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**EXPERIMENTAL ARTICLES**

# Investigation of the Cell Surface Structures of Agrobacteria Involved in Bacterial and Plant Interactions

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**Abstract**—Agrobacterial cells produced straight microfibrils not only when in contact with wheat seedling roots, but also when in contact with each other. After 2 h of incubation, agrobacterial cells were found to form aggregates, in which the cells were in contact either directly or through thick straight microfibrils (bridges) of an unknown composition. The majority of the microfibrils were susceptible to attack by cellulase, although some of them showed resistance to this enzyme. Like the wild-type flagellated agrobacteria, their bald mutants produced long straight microfibrils. The cell surface structures of agrobacteria were examined by labeling them immunocytochemically with colloidal gold–conjugated antibodies against *O*-specific lipopolysaccharides, Vir proteins, and cellulase. Agrobacterial cells treated with acetosyringone and brought into contact were found to contain subpolar and polar cell surface structures. Antibodies against the VirB2 protein were able to interact with a tuft of thin microfibrils located on one pole of the agrobacterial cell whose *vir* genes were induced by acetosyringone but were unable to interact with the surface structures of the agrobacterial cells aggregated in liquid medium in the absence of wheat seedlings.

*Key words*: agrobacteria, VirB2 protein, microfibrils.

Bacteria of the genus *Agrobacterium* can induce tumorous growth on a wide variety of dicotyledonous plants due to the Ti plasmid (T-DNA) transfer to the plant genome [1]. The hypothesis that T-DNA transfer to the plant cell and the conjugal transfer of plasmids between bacterial cells are similar processes [2] recently received strong experimental underpinning [3]. The transfer of T-DNA is probably preceded by the stage of bacterial cell attachment to the surface of the recipient plant cells: the *A. tumefaciens* mutants with affected adherence to plant cells were found to be nontumorigenic [4, 5]. At the same time, the attachment-affected exoC mutant of the bacterium *A. tumefaciens* A6-1h was capable of T-DNA transfer to the nuclei of tobacco cells when introduced into their cytoplasm [6]. It is believed that the tight attachment of agrobacterial cells to the surface of plant cells is due to microfibrils containing cellulose, a linear plant polysaccharide composed of glucose residues linked by β-1,4 bonds [5]. The loss of these microfibrils reduces the number of agrobacterial cells attached to the surface of dicotyledonous plants and diminishes the tumorigenic ability of these cells [5]. Some authors have described the formation of polysaccharide filiform appendages on the surface of agrobacterial cells attached to the wheat seedling roots [7, 8]; however, they failed to provide experimental evidence that these structures are made of cellulose. It has been established that the first stage of bacterial attachment to plant cells is mediated by rhicadhesin with a molecular mass of 14 kDa [9] and a polypeptide with a molecular mass of 34–38 kDa [16]. To adhere to biotic and abiotic surfaces, bacterial cells use various surface structures, such as pili or microfibrils.

The involvement of VirB proteins in the conjugal contact between agrobacterial cells and between agrobacterial and plant cells, as well as in the formation of bacterial pili, was first suggested by Engstrom *et al.* [10]. Later, the involvement of the pili in the conjugal transfer of plasmid pML122 was confirmed experimentally [11]. Lai and Kado showed that the *virB2* gene encodes the synthesis of propilin, a structural protein involved in the assembly of pili in agrobacteria [12]. The C-terminal part of the VirB1 protein, VirB1*, was found to possess the properties of propilin and to be likely involved in the primary contact of agrobacterial and plant cells [13].

The aim of the present work was to study the surface structures responsible for contact of agrobacterial cells with each other and for their attachment to plant cells.

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## MATERIALS AND METHODS

**Bacterial strains.** *Escherichia coli* strain S17-1 harboring plasmid pSUP5011 with the Tn5-*mob* transposon was made available by from B. Simarov, All-Russia Research Institute of Agricultural Microorganisms. *Acinetobacter radiobacter* strain 5D-1 was isolated from wheat seedling roots [8]. The transposon mutants 207, 225 through 229, and 362 were derived in our laboratory. *Agrobacterium tumefaciens* strains LMG187 and LMG196 and *Agrobacterium rhizogenes* strains LMG140 and LMG150 were a generous gift from DeLey (Belgium). *A. tumefaciens* strain GV3101 harboring plasmids pPM6000 and pTd33 was kindly donated by B. Hohn (Switzerland).

**Media and conditions of cell growth and preparation.** Agrobacteria were grown in a UBMT-12-250 fermentor at 28°C for 18 h. The growth medium was either a liquid medium 16 containing 10 g glucose, 10 g yeast extract, 1 g $(NH_4)_2SO_4$, and 0.25 g $K_2HPO_4$ in 1 l of tap water (pH 7.0) or a tryptone–soybean medium containing (g/l) tryptone, 5; peptone, 10; and NaCl, 0.9. The grown cells were harvested by centrifugation at 6000*g* for 15 min and washed twice with phosphate buffer (pH 7.0) under the same conditions. The optical density of the final cell suspension was adjusted to 0.1–0.4. Optical density was measured at 600 nm on an SF-46 spectrophotometer using 1-cm-pathlength cuvettes.

**Plant experiments** were performed using wheat *Triticum aestivum* L. seeds and tobacco *Nicotina tabacum* seeds (the latter were obtained from B. Hohn, the Friedrich Misher Institute, Switzerland). Wheat seeds were sterilized and aseptically germinated as described earlier [8].

**Scanning electron microscopy** and the preparation of specimens for it were described in the same publication [8].

**Transmission electron microscopic studies** were carried out with cells of the antibiotic-resistant strains *A. tumefaciens* GV3101 (pPM 6000)$^{Ap50}$, *A. radiobacter* 5D-1$^{Rif75}$, and *A. tumefaciens* UBAPF-2$^{Sm400}$ grown at 25°C in the tryptone–soybean medium supplemented with the respective antibiotics.

Ten microliters of the cell suspension containing $10^8$ cells/ml was placed on a parafilm M sheet, overlaid with a Formvar-coated grid (300 mesh), and incubated at room temperature for 2 h. Cells were then fixed by heating them for 5 min and treated with a blocking solution (20 mM Tris–HCl buffer (pH 8.2) containing 150 mM NaCl, 1% BSA, and 0.02% Tween-20) for 30 min. Antibodies against *A. radiobacter* 5D-1 cells grown in liquid or on solid media with glucose were prepared by immunizing a rabbit with a suspension of these cells treated with 1% glutaraldehyde (such a treatment of the cells cross*links* their surface proteins, as a result of which rabbit antibodies are primarily raised against bacterial surface antigens of a carbohydrate nature). As a marker for particular cell surface constituents, we used the conjugates of colloidal gold with ConA (a lectin specific for glucose), cellulase (direct labeling), and protein A (indirect labeling, when the protein is added to cells after their incubation with homologous antibodies). The synthesis of these conjugates was described in detail earlier [14]. *A. radiobacter* 5D-1 cells were incubated for 1 h with the rabbit antibodies (10 μg/ml) raised against the surface of these cells. The cells were treated with cellulase (Fluka), taken at a concentration of 1.4 μg/ml, for 30 min and then washed with buffer. After treatment with the antibodies, the cells were washed thrice by incubating them in a blocking solution for 15 min. Grids with the washed cells were placed for 30 min in a solution of the gold colloid–protein A conjugate.

Analysis of the surface properties of *A. radiobacter* 5D-1 and its transposon mutants 225, 227, and 362

| Test | Strain | | | |
|---|---|---|---|---|
| | 5D-1 | 225 | 227 | 362 |
| Calcofluor binding | + | – | – | + |
| The presence of flagella | + | + | + | – |
| Antibody + gold colloid conjugate | + | – | + | – |
| Cellulase + gold colloid conjugate | + | – | + | – |

To reveal Vir proteins on the surface of agrobacterial cells pretreated with acetosyringone (100 μM), 10 μl each donor and recipient cell was mixed on parafilm M and incubated at room temperature for 2 h. Formvar-coated grid (300 mesh) for electron microscopy was overlaid on a mixed-culture drop immediately after the beginning of conjugation. Following the conjugation, the cells were fixed by heating them for 5 min, treated with the blocking solution (see above) for 30 min, and incubated for 1 h with the VirB1 protein antibodies (10 μg/ml). After such treatment, the cells were washed thrice by incubating them in the blocking solution for 15 min. Grids with the washed cells were placed for 30 min in a solution of the protein A–colloidal gold (20 nm) conjugate ($A_{520}$ = 0.01).

Specimens were examined in a BS-500 electron microscope (Czech Republic) operated at 60 kV.

## RESULTS AND DISCUSSION

### *Formation of Filiform Structures on the Surface of Agrobacterial Cells Incubated without Wheat Seedlings.*

Agrobacterial cells were found to form multiple microfibrils connecting neighboring cells not only when incubated in the presence of wheat seedlings but also when incubated in the absence of these seedlings (Fig. 1a). It is known that agrobacteria can produce glucans with β-1,2, β-1,3, β-1,4, and β-1,6 bonds [9, 10, 13], particularly β-1,4-glucan (cellulose) [5]. Using the Tn[5] transposon mutagenesis, we obtained several *A. radio-*

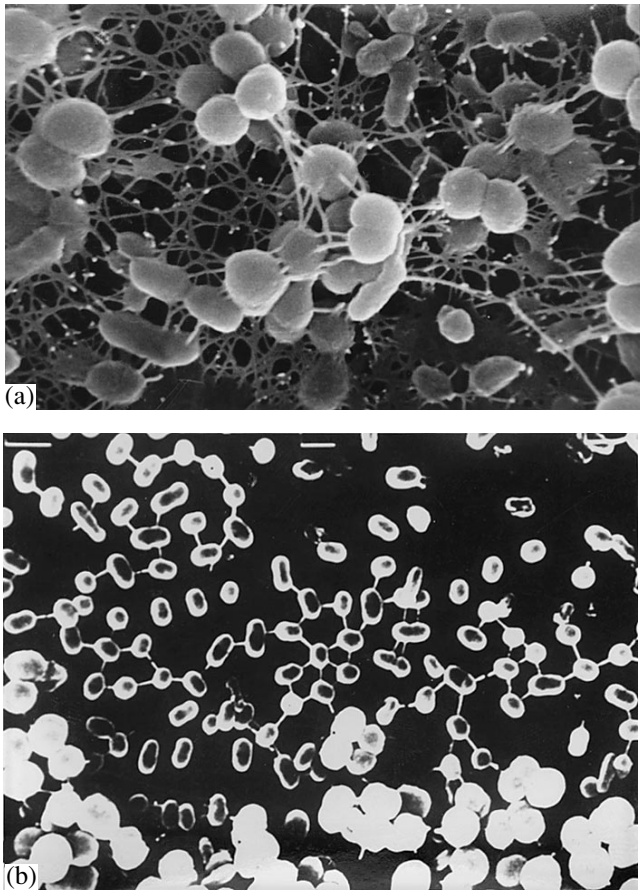


**Fig. 1.** Scanning electron microscopy of the microfibrils of *A. radiobacter* 5D-1 cells incubated on polyester filters: (a) before cellulase treatment (magnification, 5300×); and (b) after 2 h of treatment with cellulase at room temperature (magnification, 6500×).

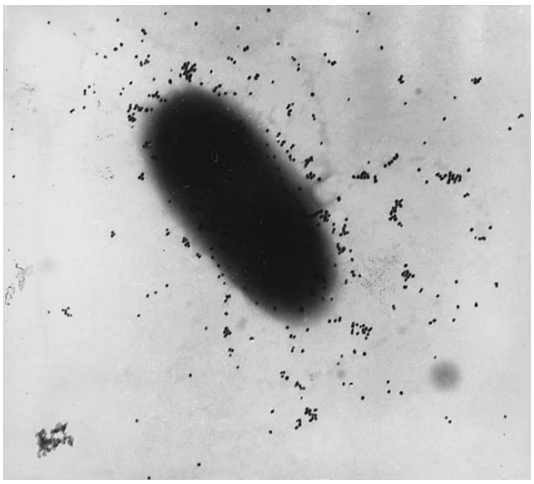

**Fig. 2.** Transmission electron microscopy of a mutant *A. radiobacter* 5D-1 362 (*fla*$^-$) cell labeled with the cellulase–colloid gold conjugate (magnification, 20800×). The mutant was grown in a liquid medium with glucose.

*bacter* 5D-1 mutants defective in the synthesis of calcofluor-binding exopolysaccharides. When grown on a calcofluor-containing solid medium, the colonies of these mutants remained dark under UV light. As was reported by Wood [15], calcofluor is specific for glucans with β-1,3 and β-1,4 bonds. Earlier, the transposon mutants of *A. radiobacter* 5D-1 with an impaired ability to bind calcofluor (the Cal$^-$ mutants) were found to produce, if any, severely shortened microfibrils upon contacting rape roots [16]. Our previous studies of the mutant *A. radiobacter* 5D-1 227 affected in the synthesis of surface polysaccharides showed that its colonies did not fluoresce under UV light when grown on a calcofluor-containing medium and had a reduced ability to adhere to wheat seedling roots and to form long microfibrils. The cellulose nature of the microfibrils did not receive strong experimental support.

In the present work, to confirm the cellulose nature of microfibrils, *A. radiobacter* 5D-1 cells grown in tryptone–soybean medium were treated with cellulase, as a result of which the majority of the cells lost their microfibrils (Fig. 1b). This can be explained by the fact that the microfibrils are mainly composed of β-1,4-glucans. The incomplete removal of the microfibrils by the cellulase may be due to (1) the resistance of some microfibrils to the cellulase because of their protein nature; (2) nonoptimal conditions for a cellulase attack; and (3) the lack of binding sites for the cellulase on the surface of polysaccharide microfibrils.

It should be noted that cellulases are typically mixtures of various glucanases acting on glucans with β-1,4 and β-1,3 bonds. As was shown by Vesper and Bauer [18], the exopolysaccharide succinoglycan can form threadlike appendages on the surface of rhizobia (rhizobial mutants defective in the synthesis of succinoglycan lack such appendages). Succinoglycan (exopolysaccharide I, EPS I), which is composed of repeating octasaccharide units containing three glucose and one galactose residues linked by β-1,4 and β-1,3 bonds, as well as acetyl and succinyl residues, can be synthesized by agrobacteria in large amounts [17].

To find out what cell surface components are susceptible to the action of cellulase, whole agrobacterial cells were analyzed using biospecific gold colloid conjugates. The liquid-phase immunoprecipitation and cell–gold immunoblotting techniques were applied to estimate the efficiency of cellulase interaction with the cell surface of *A. radiobacter* 5D-1 and its mutant affected in the binding of calcofluor. The surface of the *A. radiobacter* 5D-1 cells immobilized on nitrocellulose filters was labeled with the gold colloid conjugates of cellulase and polyclonal antibodies raised against whole 5D-1 cells. Experiments showed that 5D-1 cells did not interact with the ConA–gold colloid conjugate (see table) but interacted with the cellulase–gold colloid conjugate (Fig. 2 and the table). Electron microscopic studies showed that the cellulase–gold colloid conjugate interacted both with the cell surface and with

microfibrils (Fig. 2). The gold colloid conjugate of antibodies raised against the *O*-specific lipopolysaccharides of *A. radiobacter* 5D-1 cells interacted only with the cell surface (Fig. 3). Unlike the parent strain *A. radiobacter* 5D-1, mutants 225 and 362 did not interact with the cellulase (see table). The mutant 362 also lost its ability to synthesize flagellum (see below). At the same time, mutant 227, which is affected in the synthesis of calcofluor-specific polysaccharides, retained its ability to interact with the cellulase (see table). These data confirm the fact that the microfibrils of nontumorigenic agrobacteria incubated without plant tissues contain cellulase-specific sites.

### *Formation of Cell Surface Structures by Conjugating Agrobacteria*

After 2 h of incubation, conjugating agrobacterial cells were in close contact with each other or were connected by thin microfibrils or bridges, forming cell aggregates. It has recently been shown that agrobacteria whose *vir* genes are induced by acetosyringone can form surface protein structures (pili), which are presumably involved in the conjugal transfer of plasmids to other agrobacterial cells and in the transfer of T-DNA to plant cells [11, 12]. The transmission electron microscopic studies of specimens contrasted with uranyl acetate, which is believed to stain mainly protein structures, showed that the conjugating acetosyringone-induced donor and recipient agrobacterial cells are connected by long threadlike surface structures, or microfibrils (Fig. 4). The microfibrils varied in length and diameter, which may be due to (1) their different nature; (2) different conformations of the same microfibrils at different stages of cell interaction; and (3) different stages of the microfibril assembly. After 2 h of incubation, conjugating agrobacterial cells were connected by one pilus or, rarely, by two pili (Fig. 4).

Baron *et al.* suggested that the VirB1 protein can form aggregates on the cell surface [13]. The labeling of noncentrifuged agrobacterial cells on a nitrocellulose membrane with the gold colloid conjugate of the antibodies raised against the VirB1 protein showed that the label binds to the cells, to be more specific, to short appendages and aggregates on the cell surface (data not shown). At the same time, the gold colloid conjugate of the antibodies raised against the VirB1 and VirB2 proteins failed to bind to the microfibrils connecting conjugating agrobacterial cells, unless the donor cells were induced with acetosyringone. The transmission electron microscopy of immunolabeled cells provided direct evidence of the interaction of VirB2 protein antibodies with the thin filaments appearing at one pole of the cells (Fig. 5). Such surface structures were not observed if agrobacterial cells lacked the Ti plasmid or were not treated with acetosyringone. The functional role of the detected surface structures is to be elucidated.

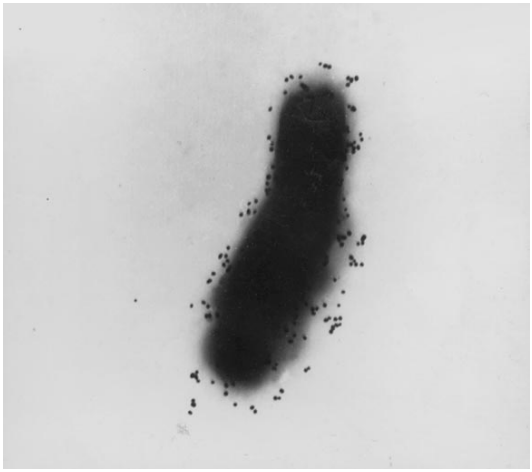

**Fig. 3.** Transmission electron microscopy of an *A. radiobacter* 5D-1 cell labeled with the antibody–colloid gold conjugate (magnification, 26100×). The strain was grown in a liquid medium with glucose. The antibodies were raised against whole *A. radiobacter* 5D-1 cells.

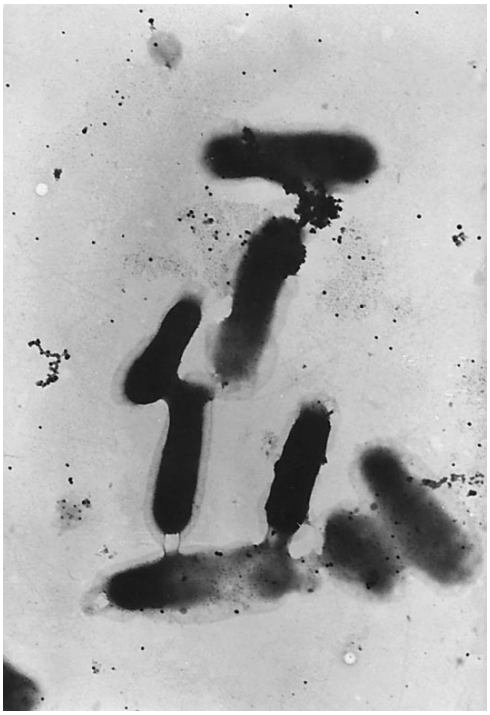

**Fig. 4.** Polar and subpolar contacts in a mixed culture of conjugating *A. tumefaciens* strains (transmission electron microscopy at a magnification of 19600×). Before conjugation, the donor strain GV3101 was incubated with acetosyringone for 2 h. The recipient strain UBAPF2 was not incubated with acetosyringone.

Thus, conjugating agrobacterial cells produce, in addition to cellulose microfibrils, two types of protein surface structures: *vir*-dependent pili and *vir*-independent bridges.

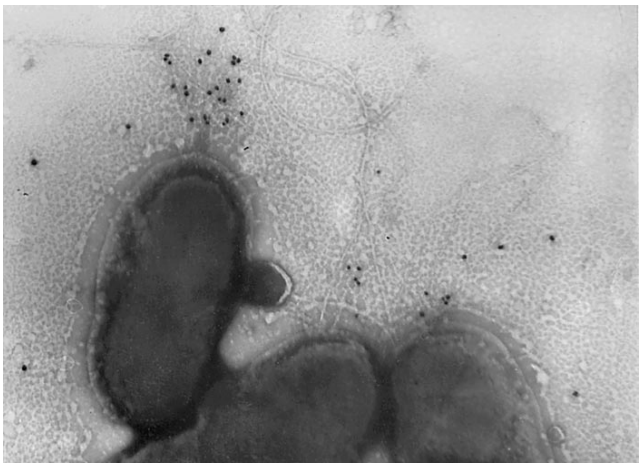

**Fig. 5.** Polar pili of an *A. tumefaciens* cell incubated in CIB medium with 100 μM acetosyringone (transmission electron microscopy at a magnification of 4600×). The antibodies against the VirB2 protein conjugated with colloidal gold interacted with long and flexible structures on one pole of the cell.

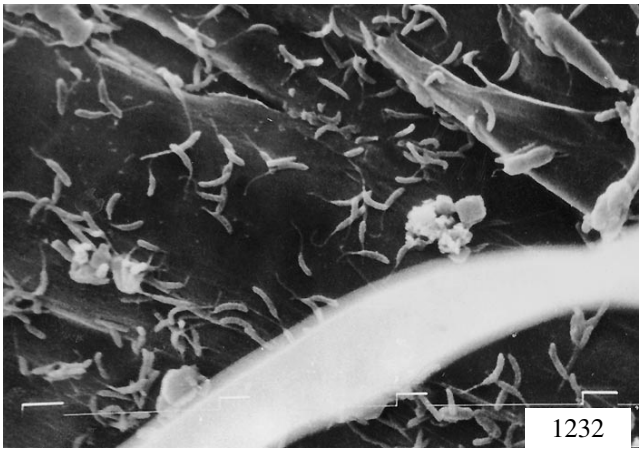


**Fig. 7.** Scanning electron microscopy of a wheat seedling root incubated with *A. radiobacter* 5D-1 cells for 2 h (magnification, 3300×). After cellulase treatment, agrobacterial cells retained their polar surface structures.

### *Formation of Fibrillar Surface Structures by Agrobacterial Cells Colonizing Wheat Seedling Roots*

When incubated with wheat seedlings, agrobacterial cells adhere to both the root epidermis and to root hairs [7–9]. Depending on the conditions and the duration of incubation with the seedlings, a number of agrobacterial cells produce surface threadlike appendages which connect bacterial cells with each other or with plant cells [7, 8] and resemble the surface structures of rhizobia interacting with legume root hairs [9] and of tumorigenic agrobacteria interacting with the surface of dicotyledonous plants [5, 8]. In the present work, we showed that agrobacteria incubated without wheat seedlings can synthesize cellulose, and that their

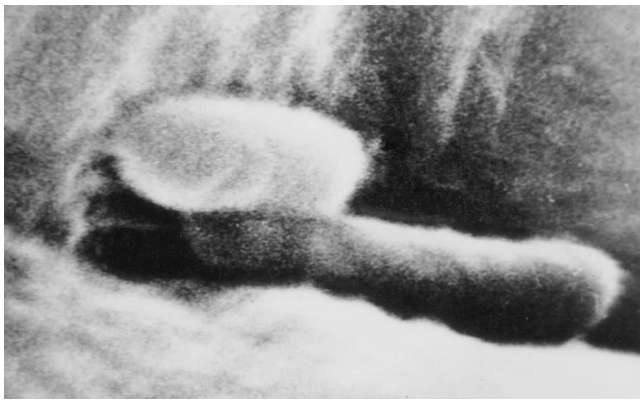

**Fig. 6.** Absence of microfibrils during the colonization of wheat seedling roots by the transposon mutant *A. radiobacter* 5D-1 227 affected in the synthesis of calcofluor-binding polysaccharides (transmission electron microscopy at a magnification of 25700×). Cells were incubated with wheat seedling roots at room temperature for 2 h.

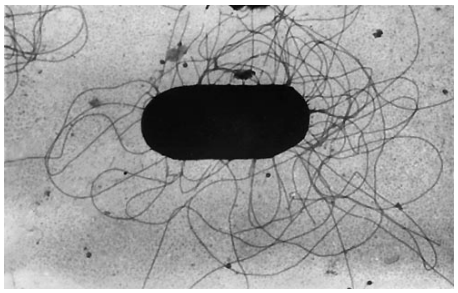

**Fig. 8.** Multiple flagella of an *A. radiobacter* 5D-1 cell incubated in tryptone–soybean medium (transmission electron microscopy at a magnification of 14000×).

mutants defective in the cellulose synthesis are unable to form microfibrils. When attached to the surface of dicotyledonous plants, agrobacteria also produce such microfibrils [7]. However, there is no direct evidence that the microfibrils involved in attaching the agrobacteria to the plant surface are made of cellulose.

When attached to the wheat seedling roots, the $Tn^5$ transposon mutants of *A. radiobacter* 5D-1 defective in calcofluor binding, i.e., mutants 227 and 228, failed to produce long microfibrils (Fig. 6). This finding may suggest that the long microfibrils formed during the incubation of agrobacteria with wheat seedlings are made of calcofluor-binding polysaccharides or cellulose. This suggestion is confirmed by the following observation: when strain 5D-1 was incubated with wheat seedlings in the presence of cellulase, the thin and long microfibrils typical of attached agrobacterial cells were not formed (Fig. 6).

On the other hand, the possibility of an involvement of flagella and pili in the attachment of agrobacteria to plants cannot be excluded. For instance, the pili of *Bradyrhizobium japonicum* were found to be involved in the attachment of this bacterium to the legume plant

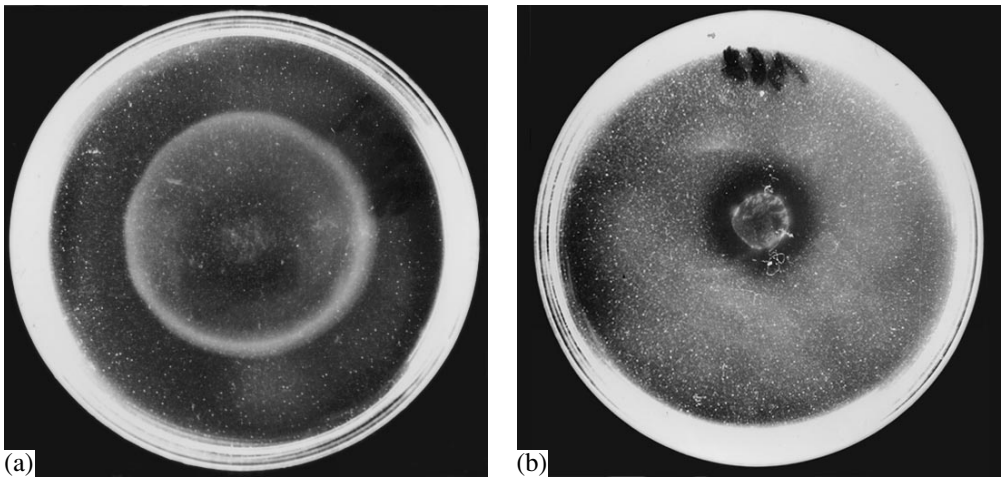


**Fig. 9.** Chemotactic rings of (a) *A. radiobacter* 5D-1 and (b) the $Tn^5$ mutant 362 (*fla*$^-$) on a 0.03% soft agar containing 1 mM glucose. 100 μl of cell suspensions ($10^9$ cells/ml) were placed at the centers of the petri dishes and incubated at 28°C for 48 h.

surface [18]. However, respective experimental data for agrobacteria are absent.

It is generally believed that the flagella of agrobacteria are not responsible for their phytopathogenicity, since *A. tumefaciens* mutants lacking flagella are virulent and are able to adhere to the mesophilic tissues of *Zinnia* [19]. However, Chesnokova *et al.* showed that the loss of flagella was associated with a 38% reduction in the virulence of agrobacteria [20]. In our experiments, the number of cells of the flagella-lacking mutant 362 of *A. radiobacter* 5D-1 attached to the wheat seedling roots was 3.5 times lower than in the case of the parent strain. After cellulase treatment of the *A. radiobacter* 5D-1 cells attached to the wheat seedling roots, one could observe only rare, flexible, polarly arranged surface structures resembling polar flagella (Fig. 7). It should be noted that, so far, the involvement of flagella in the attachment of agrobacteria to plant cells has not been described.

Thus, the role of various surface structures of agrobacteria in bacterial and plant interactions remains poorly understood.

### *Are the Flagella of Agrobacteria Involved in Interactions with Wheat Seedling Roots?*

When cultivated in liquid and on solid media, agrobacteria produce numerous flagella, which can be observed with transmission and scanning electron microscopes (Fig. 8). The experimental data described below suggest that the flagella of agrobacteria may play a role in the interactions with plant cells. Indeed, the 362 mutant of *A. radiobacter* 5D-1 altered in chemotaxis and motility (Figs. 9a, 9b) was found to have no flagella (Fig. 10). The mutation of the *exoC* gene of agrobacteria led to the loss of flagella and the ability to synthesize β-1,2-glucan and succinoglucan. The pleiotropic character of mutant 362 suggests that it may also

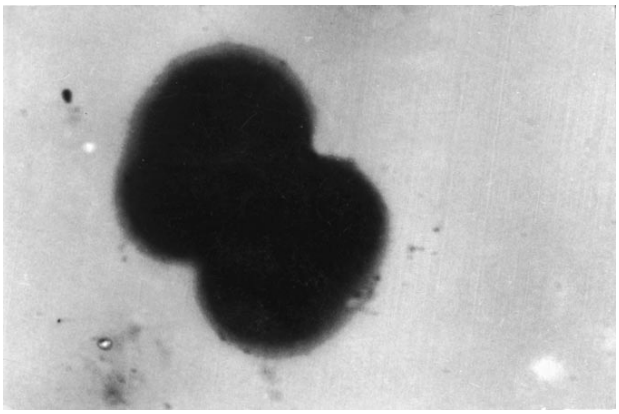

**Fig. 10.** Absence of flagella on the $Tn^5$ mutant *A. radiobacter* 5D-1 362 cells grown in tryptone–soybean medium at 28°C (transmission electron microscopy at a magnification of 40000×).

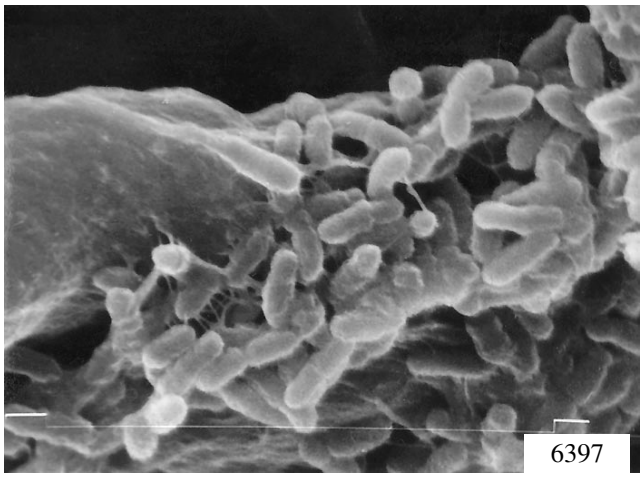


**Fig. 11.** Microfibrils of the $Tn^5$ mutant *A. radiobacter* 5D-1 362 cells incubated with wheat seedling roots for 2 h (transmission electron microscopy at a magnification of 7200×).

be defective in the synthesis of surface polysaccharides. However, the analysis of colonies of nonmotile mutants grown on calcofluor-containing solid media showed that they fluoresce under UV light (as does the wild-type strain). The lipopolysaccharide compositions of strain 5D-1 and mutant 362 were also identical. Therefore, the loss of flagella and the inability of mutant 362 to synthesize calcofluor-binding exopolysaccharides are not related. Scanning electron microscopy showed that the bald mutant 362 formed a net of thin and long microfibrils when it was incubated with wheat seedlings (Fig. 11). Cellulase treatment of the wild-type 5D-1 cells attached to wheat seedling roots led to the disappearance of all straight microfibrils except for a polar flagellum (Fig. 7). The absence of flagella correlated with a decrease in the ability of *A. radiobacter* 5D-1 cells to adhere to the wheat seedling roots. Therefore, the flagella of agrobacteria are involved in their interactions with plants.

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