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Role of Pili (Fimbriae) in Attachment of *Bradyrhizobium japonicum* to Soybean Roots†

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Pili (fimbriae) were observed on cells of each of the five strains of *Bradyrhizobium japonicum* and the one strain of *Rhizobium trifolii* examined. Pili on *B. japonicum* were about 4 nm in diameter and polarly expressed. Piliated cells were estimated by transmission electron microscopy and hydrophobic attachment to polystyrene to constitute only a small percentage of the total population. The proportion of piliated cells in these populations was dependent on culture age in some strains. Piliated *B. japonicum* cells were selectively and quantitatively removed from suspension when cultures were incubated with either soybean roots or hydrophobic plastic surfaces, indicating that pili were involved in the attachment of the bacteria to these surfaces. Pili from *B. japonicum* 110 ARS were purified and found to have a subunit molecular weight of approximately 21,000. Treatment of *B. japonicum* suspensions with antiserum against the isolated pili reduced attachment to soybean roots by about 90% and nodulation by about 80%. Pili appear to be important mediators of attachment of *B. japonicum* to soybean roots under the conditions examined.

The means by which rhizobia adhere to host root surfaces and the specificity of this adhesion have been a matter of interest and uncertainty for some time (36). Several workers have suggested that attachment of rhizobia to roots is host specific and mediated by the binding of lectins on the surface of the host root to specific saccharide receptors on the bacterial cell surface (2, 12–14, 40). However, a number of studies (1, 7, 10, 27, 31, 35, 36, 39, 44) have provided evidence that rhizobia attach nonspecifically, with no involvement of host lectin, to various surfaces, including the roots of their host plants. This suggests that mechanisms other than host lectin binding must determine or contribute importantly to root attachment of rhizobia.

In an earlier study (44), we identified three subpopulations of cells in *Bradyrhizobium japonicum* cultures which were distinct from each other with respect to their attachment capabilities. One class of cells adhered to soybean root surfaces and remained attached despite vigorous rinsing. This was defined as the firmly attaching subpopulation. A second class of cells adhered to soybean roots, but the cells were readily removed by rinsing. These cells were defined as the weakly attaching subpopulation. The third subpopulation consisted of cells that did not adhere to root surfaces. At the culture age optimal for attachment of *B. japonicum* 110 ARS, about 4 to 5% of the cells attached firmly to soybean roots, and 10 to 12% attached weakly. Thus, approximately 16% of the cells were competent to attach in some manner. The remaining 84% of the cells in these cultures gave no indication of any attachment capability (44).

In this paper, we describe the identification and isolation of pili from *B. japonicum* and examine their role in attachment of the bacteria to soybean roots. Pili (fimbriae) are proteinaceous, fibrillar structures on the surfaces of many gram-negative bacteria (33). They commonly mediate attachment to other bacteria for conjugation (sex pili) but can also mediate attachment to host tissues and other substrates, including plant roots (attachment pili) (19, 25, 33, 47). Piluslike structures about 3 nm in diameter have been reported on the slow-growing symbiont *Rhizobium lupini* (21, 29) and on *B. japonicum* (P. Stemmer and L. Sequiera, Phytopathology **71**:906, 1981).

MATERIALS AND METHODS

Bacterial cultures. *B. japonicum* USDA 138 was obtained from D. Weber, U.S. Department of Agriculture, Beltsville, Md., and other strains were obtained as described previously (44). Isolates were stored and cultured as described previously (11).

Transmission electron microscopy. Portions of the cultures to be examined were placed onto grids coated with Formvar and carbon, allowed to incubate for 30 min, blotted with a filter paper wick, and negatively stained with 1% aqueous potassium phosphotungstic acid (pH 7.0). Observations were made with a Philips model 200 electron microscope.

Determination of piliated cells in cultures of rhizobia. Two techniques were used to estimate the percentage of piliated cells. Direct counts were made by examining 100 randomly selected cells by transmission electron microscopy. Where clumps of cells occurred, only those cells that could be clearly examined were counted. The percentage of piliated cells was also estimated indirectly by a method based on the selective attachment of piliated cells to hydrophobic solvents (38). Instead of solvents, polystyrene petri dishes were used as substrates, based on a modification of the assay for measuring the hydrophobicity of cells described by Fletcher (18). Portions of the cultures were placed in standard 9-cm glass and polystyrene petri dishes (catalog no. 3488-B28; Thomas Scientific) so that the liquid just covered the bottom. The dishes were then kept at 6°C for 4 h, allowed to come to room temperature, and gently rocked to suspend unattached bacteria. The bacterial suspensions were decanted, and their optical densities were measured at 620 nm. The percentages of attached, possibly piliated cells were calculated from the differences in optical density between cultures exposed to glass and plastic surfaces.

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The proportion of cells with the capacity to attach to soybean roots after exposure to plastic was also determined. An early-log-phase culture of strain 110 ARS was exposed to glass and plastic petri dishes as described above. When no more cells could be measured binding to the plastic dish (at 2 h), a sample of the supernatant was diluted with YEMG medium (6) to a density of approximately 10^5 bacteria per ml. More than 150 root segments, which were excised from the infectible region of roots of young seedlings of soybean (*Glycine max* (L.) Merr. cv. Williams) as previously described (44), were added to 15 ml of this diluted inoculum, and the mixture was placed on a rotary shaker at 50 rpm. The bacteria remaining free (i.e., unattached) in the culture were then monitored for 1 h by withdrawing and plating replicate samples of the suspension. The results were compared with the results of a similar test of a culture that was not preexposed to plastic.

In order to distinguish pili from cellulose microfibrils, samples of *B. japonicum* 110 ARS cultures at early and mid-log phases were examined by treating cells with Calcofluor white ST (American Cyanamid Co., Pearl River, N.Y.) at pH 8.0 (29). Fungal hyphae were used as positive controls to test whether the Calcofluor white was staining cellulose. For some tests, surface-sterilized carrot disks were added to the culture for coincubation.

Effect of removing bacterial surface structures on attachment. Fifty-milliliter early-log-phase cultures of strain 110 ARS at a density of 2×10^7 cells per ml were blended in a homogenizer (The VirTis Co., Inc., Gardiner, N.Y.) for 5 min. The capacity of the bacteria to attach to soybean root segments was determined at various times after blending. In some cases, chloramphenicol was added immediately after blending to give a final concentration of 5 μg/ml. Attachment to root segments was assayed by direct plate counting of bacteria released by sonic vibration from the surfaces of rinsed segments, as described previously (44). The blended cultures were also examined by transmission electron microscopy for the presence of pili as described above and by binding of fluorescein isothiocyanate-labeled soybean lectin (Vector Laboratories, Burlingame, Calif.) for detection of bacterial capsules as described by Bhuvaneswari et al. (4, 5).

Pilus purification. Pili were isolated from cultures of *B. japonicum* USDA 110 ARS grown on a rotary shaker at 100 rpm and 28°C in 2.8-liter Fernbach flasks containing 2 liters of either YEMG medium (6) or TY broth (3). Cells were harvested after 10 to 12 days by centrifugation at 9,000 × *g* for 10 min. The pelleted cells were suspended in an equal volume of fresh YEMG medium and cultured for 24 h. These cultures were centrifuged, and the pellets were suspended in 100 ml of fresh YEMG medium. The suspension was blended in a model 45 VirTis homogenizer at a setting of 30 for 5 min and then centrifuged at 10,000 × *g* for 15 min. The supernatant containing pili was collected, sodium azide was added to a final concentration of 0.1%, and the suspension was stored at 6°C. The bacterial pellets were combined and suspended in fresh YEMG medium, maintaining aseptic conditions, and placed on a rotary shaker at 100 rpm. Following incubation for 24 h, the pilus collection procedure was repeated.

Pili were purified from the suspensions by using the method of Korhonen et al. (24). Pili were precipitated with ammonium sulfate, solubilized with sodium deoxycholate, fractionated by sucrose density gradient centrifugation, and further purified by gel filtration on Sepharose 4B.

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate was performed in 1-mm slab gels containing 12.6% acrylamide by using a modification of the method of Laemmli (26). The marker proteins used (Sigma Chemical Co., St. Louis, Mo.) were trypsinogen (bovine stomach mucosa), β-lactoglobulin (bovine milk), and lysozyme (egg white). The gels were stained with silver nitrate (49).

Antibody production. Antibodies were produced against purified pili from strain 110 ARS in Australian white rabbits by a series of four subcutaneous injections. The injections, each containing approximately 30 μg of protein in Freund incomplete adjuvant, were given at 3-week intervals. The immunoglobulin G (IgG) fraction was purified by affinity chromatography with a protein A-Sepharose column (22). The anti-pilus and nonimmunized rabbit sera were checked for reaction against the purified pili and against 1% bovine serum albumin with an Immun-Blot (GAR-HRP) Assay Kit according to the directions of the supplier (Bio-Rad Laboratories, Richmond, Calif.).

Immunofluorescent localization of pili. Portions (30 ml) of early-log-phase cultures of strains 110 ARS, USDA 138, and 61A76 were centrifuged at 10,000 rpm for 10 min. The pellets were washed with YEMG medium, and the resulting preparations were pelleted. The anti-pilus antiserum or control serum was diluted in HEPES buffer (2.3 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [Sigma] per liter, 8.0 g of NaCl per liter, adjusted to pH 7.4) to a concentration of approximately 100 μg of protein per ml and was added to each pellet; the preparation was vortexed briefly and then placed on a shaker (50 rpm) at 28°C for 0.5 to 1 h. The anti-pilus or control serum-treated cells were then pelleted by centrifugation at 10,000 rpm for 10 min, washed in phosphate-buffered saline, and pelleted again. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Calbiochem-Behring, La Jolla, Calif.) was added at a concentration of 0.1 to 0.2 mg of protein per pellet. The cells were then examined by using an epifluorescence method (5). Photomicrographs were made by using a Leitz Ortholux II microscope and Ilford type 800 film.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was used to monitor the occurrence of piliated cells in cultures of strain 110 ARS at various culture ages. The assay used was an indirect ELISA adapted from the protocol of Voller et al. (46). The anti-pilus antiserum was diluted 1/600 in the HEPES buffer described above containing 0.5 ml of Tween 20 per liter. All washes were done with HEPES buffer. Alkaline phosphatase-conjugated antibody (from goat specific) to rabbit IgG (Litton Bionetics, Kensington, Md.) was used as the enzyme-linked antiglobulin. The enzyme substrate used was type 104 phosphatase substrate tablets (Sigma). Color development was measured at 410 nm with a Microelisa minireader (model MR590; Dynatech Instruments, Torrance, Calif.).

Effect of anti-pilus antibodies on attachment and nodulation. Anti-pilus antiserum, antiserum from a nonimmunized rabbit, or phosphate-buffered saline was added to 100 ml of a suspension containing 5×10^6 *B. japonicum* 110 ARS cells per ml and allowed to react for 2 h on a rotary shaker at 50 rpm and 28°C. The ability of the bacteria in these suspensions to attach to soybean root segments during a 15-min incubation period was determined as described previously (44). Protein determinations were made by using the method of Bradford (8). Purified anti-pilus IgG was tested in a similar manner.

To test the effect of anti-pilus antibodies on nodulation, the roots of 50 soybean seedlings that were germinated from surface-sterilized seeds for 48 h on filter paper were dipped into suspensions of rhizobia (10^4 cells per ml) containing the antiserum or IgG as described above. Control seedlings were

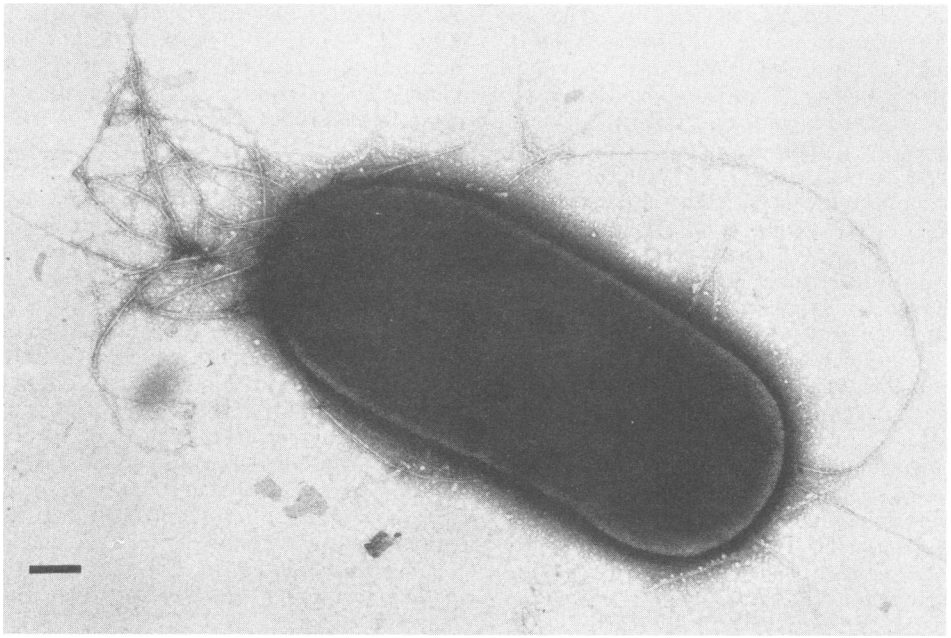

FIG. 1. Pili on *B. japonicum* 123. Bacteria were grown in YEMG medium to early log phase and then negatively stained with phosphotungstic acid and examined by transmission electron microscopy. Bar = 100 nm.

similarly treated with nonimmune serum or purified IgG from nonimmune serum. After 15 min, the seedlings were rinsed by vigorous shaking in 700 ml of sterile water for 10 s. Inoculated seedlings were transferred to disposable plastic growth pouches (Northrup King Seed Co., Minneapolis, Minn.) which had been prewetted with 10 ml of half-strength, N-free Jensen plant growth medium (45). The subsequent development of nodules on the primary roots was measured as described previously (6).

RESULTS

Appearance of piluslike structures. Fine fibrillar structures (Fig. 1) tentatively identified as pili were present on cells of each of the several *B. japonicum* strains examined. These structures were quite similar in appearance among the different strains, having an approximate diameter of 4 nm, appearing to originate primarily in polar to subpolar positions, and varying in length from just visible to several micrometers (Fig. 1 and Table 1). Relatively few (1 to 28) of the fine fibrillar structures were present on any given bacterium (Table 1), and the great majority of the bacteria in cultures of these strains appeared to have none. The bacteria with these fibrillar structures were frequently observed in association with other cells in clumps. The fine fibrillar structures were easily distinguished from flagella, which were 12 to 14 nm in diameter and sinusoidal.

TABLE 1. Characteristics of pili on strains of rhizobia

Strain	Pilus diam (nm)	Avg no. of pili per cell
B. japonicum		
123	3.8 ± 0.4[a]	16
USDA 138	3.8 ± 0.4	20
110 ARS	3.8 ± 0.4	18
61A76	3.6 ± 0.5	16
83	3.6 ± ND[b]	ND
R. trifolii TA1	5.0 ± 1.0	12

[a] The values given are the averages of 10 measurements ± standard deviations.
[b] ND, Not determined.

No fibrillar structures fluorescing with Calcofluor white were observed on *B. japonicum* 110 ARS grown in YEMG medium, although the cells themselves did fluoresce weakly. Some fluorescing fibrillar structures were found in cultures that were coincubated for 4 days with carrot root pieces. These fibrils generally appeared in large ropelike structures or masses.

Hydrophobic attachment assay. *B. japonicum* cells attached to ordinary polystyrene petri dishes within a 4-h period at 6°C. Attachment was rapid during the first 20 min, but no further attachment was seen after 80 to 100 min (Fig. 2). About 84% of the bacteria in the original suspension of early-exponential-phase cultures of strain 110 ARS remained unattached during prolonged incubation. No bacterial cells with the fine fibrillar structures were detected by electron microscopy among these unattached rhizobia. Attachment of rhizobia to glass petri dishes was negligible over comparable periods of time (data not shown). Cultures of strain 110 ARS which had been exposed to a plastic petri dish for 2 h were tested for subsequent attachment to soybean roots. Less than 3% of the cells from such preexposed cultures of *B. japonicum* 110 ARS attached firmly or weakly to the root segments, whereas approximately 14% of the cells from nonexposed cultures were capable of attaching (firmly plus weakly).

Culture age effects. The percentage of bacteria with the fine fibrillar structures varied substantially with culture age for some strains. Electron microscopic examinations indicated that the strains with the largest subpopulations of apparently piliated bacteria were *B. japonicum* 110 ARS and 123 in the early to mid-log phases of growth (Fig. 3A). Less than 1% of the cells in stationary-phase cultures of these two strains and less than 1% of the cells of strain 83 or 61A76 regardless of culture age appeared to possess the fine fibrillar structures (Fig. 3A).

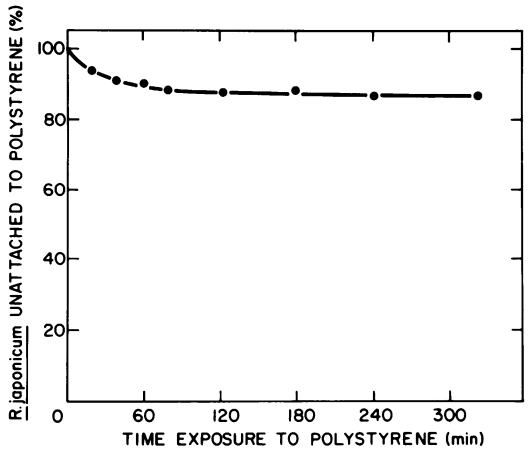

FIG. 2. Time-dependent attachment of *B. japonicum* 110 ARS to polystyrene. Samples (5 ml) of a suspension of *B. japonicum* 110 ARS (optical density at 620 nm, 0.15) were added to plastic petri dishes and incubated at 6°C. At different times three petri dishes were selected, and the extent of hydrophobic attachment was measured as described in Materials and Methods.

The sizes of the subpopulations of bacteria capable of attaching to hydrophobic surfaces also changed with culture age. For each strain the culture age dependence of hydrophobic attachment was similar to the effects of culture age on the proportion of cells with the fine fibrillar (pilus) structures (Fig. 3B). The size of the subpopulation capable of attachment to hydrophobic plastic dishes was generally two to three times larger than the size of the subpopulation estimated by electron microscopy to have the fine fibrillar structures (Fig. 3A and B). However, a closer correspondence was found for *Rhizobium trifolii* TA1 (Fig. 4).

B. japonicum cells survive in water suspensions at ambient temperature for long periods of time without loss of symbiotic infectiveness (11). Approximately 8% of the cells of strain USDA 138 were found by electron microscopy to have fine piluslike structures shortly after suspension in water. This proportion increased to approximately 12% after 10 weeks. Approximately 10% of the cells of strain 110 ARS and about 2% of the cells of strain 61A76 possessed piluslike structures after 1 year in water suspension.

Effect of miscellaneous compounds on hydrophobic attachment. Compounds observed to affect the attachment of *B. japonicum* to soybean roots (44) were tested for their effect on attachment to hydrophobic surfaces. Both sodium chloride (10 mM) and potassium nitrate (15 mM) promoted attachment (31 ± 9 and 30 ± 11%, respectively) in the hydrophobic assay; galactose (30 mM) had no effect, and urea (10 mM) significantly inhibited hydrophobic attachment (by 38 ± 8%).

Effect of removing bacterial surface structures. *B. japonicum* suspensions were blended in order to remove surface structures from the bacterial cells. Blended *B. japonicum* suspensions lost approximately 90% of their attachment capacity but regained 70% of this capacity within 60 min (Table 2). Cells with the piluslike structures were detected by electron microscopy within about 30 min after blending. Chloramphenicol almost completely inhibited the ability of the bacteria to attach after blending (Table 2). Capsules detectable by binding of labeled soybean lectin were also removed by the blending treatment. The size of the subpopulation of bacteria with detectable capsules (70 to 75%) returned to normal within approximately 20 min, although the regenerated capsules were smaller than the original capsules.

Isolation and purification of pili. Attempts were made to isolate the piluslike structures from *B. japonicum* 110 ARS by methods used for the purification of pili from other

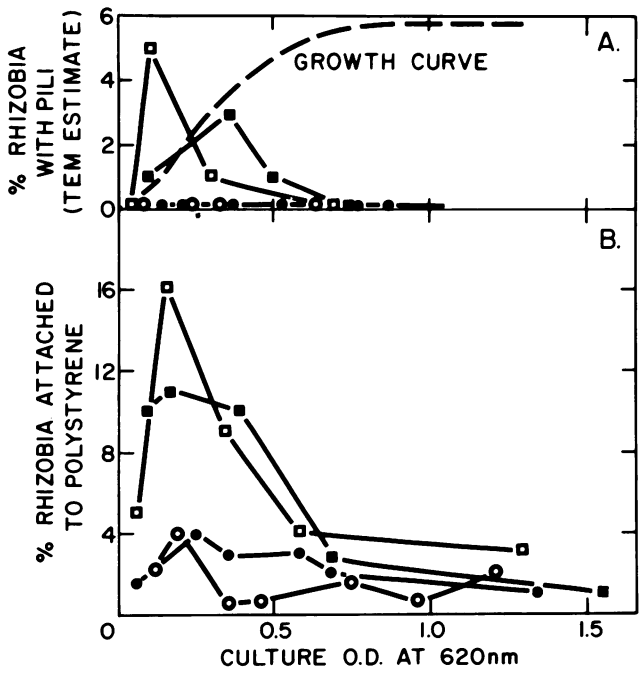

FIG. 3. Culture age effects on pilus expression by *B. japonicum* strains. Cultures of strains 110 ARS (□), 123 (■), 61A76 (○), and 83 (●) were grown in YEMG medium. At various culture ages, cells were negatively stained and examined by electron microscopy (A) or by the hydrophobic attachment assay (B) as described in Materials and Methods. The dashed line in panel A indicates an average growth curve. TEM, Transmission electron microscopy; O. D., optical density.

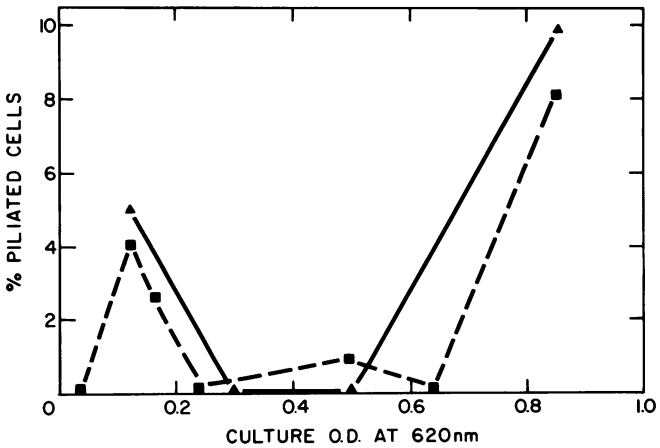

FIG. 4. Culture age effect on pilus expression by *R. trifolii* TA1. *R. trifolii* TA1 was grown in YEMG medium to various culture ages. Samples of the culture either were stained for transmission electron microscopy and a random count was made of piliated cells (▲) or were quantitated by the hydrophobic attachment assay (■). O. D., Optical density.

bacteria (24). Three distinct peaks of material absorbing at 280 nm were found during an examination of the various fractions from the sucrose step gradient used to purify solubilized pili. Fractions from each peak were checked for the presence of the piluslike structures by electron microscopy after removal of deoxycholate. The first peak, at low sucrose concentrations, contained no identifiable structures. The second peak (28 to 40% sucrose) contained what appeared to be aggregates of the fine fibrillar structures observed on the bacteria. The third peak, at higher sucrose concentrations, contained flagella but no other identifiable structures. Only one peak absorbing at 280 nm was found when the pooled fractions containing the piluslike structures were subjected to gel filtration. Approximately 100 g (fresh weight) of cells yielded 100 μg of purified protein.

The purified protein, which was apparently derived from the fine fibrillar piluslike structures, could be separated into two bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of the major band was approximately 21,000, and there was a minor band, perhaps a doublet, at a molecular weight of approximately 18,000 (Fig. 5).

Effect of anti-pilus antiserum and purified IgG on attachment and nodulation. Antiserum raised against the protein obtained by pilin purification techniques reacted with the purified protein in the Bio-Rad Immun-Blot (GAR-HRP) assay, as did the IgG fraction. Neither this serum nor nonimmunized rabbit serum (nor IgG fractions obtained from these sera) reacted with bovine serum albumin. Nonimmune serum did not react with the protein material purified as pili.

Immunofluorescence microscopy revealed that only a small subpopulation (1 to 8%) of the bacteria in cultures of strains 110 ARS, USDA 138, and 61A76 reacted with the antiserum against the protein purified as pili (Fig. 6). The reactive subpopulation of bacteria generally exhibited bright fluorescence only at one cell pole.

When cultures of strain 110 ARS were examined in an ELISA for reaction to the anti-pilus antiserum, the reaction followed the same pattern of culture age dependency observed in the microscopic assay of piliated cells. The greatest reaction to the anti-pilus antiserum occurred at the early log phase of culture growth, and the reaction was significantly reduced in older cultures. The ELISA-based optical density reading (at 410 nm) dropped from 0.56 ± 0.01 at early log phase to 0.20 ± 0.02 at stationary phase.

TABLE 2. Effect of blending on lectin binding and attachment[a]

Treatment	Time after blending (min)	% of cells with lectin-binding capsules	No. of bacteria attached per segment (10^{-3})
Nonblended		75 ± 9[b]	5.72 ± 0.5[b]
Blended	0	20 ± 26	0.74 ± 0.05
Blended	20	75 ± 4	ND[c]
Blended	60	72 ± 3	3.41 ± 0.2
Blended + chloramphenicol (5 μg/ml)	60	ND	0.83 ± 0.03

[a] Log-phase *B. japonicum* 110 ARS cultures diluted to a density of 2×10^7 cells per ml were blended in a VirTis homogenizer at a setting of 30 for 5 min. Attachment to soybean root segments was measured over a 15-min interval at the times indicated, as described in Materials and Methods.

[b] The values given are the averages of three replicates ± standard deviations.

[c] ND, Not determined.

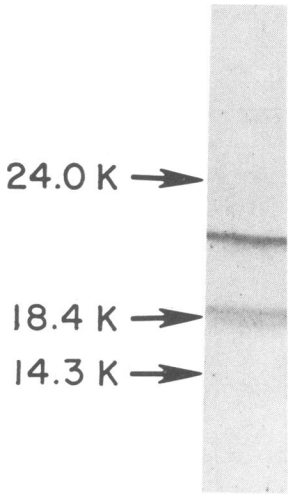

FIG. 5. Molecular weight determination for pilus protein isolated from *B. japonicum* 110 ARS. The standard proteins used were trypsinogen (molecular weight, 24,000), β-lactoglobulin (18,400), and lysozyme (14,300). The sample well contained approximately 30 μg of purified pilin.

Crude antiserum inhibited both attachment and nodulation, as did the IgG fraction (Table 3). None of the treatments significantly affected cell viability or the dispersion of bacteria on plates for colony counts. Serum or IgG from a nonimmunized rabbit had no effect on attachment or nodulation.

DISCUSSION

Several kinds of evidence indicate that the fine fibrillar structures on *Bradyrhizobium* cells are correctly identified as pili. The diameters of the piluslike structures from rhizobia (Table 1) and the subunit molecular weight of the material isolated from *B. japonicum* 110 ARS by standard methods of pilus purification appear to be comparable to the values reported for pili of other bacteria (34, 48). The minor band(s) seen in gels of the purified pilus preparations may indicate the presence of a second pilus type on cells of this strain. However, it is also possible that the minor band represents a protease cleavage product (17) of the major polypeptide or an artifact of reaction with mercaptoethanol (23).

Quantitation of cells with the piluslike structures by electron microscopy was made difficult and somewhat uncertain by the low frequency of such cells in the cultures (Fig. 3A), by the low number of the piluslike structures on these cells (Table 1), and by the frequent occurrence of cells with such structures among clumps of the bacteria. Assuming that the fine fibrillar structures observed by electron microscopy were in fact pili, it seemed possible that attachment to hydrophobic surfaces could be used as a more convenient method of estimation, as suggested by reports of the selective binding of piliated bacterial cells to polystyrene or to droplets of hydrophobic solvents (37, 38). While the surface of a typical bacterium is generally hydrophilic (48), pili normally have a high percentage of hydrophobic amino acids in their structure (23, 48). This hydrophobic character appears to make an important contribution to pilus attachment (20, 38, 48). We found that exposure of *Bradyrhizobium* cultures to a hydrophobic plastic surface removed essentially all of the cells with the piluslike structures from suspension even though the large majority of the bacterial

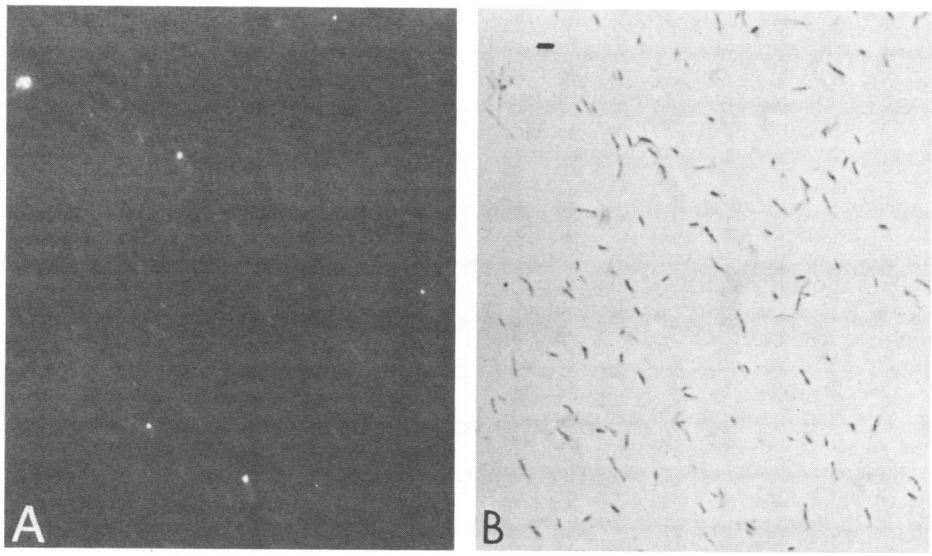

FIG. 6. Immunofluorescence examination of pilus expression in *B. japonicum*. A field of *B. japonicum* cells is shown. (A) Immunofluorescence. (B) Phase-contrast microscopy. Cells from an early-log-phase culture of strain 110 ARS were allowed to react with anti-pilus antiserum for 30 min to 1 h and then reacted with fluorescein isothiocyanate-labeled goat anti-rabbit IgG as described in Materials and Methods and examined by using epifluorescence. Bar = 1 μm.

population did not attach to the plastic. The selective and quantitative attachment of those cells with the piluslike structures to hydrophobic plastic surfaces supports the notion that these structures are attachment pili of the kind observed on other bacterial species (37, 38). It appears that the hydrophobic attachment assay tends to overestimate the proportion of piliated cells in a culture of *B. japonicum*, perhaps because surface structures other than pili contribute to hydrophobic attachment (18).

Cellulose microfibrils are produced by several *Rhizobium* species (15, 32) and are involved in host attachment in the closely related genus *Agrobacterium* (28). Therefore, we sought to determine whether the fine fibrillar structures observed by electron microscopy were cellulose microfibrils rather than pili. When Calcofluor white was used as a stain for cellulose microfibrils as described by Matthysse et al. (28), no staining of polar fibrillar structures on cells of strain 110 ARS was observed, even at the culture age which produced the greatest number of cells with the fine fibrillar structures. Since positive fungal controls were stained by Calcofluor white, these results suggest that the rhizobia did not produce abundant cellulose microfibrils. Only when *B. japonicum* was coincubated with carrot root pieces for several days, as described by Matthysse et al. for *Agrobacterium*, was any staining of the bacteria with Calcofluor white discernible, indicating appreciable synthesis of cellulose by the rhizobia under these conditions. Deinema and Zevenhuizen (15) reported that the cellulose microfibrils produced by *Rhizobium* cells were between 11 and 13 nm in diameter, roughly three times larger than the fine fibrillar structures identified as pili in this study. This discrepancy in diameter, the lack of Calcofluor white staining, the purification and immunological properties of the isolated structures, and the strong, selective affinity of cells with these structures for hydrophobic plastic surfaces all indicate that these structures are probably pili and not cellulose microfibrils.

TABLE 3. Effect of anti-pilus serum and IgG on attachment and nodulation by *B. japonicum* 110 ARS[a]

Treatment	% Inhibition of attachment[b]	% Inhibition of nodulation[b]	% of viable cells[b]
None	0	0	100
Whole anti-serum			
10 μg of protein per ml	77 ± 4[c]	ND[d]	90 ± 8
100 μg of protein per ml	93 ± 4[c]	80 ± 11[c]	98 ± 3
Purified IgG from a preparation containing 100 μg of crude serum protein per ml	88 ± 7[c]	71 ± 16[c]	99 ± 4

[a] The IgG fraction from anti-pilus antiserum or nonimmunized rabbit antiserum was purified by protein A-Sepharose affinity chromatography. The antiserum and purified IgG were allowed to react (2 h and 30 min, respectively) with an early-log-phase culture of strain 110 ARS diluted to a density of 10^6 cells per ml. The treated cells were then tested for their attachment ability and nodulating capacity as described in Materials and Methods.

[b] The values given are the averages of three replicates ± standard deviations.

[c] Significantly different from the control value at the 95% confidence level, as determined by the F test.

[d] ND, Not determined.

Several lines of evidence indicate that pili probably play a significant role in the attachment of *B. japonicum* to soybean roots. One indication is the reasonably good quantitative correspondence between the size of the piliated subpopulation of *B. japonicum* strains and the size of the subpopulation that is capable of firmly attaching to soybean roots within a period of 20 to 30 min (44). Electron microscopy (Fig. 3A) and reaction with anti-pilus antiserum (Fig. 6) indicated that only about 1 to 5% of the cells in cultures of strains 110 ARS and 61A76 were piliated. Attachment studies with cultures of these strains at comparable culture ages established that approximately the same small percentages of cells in these cultures were capable of firmly attaching to soybean roots (44). On this basis, it appears that pili are not involved in weak attachment, although much remains to be learned about the nature of weak attachment.

A second indication that firm attachment to roots is related to piliation is the correspondence between the effects of culture age on the size of the piliated subpopulation and the

effects of culture age on the rate of bacterial attachment to roots. Various *Bradyrhizobium* strains differed substantially from one another in the effects of culture age on pilus expression (Fig. 3A and B). Quantitatively and qualitatively similar culture age effects on the rate of bacterial attachment to soybean roots have been observed with each of these strains (44).

A third kind of evidence indicating that pili may be responsible for much of the attachment of rhizobia to host roots observed in these studies is the essentially complete and highly selective removal of the piliated subpopulation of bacteria from suspension upon incubation with soybean root segments. After a culture of strain 110 ARS with a significant subpopulation of piliated cells was allowed to attach to a large number of root segments, virtually no unattached piliated cells could be detected by electron microscopy.

Two other kinds of observation are consistent with the notion that pili are significantly involved in root attachment of rhizobia. Various investigators have reported that *B. japonicum* cells attach most frequently to soybean roots in a polar orientation (7, 40, 42, 43). This is consistent with the predominantly polar to subpolar location of pili on the bacterial cell surface, as observed by electron microscopy. Blending of *Bradyrhizobium* cultures reduced their attachment capacity by approximately 90% (Table 2). The kinetics of reacquisition of *Bradyrhizobium* attachment capacity was similar to the kinetics of pilus synthesis by a blended culture of *Escherichia coli* (16). Chloramphenicol treatment, which completely inhibited the synthesis of type 1 (attachment) pili in *E. coli* (16), also eliminated the reacquisition of the attachment capacity of a blended *Bradyrhizobium* suspension (Table 2).

It is of interest that 30 mM galactose substantially inhibited attachment of *B. japonicum* to soybean root segments (40, 44) but had no significant effect on attachment of the same bacteria to plastic petri dishes. This raises the possibility that the pili of *B. japonicum* may be lectinlike proteins capable of binding to carbohydrate structures on the host root surface. It has been reported that simple sugars can specifically inhibit the pilus-mediated attachment of various bacteria to host surfaces (48). We note that added salts and 10 mM urea had quite similar effects on attachment of *B. japonicum* to both plastic petri dishes and excised soybean root segments (44).

Further evidence for pilus-mediated attachment of *B. japonicum* to soybean roots is provided by the substantial inhibition of attachment and nodulation obtained when the bacteria were exposed to antibodies prepared against purified pilus protein (Table 3). Viability tests (Table 3) indicated that such inhibition of attachment by the anti-pilus antibodies was not an artifact of reduced viability or simple agglutination of the bacteria. The specificity of the antiserum for pili at the dilution tested was indicated by the binding of fluorescein isothiocyanate-labeled goat anti-rabbit antiserum primarily to polar locations on a small percentage of the bacterial cells (Fig. 6).

The evidence for pilus-mediated attachment of *B. japonicum* described above is consistent with an earlier report by Bohlool and Schmidt indicating that "extracellular polar bodies" may be involved in attachment of this species (7). These structures were observed by fluorescent antibody techniques to be present on subpopulations of *B. japonicum* cells in cultures of various strains, with the proportions dependent on both strain and culture age. The extracellular polar bodies were located at only one end of the bacterial cells and were always present at that end of the cell which was involved in the attachment of the bacteria, either to other bacteria or to the hyphae of fungi copropagated in soil (7). Microfibrillar structures were associated with the polar bodies (7). It will be of considerable interest to determine whether these extracellular polar bodies are actually the same structures as the pili described in this paper.

The environmental factors and genetic mechanisms governing pilus expression in bacteria are not well understood. There is evidence that pili are expressed in some cells but not others within populations of *Neisseria gonorrhoeae*, depending on the orientation of specific insertion sequences (30). It remains to be determined whether similar regulatory mechanisms govern the expression of attachment pili in rhizobia and whether pilus expression is affected by the presence of the host root.

The apparent involvement of pili in the attachment of various rhizobia to soybean roots does not exclude the possibility of attachment mediated by binding of soybean lectin (40). However, a variety of recent evidence (9, 27, 35, 36, 41, 44) indicates that the attachment of rhizobia to soybean roots is not normally host specific or host selective and, furthermore, that binding of soybean lectin to *Bradyrhizobium* cell surfaces does not play a major role in overall attachment to soybean roots under the conditions examined. In the future, it will be important to learn what contributions pili, lectins, or other molecular and cellular structures make to the attachment of *B. japonicum* to soybean roots in the soil.

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