca^{2+} limitation is not due to loss of flagella or motility.

Attachment of nonmotile mutants affected in lipopolysaccharide composition. Twelve nonmotile mutants were found to lack an 18-kDa band as judged by SDS-PAGE of crude flagellum preparations derived from these strains (Fig. 1, lanes D through F). These nonmotile mutants appeared to be LPS mutants. LPS was isolated, according to the method of Westphal and Jann (15), from the wild-type strain and from a number of class 2 mutants. Analysis of LPS from *R. leguminosarum* 248 by SDS-PAGE revealed two bands of differing molecular masses running at positions of proteins with apparent molecular masses of 18 and 12 kDa, respectively (Fig. 2, lane A). Similar results were described by Carlson et al. (3, 4) for LPS of *R. leguminosarum* biovars *trifolii* and *phaseoli*; in their studies, the lower-molecular-weight band appeared to represent the lipid A and core part of the LPS and the higher-molecular-weight form appeared to represent the complete LPS, consisting of lipid A, core, and O-antigenic polysaccharide. LPS isolated from the class 2 mutants RBL1496, RBL1497, and RBL1500 appeared to lack the high-molecular-weight form of the LPS (Fig. 2, lanes B and C) and therefore most likely the O-antigenic polysaccharide part of the LPS and perhaps part of the core. Mutant

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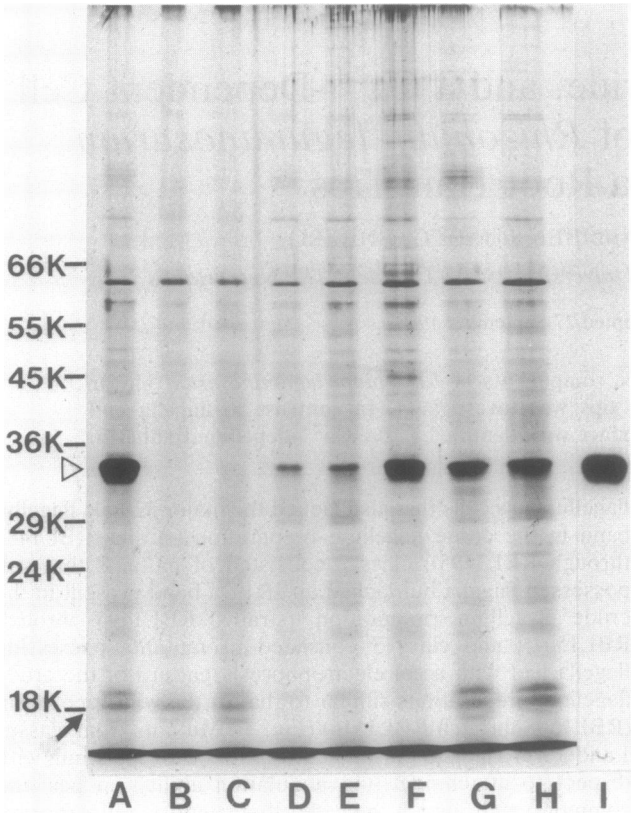

FIG. 1. SDS-PAGE of crude flagellum preparations of *R. leguminosarum* 248 and some nonmotile mutants. Lanes: A, *R. leguminosarum* 248; B, RBL1484 (class 1); C, RBL1485 (class 1); D, RBL1496 (class 2); E, RBL1497 (class 2); F, RBL1500 (class 2); G, RBL1508 (class 3); H, RBL1509 (class 3); I, purified flagella of *R. leguminosarum* 248. Positions of molecular mass markers are indicated at the left (size given in kilodaltons). Symbols: ▷, flagellin; ➡, higher-molecular-weight form of the LPS. Note that the staining procedure used (2) stains both proteins and LPSs.

TABLE 1. Influence of various Ca^{2+}-dependent cell surface components from *R. leguminosarum* 248 on attachment of *R. leguminosarum* 248 cells to pea root hair tips[a]

Deviation from standard assay	% Attachment in class:[b]			
	1	2	3	4
None	10	28	12	50
Flagella	8	25	13	54
LPS	6	28	16	50
CSP[c]				
7.0 mM Ca^{2+}	47	26	19	8
0.35 mM Ca^{2+}	13	19	9	59
CSP, 7.0 mM Ca^{2+}				
Heat treated[d]	9	30	6	55
Protease treated[e]	3	31	11	55
Protease[e]	15	16	11	58
CSP 7.0 mM Ca^{2+}[f]				
>30 kDa	17	16	12	55
<30 kDa	50	22	11	17
>5 kDa	39	33	12	16
<5 kDa	12	19	9	60

[a] Bacteria was harvested at an A_{620} of 0.70, suspended, and added to the pea roots in a final concentration of 1.5×10^8 to 2.0×10^8 cells per ml (12). Roots were incubated with flagella (100 μg/ml), LPS (250 μg/ml), cell surface preparation (CSP), proteinase K (200 μg/ml), or potassium phosphate buffer for 60 min, washed, and incubated with the bacteria.

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

[c] Cell surface preparations (200 μl) derived from 10 ml of *R. leguminosarum* 248 culture, grown at Ca^{2+} concentrations of 7.0 and 0.35 mM, were added to the roots.

[d] Cell surface preparation derived from rhizobia grown under normal Ca^{2+} conditions was incubated at 100°C for 5 min before incubation with the roots.

[e] Cell surface preparation was incubated with proteinase K (1 mg/ml) at 37°C for 60 min before incubation with the roots. As a control, roots were incubated for 60 min at room temperature with proteinase K before incubation with bacteria.

[f] Cell surface preparations were separated into two fractions by ultrafiltration, using a 30- and a 5-kDa membrane. Equal amounts corresponding to a cell surface preparation derived from 10 ml of culture were used in the experiments.

RBL1500 was an exception in that it was found to yield an additional band (Fig. 2, lane D). This result might be attributable to a reduced length of the O-antigenic polysaccharide part of the LPS or to a lack of putative side chains in the O-antigenic repeating unit. These results indicate that the LPS of *R. leguminosarum* is involved in motility of the bacteria, as has been found for LPS mutants of other gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium* (1, 7).

Since LPS has repeatedly been proposed to be involved in attachment of members of the family *Rhizobiaceae* to host plant cells (8, 10, 16) and since the 18-kDa band in crude flagellum preparations was found to strongly increase under Ca^{2+}-limiting conditions, we studied the possibility that LPS is involved in attachment to pea root hairs. LPS isolated from *R. leguminosarum* 248 and from strains RBL1496, RBL1497, and RBL1500, added to the roots in concentrations of up to 250 μg/ml before the attachment assay, did not inhibit attachment of *R. leguminosarum* 248 (Table 1). In attachment assays in which the LPS was added during the attachment assay, the size of the caps (class 4 attachment; 12) was even increased (data not shown). With one exception, attachment of LPS mutants of *R. leguminosarum* was similar to that of the wild-type strain 248. However, since LPS mutants were found to adhere optimally to pea root hairs at earlier phases during growth in batch culture than did the wild-type strain, the LPS might be involved indirectly in attachment, e.g., in masking of adhesins on the cell surface of the bacteria. Comparable results were found for O-antigen-less LPS mutants of uropathogenic *E. coli* (14). The LPS mutants nodulated pea and common vetch, although nodulation on the latter host plant was delayed for 3 to 7 days. Taken together, these results demonstrate that LPS is not directly involved in the attachment process.

One LPS mutant, strain RBL1500, showed a reduced ability to attach to pea root hair tips. This strain was found to be affected in the second step of the attachment process, and since cellulose fibril isolation (13) revealed that this mutant does not produce cellulose fibrils, it is very likely that this pleiotropic effect causes the altered phenotype with respect to attachment.

The Ca^{2+}-dependent adhesin of *Rhizobium* appears to be a soluble surface protein. The supernatant, and not the flagellum-containing pellet, obtained after the ultracentrifugation step in flagellum purification appeared to possess attachment-inhibiting activity (Table 1), which indicated that this fraction contained an adhesin which was detached from the bacteria together with the flagella. This fraction is called the cell surface preparation. Adhesin activity was found both when the cell surface preparation was incubated with the pea roots before the attachment assay as well as during the

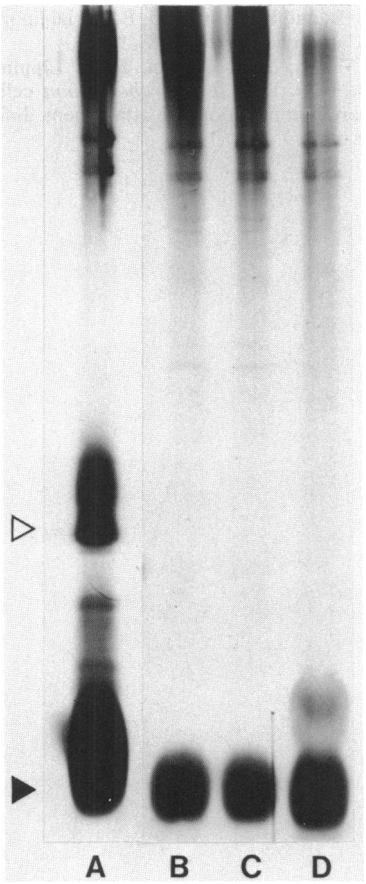

FIG. 2. SDS-PAGE of isolated LPS of *R. leguminosarum* 248 (lane A), RBL1496 (lane B), RBL1497 (lane C), and RBL1500 (lane D). Equal amounts (2.5 μg) of LPS were applied in all slots. Symbols: ▷, higher-molecular-weight form; ▶, lower-molecular-weight form.

attachment assay, although in the former case attachment-inhibiting activity was higher, a result most likely due to a lack of competition between the adhesin and the bacteria. The attachment-inhibiting factor resulted in a high percentage of root hairs without attached bacteria (Table 1), which indicated that this factor is involved in the first step of the attachment process (see also reference 13). Cell surface preparations isolated from representatives of the three nonmotile mutant classes, including strain RBL1500, were all found to possess attachment-inhibiting activity, which indicated that none of the nonmotile mutants was affected in the synthesis of this adhesin (data not shown).

To determine whether the adhesin present in the cell surface preparation is Ca^{2+} dependent, a cell surface preparation was isolated from *R. leguminosarum* 248 grown under low-Ca^{2+} conditions. This fraction did not possess any attachment-inhibiting activity (Table 1), which makes it very likely that the adhesin present in the cell surface preparation was the Ca^{2+}-dependent adhesin which mediates the first step in *Rhizobium* attachment.

Partial characterization of the adhesin revealed that it must be a soluble surface component, since no activity was found in the pellet fraction even after prolonged ultracentrifugation for up to 4 h at 100,000 × *g*. Treatment of a cell surface preparation by heat for 5 min at 100°C completely abolished the ability of the preparation to inhibit attachment of *R. leguminosarum* to pea root hair tips (Table 1). Treatment of the cell surface preparation with proteolytic enzymes for 60 min at 37°C also resulted in loss of attachment-inhibiting activity of the adhesin. A control incubation of the roots with protease was necessary, since protease could not easily be removed from the cell surface preparation after the treatment. This control incubation did not affect attachment (Table 1). Ultrafiltration of the cell surface preparation yielded a molecular mass for the adhesin of between 5 and 30 kDa (Table 1). Taken together, these results indicate that the adhesin is a Ca^{2+}-dependent, cell surface-located, water-soluble, heat-labile small protein.

Future research will focus on purification and characterization of the Ca^{2+}-dependent adhesin and on isolation of mutants lacking this adhesin.

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