JOURNAL OF BACTERIOLOGY, Mar. 1976, p. 1188-1194

Vol. 125, No. 3
Printed in U.S.A.

Immunofluorescent Polar Tips of *Rhizobium japonicum*: Possible Site of Attachment or Lectin Binding[1]

B. B. BOHLOOL[2] AND E. L. SCHMIDT*

Departments of Microbiology and Soil Science, University of Minnesota, Minneapolis, Minnesota 55455

Received for publication 14 November 1975

Rhizobium japonicum USDA 31 demonstrated marked polarity by binding homologous fluorescent antibody (FA) heavily on one end of the cell. FA prepared against *R. japonicum* strains 110 and 138, and against *R. trifolii* TA1 cross-reacted with strain 31 only in the polar tip region. No polar immunofluorescing tips could be seen with FA against two other strains of *R. japonicum* or with those against several unrelated microorganisms. Common antigens localized only in a polar region were seen in many rhizobia stained with *R. japonicum* 31 FA: 22 of 23 strains of *R. japonicum*, 10 of 17 strains of *R. trifolii*, 3 of 7 strains of *R. melitolii*, 3 of 6 strains of *R. phaseoli*, and 0 of 9 strains of *R. leguminosarum* had some cells with detectable polar tips. The proportion of *R. japonicum* 31 cells with polar tips was high throughout the growth cycle. Polar tip staining was not affected by drastic cell treatments. A function was proposed for the polar tip region as a site for attachment. *R. japonicum* 31 cells attached to each other in a tip-to-tip fashion and endwise to fungal hyphae with the polar tip in contact with the hyphal wall. Binding of fluorescein isothiocyanate-labeled soybean lectin to certain strains of *R. japonicum* gave additional evidence of polarity. Polar binding of both antibody and lectin may provide insights into relationships between rhizobia and roots of host legumes.

The sequence that culminates in the nodulation of a legume root by an appropriate soil *Rhizobium* is comprised of many steps. It is a reasonable hypothesis that so specific an interaction may be preceded by mutual recognition between the partners in the rhizosphere, and by attachment of the specific bacterium to the root "nodulation site." Evidence has been presented that plant lectins may provide the means for mutual recognition of the specifically interacting partners (2, 5). Although little attention has been given to attachment, an end-on attachment of *Rhizobium trifolii* to a host legume root was seen clearly in an electron micrograph reported by Sahlman and Fahraeus (7).

We observed that cells of a particular strain of *R. japonicum* (USDA 31) stained irregularly with homologous fluorescent antibody (FA). In addition to a generalized 3+ to 4+ fluorescence, many cells exhibited a still brighter fluorescing tip on one end. This report describes immunofluorescent polar tips of *R. japonicum* and notes the possibility of a role for these tips in attachment and/or lectin binding by the cells.

[1] Paper no. 9252, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, Minn.

[2] Present address: Cawthron Institute, Nelson, New Zealand.

MATERIALS AND METHODS

Strains of rhizobia were obtained as described (9) and have been carried in our laboratory for several years, as were the other cultures used in this study. The usual culture medium for the rhizobia was a modified yeast extract-mannitol-salts solution (1). Antisera were obtained against heat-killed cells and FAs were prepared according to immunization schedules and procedures reported elsewhere (1, 9). Dilutions of 1 part FA to 3 parts 0.2 M phosphate-buffered saline were used as a stain.

Microscopy was performed with a Zeiss Universal microscope equipped for double lighting with incident fluorescence illumination from an HBO 200 Osram mercury light source, and transmitted dark-field tungsten illumination. Observations were made by epifluorescence alone or in combination with the dark-field system. The reflected system for fluorescence used an FL 500 reflector, one BG 38 and two fluorescein isothiocyanate (FITC) exciter filters, and a no. 50 barrier filter. Photomicrographs were taken at 15- to 30-s exposures with GAF 200 color slide film.

Attachment of *R. japonicum* USDA 31 to a fungus was studied by inoculating a sterile microscope slide with both the bacterium and a soil *Penicillium*, and burying the slide in various sterilized (autoclaved) soils. Preparation of such contact slides for FA examination followed usual procedures (1, 9). FITC-labeled soybean lectin was obtained and used as in a previous report (2).

RESULTS

A brightly fluorescing tip region is present on each of the two *R. japonicum* 31 cells seen in Fig. 1. The bright staining seen on the tip is in contrast to the rest of the cell, which stained poorly to not at all with the particular homologous FA used. Such results were obtained with antisera obtained from very early (1 to 2 months from immunization day zero) and late (4-month) bleedings, whereas intermediate bleedings gave a generalized 2+ or 3+ overall reaction with a 4+ polar tip reaction. We have found that most other strains of *R. japonicum* react to homologous FA with overall 4+ somatic staining, and fluorescing polar tips, if present, may be masked by the bright generalized fluorescence.

That tip staining of strain 31 cells is not merely trapping of antibody or nonspecific protein binding is shown in Table 1. Of the five fluorescent antibodies prepared against strains of *R. japonicum* other than USDA 31, three did stain a polar region of USDA 31 with 3+ fluorescence, but the other two produced essentially negative reactions. The somatic cross-reaction between strains 31 and 61A72 was reported earlier (9), so it was not surprising that the polar region of 31 was stained by 61A72 FA. Staining of the polar region of 31, and only that region, by 110 FA, 138 FA, and to a lesser extent, *R. trifolii* TA1 FA, indicates, however, a small localization of common antigens among strains that are otherwise distinctive antigenically. FA prepared from the unrelated bacteria *Azotobacter* and *Nitrobacter* and the two fungi *Pisolithus* and *Thelephora* gave negative or very weak tip staining. Trapping or nonspecific binding could at best account for but feeble fluorescence.

TABLE 1. *Immunofluorescence staining of R. japonicum USDA 31 by various FAs*

FA	Immunofluorescence[a]	
	Whole cell	Polar tip
R. japonicum USDA 31	3+	4+
R. japonicum 61A72	2+	3+
R. japonicum 61A24	–	–
R. japonicum USDA 110	–	3+
R. japonicum USDA 135	–	±
R. japonicum USDA 138	–	3+
R. trifolii TA-1	–	2+
R. trifolii CC2480	–	–
Nitrobacter agilis	–	1+
Azotobacter chroococcum	–	–
Pisolithus tinctorius	–	±
Thelephora terrestris	–	1+

[a] –, Negative; ±, trace; 1+, feeble through 4+, maximal.

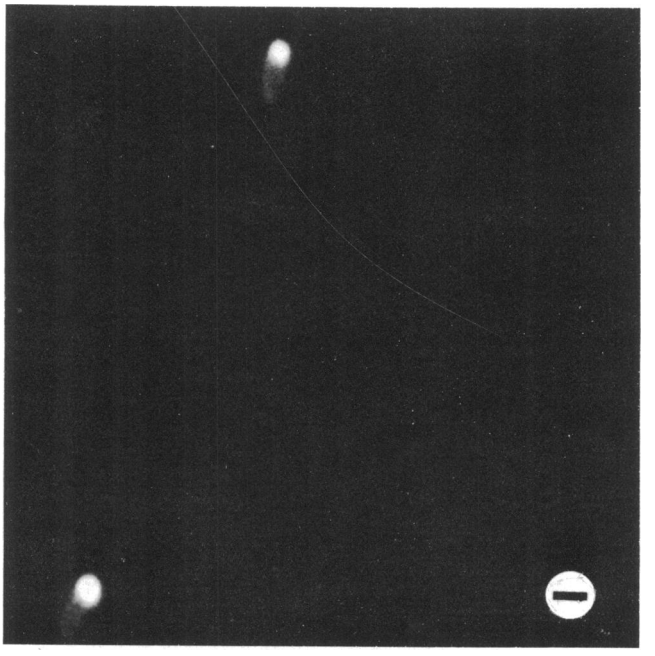

FIG. 1. *Immunofluorescent polar tips of R. japonicum USDA 31 as viewed with fluorescence microscopy after reacting with a homologous antibody that gave only a weak generalized fluorescence. Scale mark equals 2 μm.*

Further evidence on the incidence of polar immunofluorescence among the rhizobia is presented in Table 2, summarized from data obtained by staining numerous heterologous rhizobia with USDA 31 FA. Nearly all of the 23 *R. japonicum* cultures included cells with 3+ to 4+ polar fluorescence. The polar tip antigen is apparently common to most strains of *R. japonicum* but is not necessarily present to the same extent in all cells of a strain. In contrast, somatic cross-reaction again was found only in strain 61A72. The 17 strains of *R. trifolii* included 10 strains and the 7 *R. melilotii* included 3 strains that exhibited polar immunofluorescence after staining with USDA 31 FA. Tips in these species appear similar to those of *R. japonicum*, but staining intensity was less, about 2+. Polar fluorescence was seen rarely in *R. phaseoli* and not at all in *R. leguminosarum*. Two isolates of *Agrobacterium tumefaciens*, two of *A. radiobacter*, and one of *A. rubi* were also tested in view of their close relationship to the rhizobia, and all were negative.

The results of attemps to remove or inactivate the polar antibody-binding site are summarized in Table 3. None of the physical, chemical, or enzymatic treatments applied to the cells before FA staining were effective in preventing the polar tip fluorescence reaction. A noticeable decrease in intensity of both the peripheral staining and polar tip staining occurred after treatment with 0.02 M periodate in pH 5.0 phosphate buffer, as well as a decrease in the incidence of tip-staining cells. These effects were apparently unrelated to the periodate since the buffer alone behaved similarly. The decrease in incidence of fluorescing polar tips at pH 5.0 was considered to result from a general pH-related loss in fluorescence, so that only the larger bright tip areas were visible. The data indicate that the polar immunofluorescing region is closely associated with the cell surface and is relatively stable.

Microscopy counts of tip-positive cells made at regular intervals during the growth cycle of *R. japonicum* 31 in liquid shaken culture dem-

TABLE 2. *Estimated occurrence of immunofluorescing polar tips in R. japonicum other than strain USDA 31 and in other rhizobia after staining with R. japonicum USDA 31 FA*

Species	No. of strains tested	No. negative	No. of strains having a % of cells with 2+ to 4+ polar tips			
			<1	1–5	5–50	>50
R. japonicum[a]	23	1	1	4	11	6
R. trifolii	17	7	3	5	2	0
R. meliloti	7	4	0	1	1	1
R. phaseoli	6	3	3	0	0	0
R. leguminosarum	9	9	0	0	0	0

[a] Less extensive tests with *R. japonicum* strains and other FA resulted as follows. With 138 FA, 8 of 12 strains had cells with 5% or more positive for polar tips, and 4 of 12 were positive with less than 1% of the cells reacting. With 110 FA, 3 of 10 strains had polar tips in 5% or more of the cells, 1 strain had bright tips in less than 1%, and 6 were negative.

TABLE 3. *Effect of various enzymes and treatments on the polar tip fluorescence of cells from 5- to 10-day cultures of R. japonicum USDA 31*[a]

Treatment	Concn (μg/ml)	Conditions[b]	Polar tip fluorescence
Deoxyribonuclease	50	0.01 M $MgSO_4$-0.005 M PO_4, pH 7.0; 18 h	4+
Ribonuclease	150	0.01 M acetate-0.005 M PO_4; pH 6.7; 18 h	4+
Protease	300	0.01 M Tris-0.01 M $CaCl_2$; pH 7.3; 18 h	4+
Trypsin	200	0.01 M Tris-0.01 M $CaCl_2$; pH 7.3; 18 h	4+
Papain	150	0.001 M EDTA; pH 6.5; 18 h	4+
Lysozyme	300	0.03 M NaCl; 0.01 M PO_4; pH 6.8; 18 h	4+
Periodate		0.02 M; 0.05 M PO_4; pH 5.0; 18 h	1+
Buffer		0.05 M PO_4; pH 5.0; 18 h	1+
Sodium dodecyl sulfate		Vigorous agitation periodically during 1 h	3+
Heat		100 C with periodic agitation; 0.5 h	3+
Agitation		Vortex mixer, 5 min with 0.15 g of alumina/ml	3+
Hot acetate		1.2 M $NaCH_3COO$-0.02 M HCH_3COO; 100 C; pH 6.4	3+

[a] Fluorescence ranked as in Table 1. Cultures of *R. japonicum* were centrifuged, washed, and stained with homologous FA.

[b] Tris, Tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

onstrated a consistently high percentage (75 to 95%) of cells with the polar antibody-binding site, throughout the period of study (0 to 90 h). In other experiments, there was no indication of loss of tip staining in cells of 18 to 75-day cultures. Growth conditions, whether liquid or solid, static or shaken, had no consistent effect on the presence of polar tips, but the yeast extract content of the medium did influence the proportion of tip-positive cells (Table 4). Although the percentage of tip-positive cells decreased with increasing concentrations of yeast extract, at least some of the difference may have been due to selective clumping of the positive cells during static growth. Cultures with the higher amounts of yeast extract developed faster and reached a clumped condition earlier.

TABLE 4. *Incidence of polar tips in cells of R. japonicum 31 grown 15 days on yeast extract-mannitol medium with yeast extract concentration varied*[a]

Yeast extract concn (%)	% Cells with fluorescing tips[b]	
	Liquid (static)	Solid
0.01	95	70
0.05	95	70
0.1	50	70
0.5	30	NT[c]
1.0	40	40

[a] Percentage of positive cells was estimated by fluorescence microscopy after reacting with *R. japonicum* 31 FA.

[b] Proportion of tip-positive cells among unclumped individual cells in suspension.

[c] NT, Not tested.

Clumping of individual cells of *R. japonicum* 31 apparently is mediated by the polar tips. This is shown in Fig. 2 where star-shaped groups comprised of a few cells are seen together with larger clumps made up of many cells. It is clear that the aggregates were formed as the result of tip-to-tip contact between individual cells, and that the aggregates increase in size as more and more cells join the clump in "head-on" orientation. Large aggregates estimated to be made up of hundreds of cells developed during stationary-phase growth in liquid culture.

The head-on orientation observed in stars, rosettes, and aggregates occurred also in attachment of rhizobia to fungi in sterilized soil. Hyphae of certain fungi growing together with *R. japonicum* 31 on a glass slide buried in soil were observed with the bacteria attached at right angles to the fungus, and with polar tips invariably in contact with the fungus wall. Figure 3 shows *R. japonicum* 31 attached in this

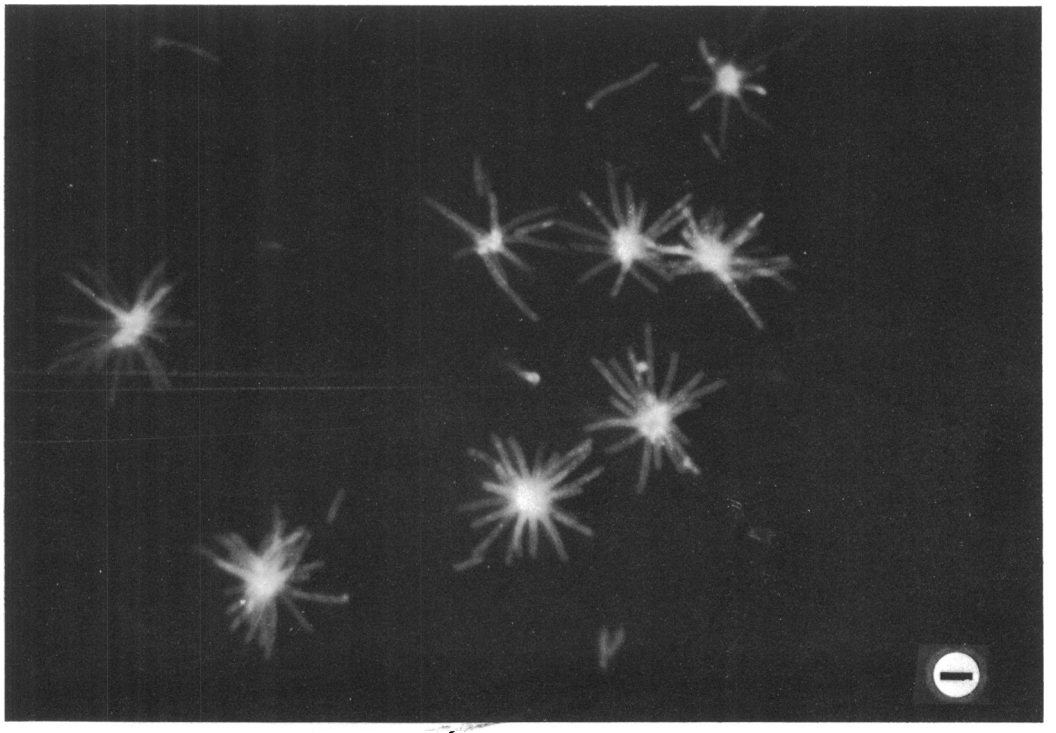

FIG. 2. *Attachment of R. japonicum USDA 31 cells to each other by means of polar tips. Contact slide-sterilized soil growth system; elongate cells are common in such preparations. Scale mark equals 3 μm.*

manner to a soil *Penicillium* in Clarion silt loam soil. In other soils similarly inoculated with the two cultures, the fungus surface was covered less extensively than seen in Fig. 3, but the endwise, polar tip orientation occurred to some extent in nearly all soils. Results of studies of the *Penicillium-Rhizobium* interaction in 12 soils are given in Table 5. At least some perpendicular attachment of bacteria to the fungus was observed in 11 of the 12 soils. A certain time dependency was noted, since after 5 days of incubation, attachment was seen in only one soil even though both microorganisms were evidenced on the contact slide. The differences seen with respect to time and soil type suggest that the attachment may involve nutritional factors related to the growth of the fungus.

A degree of polarity on the part of the *Rhizobium* cells was also observed with respect to specific binding of soybean lectin by *R. japonicum*. When lectin was placed in contact with

TABLE 5. *Attachment of R. japonicum USDA 31 to a soil Penicillium, as detected by immunofluorescence microscopy during incubation together in different sterile soils*

Soil	Extent of attachment[a]	
	5 days	18 days
Bearden	–	3+
Clarion	–	3+
Fargo	1+	1+
Glencoe	–	4+
Hayden	–	–
Lester	–	2+
Milaca	–	3+
Mora	–	3+
Nicollet	–	4+
Ronneby	–	1+
Ulen	–	1+
Webster	–	1+

[a] –, No perpendicular attachment, 1+, occasional to 4+, abundant, microscopy fields with perpendicular attachment.

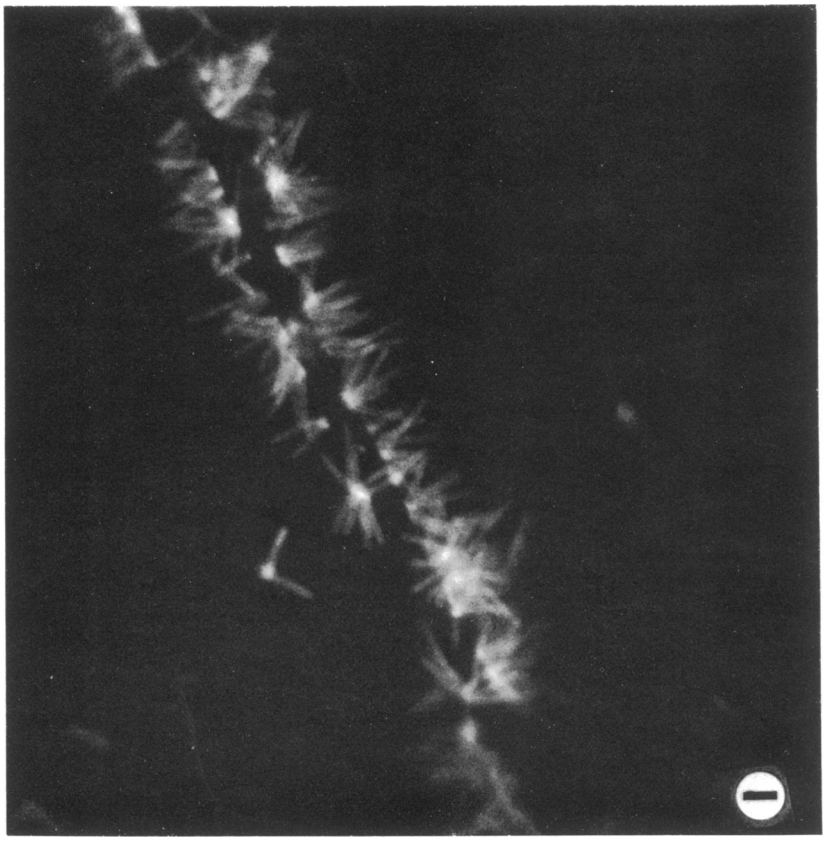

FIG. 3. *Attachment of R. japonicum USDA 31 to a hyphal filament of a soil Penicillium, in Clarion silt loam. Contact slide-sterilized soil growth system. Scale mark indicates 4 μm.*

many strains of *R. japonicum* under conditions such that the lectin-binding reaction could be observed microscopically (2), a striking localization of the lectin frequently was seen about one end of the cells. A concentration of lectin associated with only one end of the cells is shown in Fig. 4 for strain 138. In these figures, the outline of the cell was made visible by transmitted dark-field illumination, whereas the lectin was visualized by incident fluorescence microscopy and the FITC label was conjugated to the lectin. Thus, the lectin seen in the photomicrograph as large white patches partially surrounding the cells appeared as yellow-green fluorescent patches under the microscope. When individual unclumped cells were viewed by fluorescence microscopy only, characteristically about half the cell could be seen, that half binding the fluorescing lectin. The relationships between the polarity expressed in antibody tip binding (Fig. 1) and the polarity observed in lectin binding are unknown.

DISCUSSION

Antigenic heterogeneity has been well documented for the rhizobia in general and especially for *R. japonicum,* where numerous strains will react only with their homologous antisera (3, 4, 6, 9). The distinctiveness of the somatic antigens of *R. japonicum* permits serological identification of rhizobia in soybean nodules and specific detection of individual strains of *R. japonicum* in nature outside of the nodules by FA (8). Nevertheless, it now appears that many strains of *R. japonicum* have some antigens in common, which, surprisingly, are localized at one end of the cell. This polar tip localization may be visualized with FA techniques by means of which it was observed to be closely associated with the cell surface, consistent in occurrence throughout the growth cycle, and highly resistant to removal or disruption.

At least one functional feature can be associated with the marked polarity of the *R. japonicum* cell—that of attachment. Immunofluorescence microscopy clearly showed attachment between fluorescing tip and fluorescing tip, as cells joined together in tip-wise orientation to form pairs, rosettes, and clumps. Not only did the cells attach to each other in tip-to-tip fashion, but were seen to orient at right angles to the hyphae of a soil fungus with the polar tip invariably in contact with the hyphal wall. It may be that attachment of the *Rhizobium* to the fungus is mediated by the nutrition of the rhizobia, the fungus, or both since it was slow in developing, variable in intensity under different culture conditions, and not expressed at all with certain fungi.

It is interesting to speculate that attachment via polar tips analogous to that seen in Fig. 2 and 3 may occur in the legume rhizosphere as a

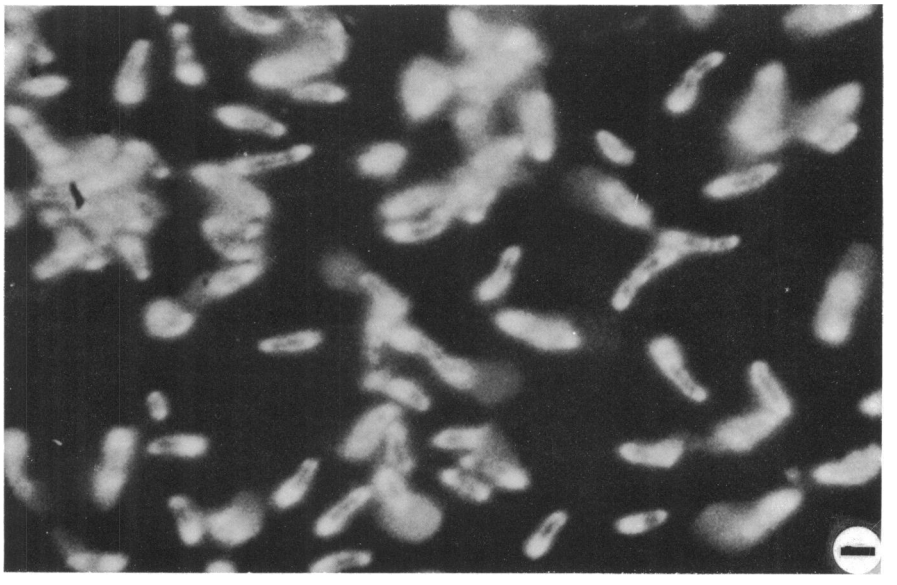

FIG. 4. *Polar binding of FITC-labeled soybean lectin to cells of R. japonicum USDA 138. Lectin is seen as diffuse patches localized about one end of cells. Photomicrograph made with both fluorescence and conventional dark-field microscopy to visualize cell outline. Scale division indicates 2 μm.*

prelude to nodulation. The electron micrograph of Sahlman and Fahraeus (7) showing end-on orientation of *R. trifolii* to a clover root lends some credence to this possibility. If the polar tips were involved in such attachment at the legume root nodulation site, it appears that the attachment process would be rather nonspecific, for at least some of the tip antigens found in *R. japonicum* were present in the polar tips of rhizobia with entirely different host preferences (Table 2).

A nonspecific mechanism for attachment at the root infection site might well be preceded by a highly specific recognition mechanism. The recent attention focused on the binding of soybean lectin specifically to cells of *R. japonicum* as a possible basis for the specificity of the *Rhizobium*-legume symbiosis (2) makes all the more interesting the observation that lectin binding, like polar tip antibody binding, also reflects cellular polarity. The marked concentration of FITC-labeled soybean lectin about one end of the *R. japonicum* cells shown in Fig. 4 was also apparent in earlier photomicrographs of lectin binding (2). It remains to be determined if polar lectin binding and polar antibody binding occur on the same end of the cells. We are now concerned with further study of the nature of the polarity in *R. japonicum* and relationships between the two manifestations of polarity reported in this study.

ACKNOWLEDGMENTS

This research was supported by grant GB29636A1 and grant BMS75-14020 from the National Science Foundation.

The authors are grateful to W. C. Lindemann for his careful technical assistance.

LITERATURE CITED

1. **Bohlool, B. B., and E. L. Schmidt.** 1970. Immunfluorescence detection of *Rhizobium japonicum* in soils. Soil Sci. 110:229–236.
2. **Bohlool, B. B., and E. L. Schmidt.** 1974. Lectins: possible basis for specificity in the *Rhizobium*-legume root nodule symbiosis. Science 185:269–271.
3. **Date, R. A., and A. M. Decker.** 1965. Minimal antigenic constitution of 28 strains of *Rhizobium japonicum*. Can. J. Microbiol. 11:1–8.
4. **Graham, P. H.** 1963. Antigenic affinities of the root nodule bacteria of legumes. Antonie van Leeuwenhoek; J. Microbiol. Serol. 29:281–291.
5. **Hamblin, J., and S. P. Kent.** 1973. Possible role of phytohaemagglutinin in *Phaseolus vulgaris* L. Nature (London) New Biol. 245:28–30.
6. **Koontz, F. P., and J. E. Faber.** 1961. Somatic antigens of *Rhizobium japonicum*. Soil Sci. 91:228–232.
7. **Sahlman, K., and G. Fahraeus.** 1963. An electron microscope study of root hair infection by *Rhizobium*. J. Gen. Microbiol. 33:425–427.
8. **Schmidt, E. L.** 1973. Fluorescent antibody techniques for the study of microbial ecology. Bull. Ecol. Res. Commun. (Stockholm) 17:67–76.
9. **Schmidt, E. L., R. O. Bankole, and B. B. Bohlool.** 1968. Fluorescent antibody approach to study of rhizobia in soil. J. Bacteriol. 95:1987–1992.