Production of Cellulose Microfibrils by *Rhizobium*

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Electron microscope examination of *Rhizobium* spp. revealed microfibrils produced by flocculating strains but not by nonflocculating strains. The microfibrils from *R. trifolii* (NA30) were isolated and identified as cellulose by enzymatic, X-ray diffraction, and infrared spectral analyses. Both infective and noninfective strains of *R. trifolii* flocculated and produced microfibrils. More infection threads were observed in clover root hairs growing in the presence of flocs in comparison with root hairs where single bacterial cells predominated.

Microbial flocculation has been defined as the adhesion of cells to form a three-dimensional matrix of cells (9). Extracellular biopolymers at critical concentrations have been reported to play an important role in the formation of natural microbial aggregates (9). Deinema and Zevenhuizen (7) examined the growth of several gram-negative bacteria in broth and correlated flocculation with the production of cellulose microfibrils. They reported flocculation and microfibril production in strains of *Rhizobium phaseoli* and *R. trifolii*, although the microfibrils from the *Rhizobium* strains were not characterized chemically. This report describes the chemical nature of extracellular microfibrils of *Rhizobium*, their relation to flocculation in broth, and their possible role in the infection process preceding the establishment of a N₂-fixing symbiosis with legumes.

MATERIALS AND METHODS

**Strains of Rhizobium.** *R. trifolii* 0403 and Bart A were obtained from P. S. Nutman, 0435 and 0435-2 from A. N. MacGregor, 2S and 2L from K. C. Marshall, and NA30 from W. F. Dudman. *R. meliloti* 2001 and 2009 were obtained from the Rothamsted Experimental Station, Harpenden, England. *R. japonicum* 311661 and *R. leguminosarum* 3HOQ1 and 3HOQ51 were obtained from D. F. Weber. *R. phaseoli* 403 was obtained from M. Alexander. Several *Rhizobium* strains within the so-called "cowpea group" were also examined. Strains 127E10 and 22A1 were obtained from J. C. Burton, 227, 229 and L1 were obtained from M. Alexander, and HR1 was isolated from nodules of *Aeschynomene americana* in our laboratory.

**Growth conditions.** Cultures were grown in either Bergeyes (BM) chemically defined medium (3) or yeast extract-mannitol (YEM) broth. YEM contains mannitol, 10 g; KH₂PO₄, 0.5 g; MgSO₄, 7H₂O, 0.2 g; NaCl, 0.2 g; FeCl₃, 6H₂O, 0.01 g; yeast extract, 1.0 g; and CaCO₃, 3.0 g in 1,000 ml of water. Cultures were incubated on a rotary shaker (150 rpm) at 22°C and then evaluated visually and microscopically for flocculation. Samples were withdrawn after 3 days of incubation and prepared for electron microscopy.

**Preparation of cellulase.** Grade C cellulase (Calbiochem, La Jolla, Calif.) was obtained in a powdered cellulose column (2 by 12 cm) equilibrated with 1 mM NaCl (pH 6.8) at 4°C. Impurities incapable of binding to cellulase, representing 99.8% of the material applied, were removed by elution with 1 mM NaCl (17). When the absorbance at 280 nm of the column effluent reached zero, the bound cellulase was eluted with 1 mM NaCl adjusted to pH 10.0 with 2 N NaOH. Fractions (3.5 ml) were collected in tubes containing 0.2 ml of 0.2 M tris(hydroxymethyl)aminomethane buffer (pH 6.8). The cellulase fraction was dialyzed against deionized water at 4°C, lyophilized, and stored at −20°C for future use.

**Cellulase digestion of flocs.** Flocs were digested with cellulase after 3 days of growth in either BM or YEM. One millilitre of a floc suspension was incubated with 22 mg of crude grade C cellulase in 2 ml of sodium citrate buffer (0.05 M, pH 4.5) at 30°C with occasional shaking. Controls consisted of flocs suspended in citrate buffer alone.

**Isolation of bacterial microfibrils.** Flocs of *R. trifolii* (NA30; infective on *Trifolium fragiferum*) grown in BM for 3 days were harvested by centrifugation at 16,000 × g for 30 min. The fibrillar fraction of the flocs was purified according to Deinema and Zevenhuizen (7). An important step in this method is an acid hydrolysis that completely removed α-glucans. The yield was 3 mg (dry weight) of microfibrils per 7 liters of culture medium.

**Analytical methods.** Cellulase assays of purified *R. trifolii* (NA30) microfibrils and pure cellulose (Nutritional Biochemicals, Richmond, Va.) were performed according to the Worthington Enzyme Manual (18), and reducing sugars were determined by the method of Kibdy and Davidson (10). Sugars released by enzymatic hydrolysis were identified by compari-

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son with authentic standards using gas-liquid and thin-layer chromatography. Hydrolysate aliquots were lyophilized, and the sugars were converted to their, trimethylsilyl derivatives using Tri-Sil (Pierce Chemical Co., Rockford, Ill., [14]). These were separated on SE-30 (Ultraphase, Pierce Co.) on Chromosorb W(HP) using N₂ carrier, 165°C isothermal column temperature, and H₂ flame ionization detection in a Packard model 803 gas chromatograph. The remainder of the hydrolysates were lyophilized, extracted with pyridine, spotted on silica gel thin-layer plates (no. 13179, Eastman Kodak, Rochester, N.Y.), developed with ethyl acetate-pyridine-water (2.5:1.0:3.5, vol/vol/vol), and sprayed with alkaline AgNO₃. X-ray diffractograms of dried, purified NA30 microfibrils and microcrystalline cellulose (Sigma, St. Louis, Mo.) were dried on glass microscope slides and scanned from 2° 2θ to 30° 2θ with a General Electric XRD Diffractometer using Ni-filtered CuKα radiation. Infrared spectra of NA30 microfibrils and microcrystalline cellulose were recorded on a Perkin Elmer model 567 spectrophotometer using a KBr pellet.

Bacterium-host interaction. T. fragiferum (strawberry clover) seeds were surface sterilized with 0.1% HgCl₂, rinsed with sterile deionized water, spread on water agar plates, and held for 48 h at 4°C (13). Seeds were germinated overnight at 22°C and transferred to Fahraeus glass slide assemblies (8) inoculated with R. trifolii (NA30). These assemblies were incubated for 3 days at 22°C with a 12-h photoperiod and examined under phase contrast for floc formation and associated root hair infection.

Transmission electron microscopy. One-week-old inoculated seedlings from the Fahraeus assemblies were fixed at 22°C for 2 h with 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 6.8) and postfixed at 22°C for 1.5 h with 1% buffered osmium tetroxide. Seedlings were dehydrated through a graded ethanol series (25, 50, 75, 95, and 100%) followed by acetone, infiltrated with Spurr resin (16), and polymerized overnight at 60°C. Sections were cut on a Sorvall MT2 Ultramicrotome equipped with a diamond knife, poststained with 2% uranyl acetate and Reynolds lead citrate (15), and examined in an Hitachi HU11E electron microscope operating at 75 kV. Bacteria were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 6.8) for 5 h and then rinsed with deionized water. Bacteria and purified microfibrils were dried on 200-mesh, plastic-coated copper grids and shadowed at approximately 45° with carbon and platinum in a Balzers BA360M freeze-etch apparatus (Balzers Co., Fursten tum, Liechtenstein).

RESULTS AND DISCUSSION

Figure 1 is a typical Rhizobium floc in broth culture. Flocculated cultures (Fig. 2) contained microfibrils resembling those reported by Deinema and Zevenhuizen (7). Cellulase treatment of flocculated cultures dispersed flocs within 4 h, and microfibrils could not be found when examined in the electron microscope. Figure 3 is a carbon-platinum-shadowed preparation of purified microfibrils isolated from R. trifolii NA30.

The effect of cellulase on the bacterial microfibrils and microcrystalline cellulose was

![Fig. 1. Floc structure of R. trifolii 2S under the phase microscope. The bar represents 50 μm.](http://aem.asm.org/Downloaded from http://aem.asm.org on January 27, 2018 by guest)
Fig. 2. Electron micrograph of *R. trifolii* 2L. The microfibrils are present in bundles. The bar represents 1 \( \mu m \).

Fig. 3. Electron micrograph of a shadowed preparation of purified microfibrils from *R. trifolii* NA30. The bar represents 0.2 \( \mu m \).

determined by measuring the amount of reducing sugar released (Table 1). One unit of enzyme is defined as the amount which releases one nanomole of reducing sugar from pure cellulose in 2 h at 37 C under the specified conditions (18). The bacterial microfibrils and the microcrystalline cellulose released approximately 40 nmol of reducing sugars, indicating
the substrates were β-1,4-glucans. Small but measurable amounts of reducing sugar were detected in the control substrates lacking cellulase. Glucose was the only monosaccharide present in the enzymatic hydrolysate as shown by thin-layer and gas-liquid chromatography.

X-ray diffraction patterns of the bacterial microfibrils and microcrystalline cellulose are presented in Fig. 4. Microcrystalline cellulose gave two reinforcement peaks, one at 22.5° 2θ corresponding to an intramolecular d-spacing of 39 nm and a smaller peak at 14.5° 2θ corresponding to a d-spacing of 61 nm. These peaks are characteristic for cellulose I, the polymorphic form of cellulose found in plants (1). The broad peak at 9° was due to the glass slide supporting the microfibrils. The X-ray diffraction pattern of the bacterial microfibrils had peaks which were reduced in intensity but occurred at the same angles as the peaks for microcrystalline cellulose. These differences may be attributed to uneven spreading of the bacterial microfibrils on the glass slide or reduced crystallinity.

The infrared spectra of the bacterial microfibrils and microcrystalline cellulose (Fig. 5) exhibited absorption bands characteristic of the chemical groups and bonds in the cellulose molecule. The band at 890 to 900 cm⁻¹ is characteristic of a β-glucosidic linkage and absence of a band at 870 cm⁻¹ indicates absence of an α-glucosidic linkage (2). The spectrum of the bacterial microfibrils has a band absorbing at 1,500 cm⁻¹ which is absent in the spectrum of microcrystalline cellulose and could be due to a contaminating compound associated with the microfibrils.

The results of these three tests indicate that the microfibrils produced by *Rhizobium* are composed of cellulose.

Table 2 is a list of the rhizobial strains examined for flocs, microfibrils, and infectivity. An absolute correlation between flocculation and microfibril production was observed. Flocculation occurred in the synthetic (BM) and complex (YEM) media. A large percentage (76%) of the strains flocculated. Some strains, e.g., *R. meliloti* 2001, 127E10 and 229 and *R. leguminosarum* 3HOQ1 failed to flocculate initially. Electron microscope examination of these cultures revealed amorphous material at the poles of the rods. After repeated 72-h transfers of large inocula for 2 weeks, these cultures flocculated, and shadowed preparations revealed microfibrils. Strains L1, 227 and 22A1 and *R. leguminosarum* 3HOQ51 failed to flocculate even after repeated transfer.

Microfibrils were found during all stages of the growth cycle of *R. trifolii* NA30 (Fig. 6). When a 72-h stationary culture with microfibrils was transferred to fresh YEM medium, microfibrils were found after 12 h (in lag phase), after 24 h (in exponential phase), and after 48 h (in stationary phase).

*R. trifolii* NA30 cells remained flocculated after transfer to the simulated rhizosphere (Fahraeus assemblages) of the clover host. Bacterial cells were observed predominantly in flocs, but single cells were also found. Figure 7a shows a typical floc structure 3 days after seedling inoculation. Flocs occurred as compact structures. Figure 7b shows flocs 2 weeks later near the growing root tip. These flocs were not compact; the matrix had reduced rigidity under mechanical agitation. Bacterial cellulose synthesis may not have occurred within the rhizosphere, and the change in floc morphology may be a reflection of reduced cellulose production. Although *Rhizobium* does not produce cellulase (11), this enzyme may be released from the plant root (5) and may contribute to the deflocculation of *Rhizobium* cells in the rhizosphere.

Both infective and noninfective strains of *R. trifolii* flocculated and produced microfibrils,

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**Table 1. Hydrolysis of cellulose by cellulase**

<table>
<thead>
<tr>
<th>Cellulose substrate (mg)</th>
<th>Units of enzyme</th>
<th>Reducing sugar produced* (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA30a (0.5)</td>
<td>350</td>
<td>46.8</td>
</tr>
<tr>
<td>NA30 (0.5)</td>
<td>0</td>
<td>6.3</td>
</tr>
<tr>
<td>NBCc (0.5)</td>
<td>350</td>
<td>42.7</td>
</tr>
<tr>
<td>NBC (0.5)</td>
<td>0</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* After 2 h at 37 C with shaking.

* NA30, *R. trifolii* (NA30) purified microfibrils.

* NBC, Nutritional Biochemicals Corp. pure cellulose.

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**Fig. 4. X-ray diffractograms of microcrystalline cellulose and purified microfibrils of *R. trifolii* NA30.**
only 21 infected root hairs were not associated with flocs. A chi square ($\chi^2$) statistical test of these data indicated a significantly greater frequency (at 99% level) of infected root hairs associated with flocs. Thus, the production of cellulose microfibrils by *R. trifolii* NA30, and subsequent flocculation, may facilitate the infection of clover roots. A floc may concentrate a number of infective rhizobia in one area resulting in more infection threads.

There was a high incidence of infection threads occurring in different root hairs which were in contact with the same floc (Fig. 8). In general, a root hair contains only one infection thread. However, double infection threads were found in root hairs of clover in physical contact with flocs of *R. trifolii* (NA30).

Cellulose microfibrils may serve to anchor the bacteria to the root hair surface as suggested by their polar attachment to the plant cell wall. In ultrathin sections the attachment point consists of fibrillar material (Fig. 9). This maintenance of intimate physical association could permit the localized biochemical interactions between the bacterium and the root hair which ultimately results in successful infection.

Dart and Mercer (6) observed "multi-flagellated swarmers" on the root hairs of alfalfa. The "flagella" did not show the characteristic sine wave and appeared thicker than flagella. However, these structures closely resemble the cellulose microfibrils reported here.

The infection thread is composed of two distinct layers, an outer fibrillar layer and an inner amorphous layer containing the bacteria (Fig. 10). Although the rhizobia are capable of producing cellulose, the outer fibrillar layer of the infection thread is probably cellulose of host origin. Ultrathin sections of the cytoplasm surrounding infection threads (Fig. 10) revealed a proliferation of rough endoplasmic reticulum, mitochondria, and dictyosomes. These structures suggest active metabolic activity, e.g.,
FIG. 6. Electron micrographs of stages in the growth cycle of *R. trifolii* NA30. (A) 72-h stationary culture inoculum; (B) 12 h in lag phase; (C) 24 h in exponential growth; (D) 48 h in stationary phase. The bar represents 1 μm.

FIG. 7. Floc structure in the clover rhizosphere under phase contrast. (A) Three days after seedling inoculation flocs (arrows) occurred as compact structures. (B) Flocs 2 weeks later, near the growing root tip, were not compact. The bar represents 50 μm.
Fig. 8. Phase contrast microscopy of root hairs in contact with the same floc and infection threads (arrows) in each root hair. The bar represents 50 μm.

Fig. 9. Electron micrograph showing polar attachment of a bacterium to the root hair cell wall and the fibrillar attachment point. The bar represents 0.2 μm.
cellulose synthesis by the host legume. This would be consistent with the view that infection thread formation is a redirection of growth at the root hair tip (12).

It is concluded from these studies that *Rhizobium* produced cellulose microfibrils in culture which resulted in flocculation of cells. It seems feasible that cellulose microfibrils produced by rhizobia in the legume rhizosphere enhance infection by causing aggregation of infective cells and mediating adsorption to the root hair surface with the aid of lectin (4).

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**LITERATURE CITED**

Pierce Chemical Co., Rockford, Ill.