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EXPERIMENTAL ARTICLES

Conjugative Transfer of Plasmid pTd33 in Agrobacteria

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Abstract—Supramembrane structures that connect conjugating agrobacterial cells were visualized for the first time by transmission electron microscopy. The primary contact of cells during conjugation was shown to occur through the formation of long pili containing no VirB1 protein. Pretreatment of agrobacterial cells with acetosyringone resulted in a six- to tenfold increase in the transfer frequency of plasmid pTd33 at 19–25°C and had almost no effect at 30°C. The transfer of plasmid pTd33 from *A. tumefaciens* strain GV3101 to plasmid-free *A. tumefaciens* strain UBAPF-2 was 16 times decreased after the centrifugation of cells. The transfer efficiency of plasmid pTd33 from *A. tumefaciens* strain LBA2525 (*virB2::lacZ*) to plasmid-free *A. tumefaciens* strain UBAPF-2 was one order of magnitude lower than the transfer from the wild-type *A. tumefaciens* strain GV3101. Treatment of donor cells with 0.01% SDS before mating decreased the transfer efficiency by a factor of 26. The role of pili in the establishment of contact between conjugating cells of agrobacteria is discussed.

Key words: Agrobacterium, conjugation, plasmid pTd33, pili, transfer.

Bacteria of the genus *Agrobacterium* are capable of inducing undifferentiated tumors in a large number of dicotyledons. The process of infection occurs by the transfer and insertion of a fragment of the plasmid pTd33 (T-DNA) from the agrobacterium into the plant genome. Agrobacteria attach to the surface of a plant cell and transfer a portion of their genetic information to it by a poorly known mechanism. It has been established that the transfer of T-DNA is promoted by *vir* genes and requires the participation of the protein products of the *virB* and *virD* operons. In particular, it has been shown that *virB1-11* and *virD4* gene products are responsible for the transfer of T-DNA from bacterial to plant cells [1–3]. The hypothesis about the similarity of the T-DNA transfer into plant cells and conjugative plasmid transfer between bacteria was proposed more than ten years ago [4, 5] and has been experimentally verified in a number of works [2, 6, 7]. It has been shown that mutations in *virA*, *virB*, and *virG* genes cause a decrease in the conjugative Ti plasmid transfer frequency in agrobacteria by two to four orders of magnitude. The transfer frequency was restored by the introduction of cosmids with the wild-type copies of *vir* genes [8, 9]. On the other hand, some authors have found no relationship between the conjugative Ti plasmid transfer and mutations in *virA*, virB, *virD*, and *virG* [10, 11]. In 1987, Engstrom *et al.* [5] suggested for the first time that VirB proteins participate in the process of conjugative contact and the formation of pili. However, pili were visualized for the first time and shown to be involved in the conjugative transfer of plasmid pML122 between agrobacteria only a decade later [2]. It has been shown that the *virB2* gene encodes propilin, which is a structural protein required for the assembly of pili in agrobacteria [1]. Baron *et al.* [3] have established that VirB1* protein, which is a C-terminal fragment of VirB1 weakly bound to the membrane, possesses propilin-like properties and, probably, is involved in the primary contact with the recipient cell. The mechanism of conjugative transfer remains unclear; in particular, it is not known which surface structures of the agrobacterial cell are involved in the primary contact and transfer of genetic information into bacterial and plant cells.

In this work, we studied (1) the influence of mating conditions on the frequency of conjugative plasmid pTd33 transfer between agrobacterial strains and (2) the cell surface structures involved in the primary contact between donor and recipient cells of agrobacteria.

MATERIALS AND METHODS

Agrobacterium strains used and their characteristics are presented in Table 1.

Determination of the plasmid transfer efficiency. Protocol 1. Agrobacteria were grown in liquid TSA medium (pH 7.0) with the corresponding antibiotics at 25°C. A 200-μl portion of an overnight culture was diluted in 1 ml of fresh liquid CIB [18] medium containing 100 μM acetosyringone (Aldrich) and allowed to grow for 3 h. Donor and recipient cells were mixed at ratios of 3 : 1–7 : 1. Cell suspensions were adjusted to a density of 10^8 cells/ml on a SF-46 spectrophotometer (Russia), pipetted at 5 or 20 μl on nitrocellulose fil-

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ters 1 or 2 cm in diameter, respectively, with a 0.45-μm pore diameter (Millipore), and placed in petri dishes with the slightly dried agar medium CIB (pH 5.0). After 0.5 and 1.5 h, filters were moistened with 10 μl of fresh liquid medium CIB. Conjugation was performed for 4 h at temperatures varying from 19 to 31°C. Transconjugants were collected from the filters in 4.5 ml of a phosphate buffer (pH 7.0), and a series of 10-fold dilutions in phosphate buffer were prepared. Transconjugants were plated on petri dishes with 1.5% agar containing the corresponding antibiotics and incubated at 28°C. Concentrations of antibiotics were as follows (μg/ml): ampicillin (Ap), 50; rifampicin (Rif), 75; streptomycin (Sm), 400, kanamycin (Km), 50–100; and carbsyncymin (Cb), 25. Colonies were counted on the third day after plating.

Protocol 2. (a) Donor cells grown for 3 h were centrifuged at 6000 g for 10 min and resuspended in the mating medium.

(b) Donor cells were incubated with 15 mM carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) (Fluka) for 30 min, then centrifuged at 5000 g for 5 min, and resuspended in CIB medium. Donor and recipient cells (D_{600} = 0.5) were mixed at a ratio of 3 : 1 in liquid CIB medium (pH 5.5), allowed to conjugate for 18 h at 25°C, and then plated at serial tenfold dilutions onto dishes with appropriate antibiotics.

Electron microscopy. For transmission electron microscopy, agrobacteria were grown according to protocol 1. Donor and recipient cells (10 μl each) were mixed on a piece of parafilm M and immediately covered by a formvar-coated metal grid (300 mesh). Conjugation was performed for 2 h at room temperature. After the termination of conjugation, cells were heat-fixed for 5 min and treated with a blocking solution (20 mM Tris–HCl, 150 mM NaCl, 1% BSA, and 0.02% Tween-20 (pH 8.2)) for 30 min. Then the samples were incubated with VirB1-specific antibodies (10 μg/ml) for 1 h. Unbound antibodies were removed by washing with the blocking buffer for 15 min three times, after which the grids were stained in a solution of protein A–conjugated colloidal gold with a 20-nm particle diameter (4–12 μg/ml) for 30 min. After staining, the grids were rinsed with distilled water and examined under a BS-500 electron microscope (Czechoslovakia) at a voltage of 60 kV.

RESULTS AND DISCUSSION

Influence of the mating temperature and acetosyringone on the transfer of the pTd33 plasmid between agrobacteria. We studied the influence of temperature (19, 25, and 31°C) on the process of the conjugative transfer of the pTd33 plasmid from *A. tumefaciens* strain GV3101 to *A. tumefaciens* plasmid-free strain UBAPF-2 and to strain 5D-1 of the nonpathogenic species *A. radiobacter*. It was established in five independent experiments that the optimal temperature for the transfer of the pTd33 plasmid between agrobacteria was 25°C. Table 2 shows the typical frequencies of the pTd33 plasmid transfer between agrobacteria. Decreasing the temperature from 25 to 19°C produced almost no effect on the transfer frequency, whereas increasing the temperature from 25 to 30°C decreased the pTd33 plasmid transfer efficiency by one order of magnitude during mating in CIB medium (Table 2). A similar regularity was observed for the mating in TSA medium. As shown by Tempe *et al.* [14], the conjugative transfer of the Ti plasmid between agrobacteria is decreased by four orders of magnitude at temperatures higher than 30°C. Fullner *et al.* [15] found that the optimal temperature for the transfer of plasmid pML122 on solid media is 19°C, and the transfer is almost completely inhibited above 29°C. The data obtained by this research group [2] indicate that pili are the common structures involved both in the transfer of T-DNA into plant cells and in the transfer of conjugative plasmids between agrobacteria. The synthesis of pili is almost absent at 30°C [15]. The similar temperature dependence of both processes, the plasmid transfer, and the expression of pili suggests that pili are involved in the process of the conjugative transfer of plasmids. Fullner *et al.* proposed that agrobacteria use

Table 1. Bacterial strains and plasmids used in the work

Designation	Characteristics of strains (plasmids)			Source
	Chromosome	Markers	Plasmids	
Bacterial strains				
A. tumefaciens GV3101	C58	Rif^R	pPM6000	B. Hohn (Switzerland)
A. tumefaciens LBA2525	C58	Rif^R	pTiB6 (*virB2::lacZ*) cured	P. Hooykaas (Netherlands)
A. tumefaciens UBAPF-2		Rif^R Sm^R	of the pTd33 plasmid	N. Novikova (Russia)
A. radiobacter 5D-1		Rif^R Sm^R		M. Chumakov (Russia)
Plasmids				
pPM6000		Ap^R	Ap^r derived from pTiAch5	
pTd33		Km^R, Gm^R	derived from pCGN1559	
pTiB6		Cb^R, *virB2::lacZ*		

Table 2. Influence of temperature and pretreatment of donor cells with 100 [mu]M acetosyringone (AS) on the efficiency of the pTd33 plasmid transfer between *A. tumefaciens* GV3101 and *A. tumefaciens* UBAPF-2

Mating temperature, °C	Transfer frequency per recipient cell on CIB medium	Transfer frequency per recipient cell on CIB medium + AS
19	2.35×10^{-6}	2.00×10^{-5}
25	4.1×10^{-6}	2.87×10^{-5}
31	9.5×10^{-7}	1.32×10^{-6}

Note: Mating was carried out on CIB medium according to protocol 1.

Table 3. Frequencies of the pTd33 plasmid transfer from *A. tumefaciens* GV3101 and *A. tumefaciens* LBA2525 to plasmid-free *A. tumefaciens* UBAPF-2 cells under *vir*-inducing conditions

Donor × Recipient	Transfer frequency per recipient cell
A. tumefaciens GV3101 Gm^{10} × × *A. tumefaciens* UBAPF-2	3.0×10^{-5}
A. tumefaciens LBA2525 (*virB2::lacZ*) × × *A. tumefaciens* UBAPF-2	3.6×10^{-7}

Note: Mating was carried out on CIB medium according to protocol 1.

Table 4. Influence of centrifugation and pretreatment with an inhibitor of protein synthesis (CCCP) on the efficiency of the pTd33 plasmid transfer between *A. tumefaciens* GV3101 and *A. radiobacter* 5D-z

Conditions		Transfer frequency per recipient cell
Centrifugation of donor cells	Preincubation of recipient cells with 15 mM CCCP	
–	–	2.0×10^{-5}
+	–	1.2×10^{-6}
–	+	1.1×10^{-7}
+	+	2.3×10^{-8}

Note: Mating was carried out according to protocols 2a and 2b.

the same *vir*-induced channel (machinery) for the transfer of T-DNA and plasmids [2].

It is known that the expression of *vir* genes is induced by acetosyringone [2, 5]. It was shown that the treatment of cells with 100 μM acetosyringone before mating caused a sevenfold increase in the transfer frequency of the pTd33 plasmid between agrobacteria [8]. We suggested that acetosyringone may affect the efficiency of the *vir*-dependent pTd33 plasmid transfer between agrobacteria and studied the influence of acetosyringone pretreatment on this process at temperatures favorable (19°C) and unfavorable (31°C) for the synthesis of pili. The induction by acetosyringone was found to increase the transfer frequency of the pTd33 plasmid by a factor of six to nine at 19 and 25°C and only by a factor of 1.4 at 31°C (Table 2). Therefore, the exposure to 31°C produces a negative effect on the *vir*-dependent transfer of the pTd33 plasmid. Interestingly, the effect of high temperature on the conjugative transfer efficiency was more pronounced than that of acetosyringone. Several authors have suggested that one or more genes localized within the *virB* region of the pTd33 plasmid are responsible for the formation of a channel that connects agrobacterial and plant cells [5]. In particular, it has been suggested [16] and experimentally confirmed [1] that the *virB2* gene (together with *virB3* and *virB9*) is involved in the synthesis of conjugative pili in *A. tumefaciens* [1]. It has been shown that the *virB1* gene controls the synthesis of propilin that participates in the transfer of the T-complex [3]. We found that the efficiency of the pTd33 plasmid transfer to plasmid-free *A. tumefaciens* strain UBAPF-2 from *A. tumefaciens* strain LBA2525 is 68 times lower than the transfer from wild-type *A. tumefaciens* strain GV3101 (Table 4). Since, in both cases, acetosyringone-induced cells were used for the transfer, the lower plasmid transfer efficiency for *virB2*-mutant strain LBA2525 indicates the participation of the VirB2 protein in the process of pTd33 plasmid transfer between agrobacteria.

Influence of centrifugation and pretreatment with CCCP on the transfer of the pTd33 plasmid between agrobacteria. According to Fullner *et al.* [2, 15], pili probably mediate the conjugative transfer of plasmid pML122 between agrobacteria. However, so far, there is no direct evidence for this suggestion. Pili (fimbria) are external cell structures, which are easily disrupted during mixing, cultivation, shaking on a vibrator, or ultrasound treatment.

It can be suggested that centrifugation of *vir*-induced donor cells will influence the transfer efficiency of the pTd33 plasmid. We found that the frequency of the pTd33 plasmid transfer to plasmid-free *A. tumefaciens* strain UBAPF-2 from *A. tumefaciens* GV3101 cells centrifuged at 6000 g was 16 times less than that for the transfer from noncentrifuged cells (Table 3). Treatment of cells with 15 mM CCCP, which inhibits the respiration and protein synthesis, resulted in a 200-fold decrease in transfer efficiency (Table 4). When donor cells were centrifuged and treated with CCCP simultaneously, the transfer of the plasmid was decreased 870 times. These data indicate that pili or other structures that participate in the transfer of the pTd33 plasmid are removed from the surface of donor cells during centrifugation and are not restored subsequently in CCCP-treated cells.

Putative removal of the surface structures from the cell surface during centrifugation suggests that they could be found in the supernatants. We treated wheat roots with the concentrated supernatant (obtained after

Table 5. Influence of pH on the efficiency of the pTd33 plasmid transfer between agrobacteria

Donor × Recipient	pH of the mating buffer	Mating medium	Transfer frequency per recipient cell
A. tumefaciens GV3101 × *A. radiobacter* 5D-1 Sm^{400}	5.5	CIB	2.1×10^{-5}
A. tumefaciens GV3101 × *A. radiobacter* 5D-1 Sm^{400}	7.0	TSA	6.7×10^{-6}

Note: Mating was carried out at 19°C according to protocol 1.

Table 6. Influence of Ca^{2+} ions on the efficiency of the pTd33 plasmid transfer from *A. tumefaciens* GV3101 to *A. radiobacter* 5D-1

Concentration			Transfer frequency (per recipient cell)	Number of transconjugants per ml	
Ca^{2+}, M	EDTA, mM	acetosyringone (AS), μm		*M*	δ
0	0	0	4.30×10^{-6}	430	14.0
0.05	0	0	5.31×10^{-6}	531	17.9
0.1	0	0	5.90×10^{-6}	590	58.1
0	0	100	2.83×10^{-5}	2832	112.9
0.05	0	100	5.80×10^{-5}	5801	171.1
0.1	0	100	2.80×10^{-5}	2803	108.6
0.21	10	100	5.92×10^{-6}	592	23.6
0	10	100	3.71×10^{-7}	37	1.1

Note: Agrobacteria were grown on CIB medium according to protocol 1 (pH 5.5). Cells from an overnight culture (200 μl) were placed into 1 ml of fresh medium supplemented with 100 μM acetosyringone and various Ca^{2+} concentrations. All subsequent procedures followed protocol 1. The sample size was 10. *M* is the arithmetic mean. δ is the root-mean-square deviation.

Table 7. Influence of 0.01% SDS on the efficiency of the pTd33 plasmid transfer from *A. tumefaciens* GV3101 to *A. radiobacter* 5D-1

Introduction of 0.01% SDS	Number of transconjugants per ml		Transfer frequency per recipient cell
	M	δ	
To the donor 30 min before mating	54	7	5.4×10^{-7}
30 min after initiation of mating	36	20	3.6×10^{-7}
60 min after initiation of mating	37	8	3.7×10^{-7}
Control (without SDS)	2431	661	1.4×10^{-5}

Note: Agrobacteria were grown according to protocol 1. Before mating, donor cells were incubated with SDS at a final concentration of 0.01% for 30 min in liquid CIB medium, washed by centrifugation at 5000 g for 5 min, and resuspended in CIB medium. Donor and recipient cells ($D_{600} = 0.5$) were mixed at a ratio of 7 : 1. Conjugation was performed at 25°C, pH 5.0. *M* is the arithmetic mean. δ is the root-mean-square deviation. The sample size was 9.

centrifugation) of agrobacterial cells and revealed a decrease in the number of agrobacteria attached to the plant surface (data not shown).

It is known that pili may be restored within 30–40 min of their breakage [19]. Since the mating time significantly exceeded the time of possible restoration, a portion of the donor cells might have restored their pili. Analysis of the effect produced by 15 mM CCCP on the centrifuged cells suggests that it blocks the restoration of pili or similar structures involved in the transfer of the plasmid. This suggestion is supported by the data presented in Table 4. Since the transfer requires the expression of a large number of genes, CCCP probably affects not only the formation of the transfer machinery but also other stages of this complex process. In addition to the blockage of pilus synthesis, the CCCP treatment may impair the synthesis of other components required for DNA transfer. The significant effect of this respiration inhibitor on the conjugative pTd33 plasmid transfer from non-centrifuged *vir*-induced cells indicates the energy-consuming character of the process or, at least, some of its stages. This fact is in agreement with the observation that the proteins VirB4 and VirB11 exhibit a moderate ATPase activity [17].

Influence of pH on the efficiency of the pTd33 plasmid transfer between agrobacteria. The influence of pH on the transfer of the pTd33 plasmid from *A. tumefaciens* GV3101 to *A. radiobacter* 5D-1 was

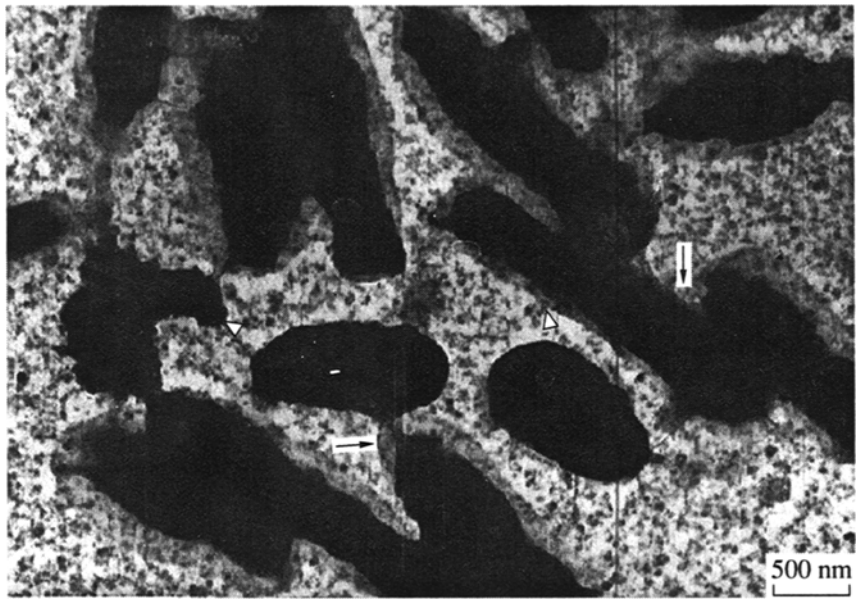

Fig. 1. Conjugating *A. tumefaciens* LBA2525 and *A. tumefaciens* UBAPF-2 cells after 2 h of co-incubation at 31°C. Single triangles indicate thin (about 10 nm in outer diameter and 50–100 nm long) pilus-like structures that connect the conjugating cells. The double triangle indicates a structure of about 50 nm in diameter and 120 nm long. The arrow shows a close contact between cells.

studied on CIB and TSA media. In four independent experiments, the higher transfer frequency was observed at the acid pH 5.5 of the medium (Table 5), which is consistent with previously reported data [8]. It should be noted that the frequency of the plasmid pML122 transfer between agrobacteria via the *vir*-independent pathway was found to decrease at low pH values [15]. The mechanism of the pH effect on the process of the conjugative transfer of the pTd33 plasmid in agrobacteria remains unknown. It may by suggested that at low pH values the surface proteins involved in the transfer have a more favorable structure.

Influence of Ca^{2+} ions on the efficiency of the pTd33 plasmid transfer between agrobacteria. Calcium ions are required for the assembly of flagella and pili. The stimulation of transmembrane DNA transfer by calcium ions has been described by Borovyagin *et al.* [18]. It has been shown that high concentrations of calcium induce membrane folding and the formation of cell contacts facilitating membrane fusion and the subsequent transfer of DNA between conjugating cells [18].

We studied the influence of two calcium concentrations in the mating medium on the process of the conjugative transfer of the pTd33 plasmid from *A. tumefaciens* strain GV3101 to plasmid-free *A. tumefaciens* strain UBAPF-2 under *vir*-inducing and noninducing conditions. In the absence of acetosyringone, both 0.1 and 0.05 M calcium only slightly increased the transfer frequency (Table 6). A similar effect was observed for a 0.1 M calcium solution under *vir*-inducing conditions. The lower concentration of calcium ions (0.05 M) caused a reliable (at 1% significance level) twofold increase in the pTd33 plasmid transfer under *vir*-inducing conditions (Table 6). It is known that EDTA chelates bivalent ions and induces the disassembly of pili [19]. In our experiments, the treatment of donor cells with EDTA before mating decreased the transfer efficiency of the pTd33 plasmid 76 times under inducing conditions (Table 6). Without the addition of calcium to the mating medium, the reduction of the transfer frequency after EDTA pretreatment was most pronounced.

Influence of SDS on the efficiency of the pTd33 plasmid transfer between agrobacteria. Isolation and study of pili in *E. coli* has revealed that certain types of pili undergo denaturation in the presence of 0.01% SDS due to the dissociation of protein subunits bound by hydrophobic interactions [19]. We found that the introduction of 0.01% SDS into the mating medium had a marked effect on the transfer of the pTd33 plasmid from *A. tumefaciens* GV3101 to *A. radiobacter* 5D-1 cells (Table 7). Treatment of the donor cells with 0.01% SDS for 30 min before mating caused a 44-fold decrease in the transfer efficiency. The addition of a 0.01% SDS solution 30 and 60 min after the initiation of mating reduced the transfer efficiency of the pTd33 plasmid from control levels by a factor of 66. The effect of the SDS pretreatment is weaker than the introduction of SDS into a suspension of mating cells, which may be explained by the fact that, after the pretreatment, donor cells were washed from SDS and, therefore, could restore their ability to conjugate during the subsequent co-incubation, whereas, in other variants, SDS remained in the medium during the whole co-incubation period.

In *E. coli*, the attachment of donor to recipient cells during mating is achieved within 5 min, whereas the

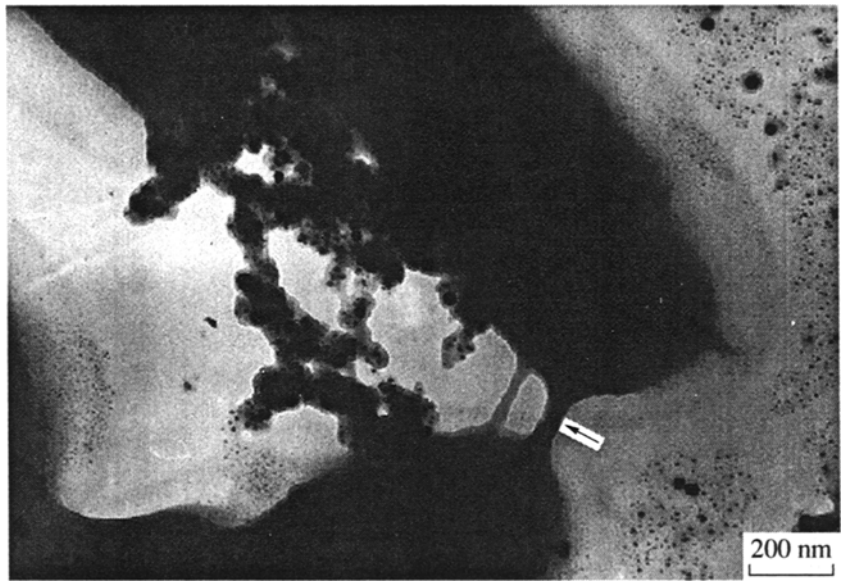

Fig. 2. Formation of a conjugation bridge between cells of *A. tumefaciens* GV3101 and *A. tumefaciens* UBAPF-2 after 2 h of incubation. The contact is mediated by two filamentous structures (with diameters of 50 and 30 nm and a length of 140–170 nm) (indicated by arrow).

whole process of the F plasmid transfer takes 150 min [20]. Sabel'nikov *et al.* reported that the conjugative transfer of plasmid ColB-R3 from *E. coli* W3110 into *E. coli* AB1157 is completed within 35 min [21]. The results presented in Table 7 suggest that the process of the pTd33 plasmid transfer between agrobacterial strains takes more than 60 min and requires the formation of pili or similar structures for the establishment of primary contact between conjugating cells, since SDS significantly affects the transfer efficiency.

Electron microscopy of conjugating agrobacterial cells. We demonstrated for the first time the formation of intracellular VIR-independent contacts between conjugating cells (Figs. 1 and 2). Transmission electron microscopy revealed the presence of long filamentous structures that connected donor and recipient cells (Fig. 1). The length and diameter of these structures varied, which might indicate the involvement of different structures in this process or the conformational variability of the same structure at different stages of interaction. Earlier, it was established that the contact of mating *E. coli* cells with the end of the pilus leads to its retraction and, therefore, thickening [20]. At the early stage of conjugation, agrobacterial cells were connected, as a rule, by a single (more rarely, two) filamentous structure (Figs. 1 and 2). After 2 h of co-incubation, most conjugating cells came into close contact, and these structures were undetectable or lacking (Fig. 1). The contact structures were localized at the polar end of the agrobacterial cells or near it (Fig. 1). One cell could be found in contact with several partners simultaneously (Figs. 1 and 2).

Staining of non-centrifuged cells with colloidal gold (carried out with VirB1-specific antibodies on nitrocellulose membranes) revealed the ability of the probe to bind to whole cell preparations (data not shown). Baron *et al.* [3] suggested that the VirB1* protein representing a C-terminal fragment of VirB1 is weakly bound to the membrane, can be secreted into the culture medium, and possesses propilin-like properties. It cannot be established by staining on a nitrocellulose membrane whether the probe interacts with the cell surface or with the protein excreted into the medium and fixed to the membrane. The probe (colloidal gold-labelled VirB1-specific antibodies) did not bind to the long filamentous structures in the acetosyringone-induced donor cells, as revealed by transmission electron microscopy. On the basis of these experiments, it is concluded that the distant contact of agrobacterial cells during conjugation occurs via the formation of long filamentous structures containing no VirB1 and VirB2 proteins.

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