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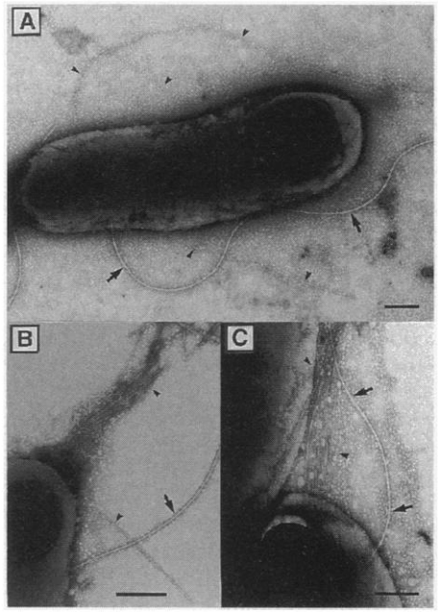

Fig. 1. *Agrobacterium tumefaciens* A348 producing pili. A348 was grown at 19°C in the presence of 200 μM acetosyringone (*10, 11*). All scale bars indicate 200 nm. Arrowheads, pili; arrows, the flagella.

Pilus Assembly by *Agrobacterium* T-DNA Transfer Genes

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Agrobacterium tumefaciens can genetically transform eukaryotic cells. In many bacteria, pili are required for interbacterial DNA transfer. The formation of pili by *Agrobacterium* required induction of tumor-inducing (Ti) plasmid–encoded virulence genes and growth at low temperature. A genetic analysis demonstrated that *virA*, *virG*, *virB1* through *virB11*, and *virD4* are the only Ti plasmid genes necessary for pilus assembly. The loss and gain of pili in various mutants correlated with the loss and gain of transferred DNA (T-DNA) transfer functions, which is consistent with the view that *Agrobacterium* pili are required for transfer of DNA to plant cells in a process similar to that of conjugation.

Agrobacterium tumefaciens is a Gram-negative bacterium with the capability of transforming eukaryotic cells. This pathogen normally infects plants, which results in the neoplastic disease crown gall, but the bacterium can also stably transform yeast cells (*1, 2*). During infection of plant cells, a segment of the *Agrobacterium* Ti plasmid, termed the T-DNA, is transferred into the plant cell nucleus, where it is integrated into the plant genome (*1*). Although this transformation system has been widely applied in studies of plant molecular biology and crop plant improvement, fundamental questions remain regarding the mechanism by which *Agrobacterium* introduces DNA into eukaryotic cells. Here we report on one crucial component of the process.

At least 10 operons, *virA* through *virJ*, on the Ti plasmid are involved in processing and transfer of T-DNA (*1*). These *virA* through *virJ* genes are regulated by the VirA-VirG two-component regulatory system and are transcribed only when the bacteria are exposed to chemical inducers found in the wound site of a plant (*3*). T-DNA is transferred to the plant cell as a single strand covalently attached to the protein VirD2 (*1*). The VirE2 protein is also transferred either separately or in association with T-DNA (*1, 4*). Transfer of T-DNA and VirE2 requires at least 12 additional proteins, VirB1 through VirB11 and VirD4 (*4, 5*), which are related to transport proteins involved in the assembly of conjugative sex pili and export of toxins by the human pathogens *Bordetella pertussis* and *Helicobacter pylori* (*6, 7*). On the basis of these homologies and subcellular fractionation studies, VirB1 through VirB11 and VirD4 are thought to assemble into a membrane-anchored transfer complex that functions similarly to bacterial conjugation (*6*). This transfer machinery performs optimally at low temperatures (*8*), as does conjugative transfer of IncH1 plasmids, which fail to form H pili when grown at the nonpermissive temperature (*9*).

To establish whether *A. tumefaciens* forms pili at low temperatures, we grew cells at 19°C on solid medium under conditions that activate *vir* gene transcription (*10*), washed the plates with 10 mM $MgCl_2$, and prepared the plates for electron microscopy (EM) (*11*). In eight separate preparations, wild-type strain A348 produced pili visible by EM (Fig. 1). The pili were 3.8 nm in diameter and were distinguishable from the flagella, which were 12 nm in diameter (Fig. 1). Pili were never seen in cells of strain A136, which lacks a Ti plasmid (Table 1), and this demonstrated that the observed structures are not cellulose fibrils, which are plant-induced structures present on A136 cells (*12*). The pili varied in length, but pili longer than the length of the bacterium were observed (Fig. 1A). In many cases, pili were present in clumps extending from a single electron-dense region on the bacterial surface (Fig. 1B). In other cases, long individual pili were observed (Fig. 1, A and C).

Pili depended on induction of *vir* genes because pili were never seen on cells grown on agar plates lacking acetosyringone (Ta-

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ble 1). When bacteria were grown at 28°C, piliated cells were rare (Table 1), which, if the assumption that pili are required for T-DNA transfer is made, is consistent with observations that tumorigenesis and T-DNA transfer are less efficient at higher temperatures (*8*, *13*). Thus, we presume that these pili have not been previously observed (*14*) because, as in the IncH1 system (*9*), the pili are rare on cells grown at the temperatures customarily used.

To determine whether the pili are essential for transfer of T-DNA, we examined mutants defective in T-DNA transfer. Strains carrying insertions within the *virB* operon or the *virD4* gene cannot transfer T-DNA strands, VirE2 protein, or the plasmid RSF1010 (Table 2) (*15*). Because *virA* and *virG* are required for transcription of *virB* and *virD4*, mutations in these genes also result in defective transfer (Table 2) (*15*). We did not find pili on cells harboring polar mutations in *virA*, *virG*, *virB*, or *virD4* (Table 2) or with nonpolar mutations in each of the 11 *virB* genes (*16*). Both the *virB4* and the *virB11* mutants were complemented in trans by the appropriate cloned gene, which demonstrated that the lack of pili was specifically due to the *virB* mutation. Thus, pili assembly required *virA*, *virG*, *virB1* through *virB11*, and *virD4*.

To assess whether other Ti plasmid genes are essential for pili formation and T-DNA transfer, we cloned *virB1* through *virB11* and *virD4* into the IncP vector pGP159, which carries the *vir* promoter regulatory genes *virA* and *virG* and a *virB*::*lacZ* reporter fusion (*17*). The resulting plasmid pKJF108 complemented A. *tumefaciens* strains harboring mutations in *virA*, *virG*, *virB*, or *virD4* for virulence on *Kalanchöe daigremontiana*. The VirA and VirG proteins produced by A136(pKJF108), which lacks the Ti plasmid, properly regulated transcription of the *virB* promoters found upstream of *lacZ*, *virB*, and *virD4* on pKJF108, as determined by β-galactosidase assays and protein immunoblot analysis. When A136(pKJF108) cells were examined by EM, pili similar to those seen for the wild-type strain A348 were observed on the cells (Table 2). These cells also functioned in *vir*-dependent transfer of DNA and protein, as measured by the ability to mobilize RSF1010 to a recipient *Agrobacterium* (Table 2) and to transfer the protein VirE2 directly to plant cells, thereby restoring virulence to a *virE2* mutant (Table 3). The mutant strain A136 carrying the vector pGP159 did not produce pili, mobilize RSF1010, or transfer VirE2 (Tables 2 and 3). Thus, *virB1* through *virB11* and *virD4*, together with *virA* and *virG*, are the only Ti plasmid genes required for both pilus assembly and transfer of T-DNA and VirE2. The only other essential transfer gene is *virE1*, which is required only for transport of VirE2 (*18*).

The pili are morphologically distinct from the thick, rigid sex pili produced from IncN, IncW, and IncP conjugative plasmids (*19*) even though many of the pilus assembly proteins exhibit amino acid sequence similarity to the VirB and VirD4 proteins (*6*). *Agrobacterium* pili appear most similar to the pili encoded by IncX plasmid R485 (*20*). However, plasmids of the IncX group can mobilize RSF1010 to other bacteria, which is consistent with the view that R485 conjugation may resemble T-DNA transfer (*21*).

The role of each VirB protein in regulation and assembly of pili remains unde-

Table 1. Environmental factors affecting pilus assembly by *Agrobacterium*. Bacteria were grown at the indicated temperatures with or without the addition of 200 μM acetosyringone (AS). Six grids for each condition were prepared and scanned by EM (*10*, *11*). The presence of pili was scored qualitatively because *Agrobacterium* naturally forms aggregates that could not be disrupted without shearing the pili, thereby preventing accurate quantitative assessments. Three experiments gave similar results. For pili on cell surface, +++ indicates wild-type piliation (10 to 20% of all cells), + indicates few piliated cells (<1% of cells), and − indicates no piliated cells seen on any of the six grids.

Strain (growth condition)	Relevant characteristics	Pili on cell surface
A348 (19°C, +AS)	Wild type	+++
A348 (28°C, +AS)	Wild type	+
A348 (19°C, −AS)	Wild type	−
A136 (19°C, +AS)	No Ti plasmid	−

Table 2. Ti plasmid genes necessary and sufficient for production of pili and mobilization of RSF1010. Six grids prepared for each strain were scanned by EM (*10*, *11*), and the presence of pili was scored qualitatively as described for Table 1. To determine the RSF1010 conjugation frequency, we introduced pML122ΔKm (*5*), a gentamicin-resistant derivative of RSF1010, into donor strains, which were then mated with *A. tumefaciens* UIA143 (no Ti plasmid, Ery^R) as previously described (*8*). Frequencies, expressed as transconjugants per input donor, are the means of triplicates from a single assay. Three experiments gave similar results. For pili on cell surface, − indicates no piliated cells and +++ indicates wild-type piliation.

Strain	Relevant characteristics	Ref.	Pili on cell surface	RSF1010 conjugation frequency (10^{-4})
A136	C58 chromosome, no Ti	(*28*)	−	<0.05
A348	A136 with pTiA6NC	(*29*)	+++	18 ± 5
At11068	A348 Δ*virA*	(*8*)	−	<0.01
At12508	A348 *virG*::Tn*3*HoHo1	(*8*)	−	<0.01
At11067	A348 *virB1*::Tn*3*HoHo1	(*8*)	−	<0.01
At12506	A348 *virD4*::Tn*3*HoHo1	(*8*)	−	<0.01
A136(pGP159)	*virA*$^+$, *virG*$^+$	(*26*)	−	<0.01
A136(pKJF108)	*virA*$^+$, *virG*$^+$, *virB1*$^+$ through *virB11*$^+$, *virD4*$^+$	(*17*)	+++	100 ± 20

Table 3. Restoration of virulence to a *virE2* mutant by coinfection. The plasmid pWD200 (*virE*) (*30*) and pUFR047 (vector) (*27*) were electroporated into Ti plasmid–deficient strain A136 carrying either pKJF108 (*virA*, *virG*, *virB*, *virD4*) (*17*) or pGP159 (*virA*, *virG*) (*26*). All overnight cultures were adjusted to OD_{600} = 0.2, and potential VirE2 protein donors were mixed at a ratio of 1:1 with either At12516 [T-DNA$^+$, Δ*virE2* (*8*)] or with culture broth (no coinfection). Leaves of *K. daigremontiana* plants were scratched with an 18-gauge needle, and 5 μl of mixed inocula was applied to the wound site. Tumor formation was scored 21 days after infection. Dashes indicate that the experiment was not done.

VirE2 protein donor	Tumor induction (leaves with tumors/leaves inoculated)	
	No coinfection	Coinfection with *virE2* mutant At12516
A348	12/12	—
At12516 (*virE2*$^-$)	0/12	—
At10002 [T-DNA$^-$, *virE2*$^+$ (*31*)]	0/8	11/12
A136(pKJF108) + *virE*	0/8	11/12
A136(pGP159) + *virE*	0/8	0/8
A136(pKJF108) + vector	0/8	0/8
A136(pGP159) + vector	0/8	0/8

fined. We postulate that VirB2 and VirB5 are the structural components of the pilus because of sequence homology with the pilin of the *Escherichia coli* F plasmid and a putative pilin subunit encoded by the IncN plasmid pKM101 (*22*). The requirement for VirD4 in pilus assembly is surprising, because its homolog in the IncP system, TraG, is not essential for the synthesis of P pili (*23*). However, construction of P pili requires TraF for which a homolog does not exist on the Ti plasmid (*24*). The requirement for VirD4 in pilus assembly may suggest that regions of VirD4 replace TraF in pilus assembly.

Once assembled, these Ti plasmid-encoded pili probably function in a manner similar to that of the classic sex pilus encoded by the F plasmid (*14*). The *Agrobacterium* pili probably attach to the recipient plant cell to establish a stable mating pair. The pili may then retract to create a channel for movement of the T-DNA strand and VirE2 protein directly into the plant cell. Therefore, further studies of *Agrobacterium*-mediated DNA transfer should lead to a better understanding of not only interkingdom but also interbacterial DNA transfer.

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Role of Postreplicative DNA Mismatch Repair in the Cytotoxic Action of Thioguanine

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It is proposed here that the delayed cytotoxicity of thioguanine involves the postreplicative DNA mismatch repair system. After incorporation into DNA, the thioguanine is chemically methylated by *S*-adenosylmethionine to form S^6-methylthioguanine. During DNA replication, the S^6-methylthioguanine directs incorporation of either thymine or cytosine into the growing DNA strand, and the resultant S^6-methylthioguanine–thymine pairs are recognized by the postreplicative mismatch repair system. Azathioprine, an immunosuppressant used in organ transplantation, is partly converted to thioguanine. Because the carcinogenicity of *N*-nitrosamines depends on formation of O^6-alkylguanine in DNA, the formation of the analog S^6-methylthioguanine during azathioprine treatment may partly explain the high incidence of cancer after transplantation.

Mercaptopurine and thioguanine are cytotoxic drugs used in the treatment of acute leukemia, and azathioprine, a pro-drug that is converted in vivo to mercaptopurine, is used for immune suppression in transplant surgery (*1*). The cytotoxicity of thioguanine and mercaptopurine involves changes in purine metabolism and, in the case of both drugs, the formation of 2′-deoxy-6-thioguanosine triphosphate and the incorporation of thioguanine into DNA (*1*). The delayed cytotoxicity and chromosome damage that are characteristic of these drugs are associated with this incorporation (*2*).

N-methyl-*N*-nitrosourea produces similar delayed cytotoxicity (*3*) and chromosome damage (*4*). An indication that the superficial resemblance between these poisons may have a deeper mechanistic basis has come from the observation that certain eukaryotic cells that are resistant to *N*-methyl-*N*-nitrosourea are also resistant to thioguanine (*5*, *6*). Both drugs produce few-

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