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Hrp pilus: An *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000

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ABSTRACT **Hypersensitive response and pathogenicity (*hrp*) genes control the ability of major groups of plant pathogenic bacteria to elicit the hypersensitive response (HR) in resistant plants and to cause disease in susceptible plants. A number of Hrp proteins share significant similarities with components of the type III secretion apparatus and flagellar assembly apparatus in animal pathogenic bacteria. Here we report that *Pseudomonas syringae* pv. *tomato* strain DC3000 (race 0) produces a filamentous surface appendage (Hrp pilus) of 6–8 nm in diameter in a solid minimal medium that induces *hrp* genes. Formation of the Hrp pilus is dependent on at least two *hrp* genes, *hrpS* and *hrpH* (recently renamed *hrcC*), which are involved in gene regulation and protein secretion, respectively. Our finding of the Hrp pilus, together with recent reports of *Salmonella typhimurium* surface appendages that are involved in bacterial invasion into the animal cell and of the *Agrobacterium tumefaciens virB*-dependent pilus that is involved in the transfer of T-DNA into plant cells, suggests that surface appendage formation is a common feature of animal and plant pathogenic bacteria in the infection of eukaryotic cells. Furthermore, we have identified HrpA as a major structural protein of the Hrp pilus. Finally, we show that a nonpolar *hrpA* mutant of *P. syringae* pv. *tomato* DC3000 is unable to form the Hrp pilus or to cause either an HR or disease in plants.**

Major groups of Gram-negative plant pathogenic bacteria belonging to genera *Erwinia*, *Pseudomonas*, *Ralstonia,* and *Xanthomonas* contain hypersensitive reaction and pathogenicity (*hrp*) genes. These genes control the ability of these bacteria to initiate interactions with plants, including elicitation of the hypersensitive reaction (HR), characterized by rapid localized death of plant cells at the pathogen infection site in resistant plants and causation of disease in susceptible plants (1, 2).

hrp genes of *Pseudomonas syringae* are expressed *in planta* as a result of a regulatory cascade involving the gene products of *hrpS* and *hrpR*, positive transcriptional regulators, and of *hrpL*, an alternative sigma factor (3, 4). HrpL recognizes a consensus sequence motif ("harp box") that has been identified in the upstream regions of many *hrp* and *avr* genes (4). The expression of *hrp* genes of many *P. syringae* pathovars can also be induced *in vitro* when bacteria are grown in defined minimal medium with low pH and containing certain sugars or sugar alcohols as carbon sources (5–7).

The 25-kb *hrp/hrmA* gene cluster of *Pseudomonas syringae* pv. *syringae* strain 61 is sufficient to enable nonpathogenic strains of *Pseudomonas fluorescens* and *Escherichia coli* to elicit the HR in nonhost plants (8). Sixteen of the 25 genes in this completely sequenced *hrp/hrmA* gene cluster are either predicted or shown to be required for secretion of $\text{harpin}_{\text{Pss}}$, a proteinaceous elicitor of the HR encoded by *hrpZ* (9, 10). Nine of these *hrp* genes, recently renamed *hrc* genes (11), are broadly conserved among *P. syringae* pathovars, *Erwinia*, *Xanthomonas*, and *Ralstonia* (9, 12–15). *hrc* genes, including *hrcC* (formerly *hrpH*; ref. 16), share sequence similarities with *ysc/lcr* genes of *Yersinia* spp. (17–19), *mxi-spa* genes of *Shigella* (20), and *inv* genes of *Salmonella* (21, 22), all of which function in secretion of proteins required for pathogenesis. The protein products of *hrc* genes and related counterparts in animal pathogenic bacteria are predicted to be components of a novel protein secretion pathway, the so-called type III secretion pathway, in Gram-negative bacteria. Some components of this secretion pathway are also used for flagellar assembly (23). In plant pathogenic bacteria the type III secretion pathway encoded by *hrp* genes is called the Hrp pathway (10, 11).

Bacterial avirulence (*avr*) genes, which are required for the elicitation of the HR and resistance in plants containing the corresponding disease resistance genes, are dependent on *hrp* genes for phenotypic expression (5, 24–26). There are no reports of Avr proteins having HR-eliciting activity when infiltrated into the intercellular space of plant leaves (27). Fenselau *et al.* (28) proposed that *hrp* genes are required for secretion of Avr proteins. Yang and Gabriel (29) showed that *Xanthomonas campestris* pv. *malvacearum* Avr/Pth proteins contain nuclear targeting signals that can direct transport of β-glucuronidase reporter protein into the plant nucleus. A recent study showed that expression of the *P. syringae* pv. *glycinea avrB* directly in leaf cells of *Arabidopsis thaliana* ecotype Columbia plants, which possess the matching disease resistance gene *RPM1*, triggers the HR (26). These results suggest that the action site of AvrB and likely *X. campestris* pv. *malvacearum* Avr/Pth proteins is inside the plant cell and that the Hrp secretion system is involved in the delivery of these proteins into the plant cell.

The mechanism by which the Hrp secretion system putatively delivers proteins through the plant cell wall is not known. In this paper, we show that *P. syringae* pv. *tomato* strain DC3000 (race 0), a virulent bacterium on tomato and *Arabidopsis thaliana*, produces a thin, pilus-like structure on solid *hrp*-inducing media. One of the major structural proteins of the Hrp pilus was identified as HrpA. The *hrpA* gene, like all other *hrp* genes, was shown to be essential for *P. syringae* pv. *tomato* DC3000 to initiate pathogenesis and to elicit the HR in plants, and for the phenotypic expression of AvrB, which is presumed to be targeted to the plant cell.

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Abbreviations: HR, hypersensitive response; EXP, extracellular protein.

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MATERIALS AND METHODS

Culture Conditions. For detection of bacterial extracellular proteins (EXPs) in liquid cultures, bacteria were first grown at 30°C to an OD_{600} of 0.8–1.0 in 50 ml King's medium B broth (30), supplemented with 100 μg/ml rifampicin. Bacteria were then pelleted and resuspended in 50 ml *hrp*-inducing broth (10) or King's medium B broth and incubated with shaking (250 rpm) at room temperature (21–23°C) for 24 hr. For preparation of bacterial surface-associated proteins, bacteria were grown on solid *hrp*-inducing medium (10) at room temperature (21–23°C) for 2 days. M9 minimal agar medium supplemented with 5 mM mannitol (31) was equally effective in inducing *P. syringae* pv. *tomato* DC3000 *hrp* genes and was used in experiments for purification of bacterial surface structures.

Analysis of Bacterial EXPs and Surface Structures. For preparation of EXPs, bacteria were removed from liquid cultures by centrifugation at 10,000 × *g* for 10 min. The supernatant was concentrated 50-fold using centricon concentrators with molecular weight cutoff of 3,000 daltons (Amicon) and 10 μl was analyzed by SDS/15% PAGE followed by staining with 0.025% Coomassie brilliant blue R-250. For preparation of bacterial surface-associated proteins, bacteria from a 76-mm agar plate containing solid *hrp*-inducing medium were resuspended in 1 ml of 10 mM sodium phosphate (pH 5.5), pelleted by centrifugation at 13,000 × *g* for 10 min to partially remove proteins not associated with cell surface structures, and then resuspended in 0.2 ml of 10 mM sodium phosphate (pH 5.5). The bacterial suspension was pushed through a 25 G needle 4 to 5 times to shear surface structures and proteins (e.g., flagella and pili) from the bacteria, and was then centrifuged at 13,000 × *g* for 10 min at 4°C. Twenty microliters of the supernatant was used for SDS/PAGE analysis.

For analysis of N-terminal amino acid sequences, proteins were separated on a large preparative SDS/15% PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was stained with 0.025% Coomassie brilliant blue R-250 and individual protein bands were excised. The amino-terminal sequence of each protein was analyzed with an Applied Biosystems protein sequencer.

Construction of the *hrpA* Mutant. For construction of nonpolar *hrpA* mutants, a 5.1-kb *Eco*RI fragment of pCPP2201 (32) containing *hrpRS*, *hrpAZBCDE*, and the 5′ half of *hrpF* of *P. syringae* pv. *tomato* strain DC3000 was subcloned into pBluescript SK(−) (Stratagene) and used as the template for the PCR. A 1.5-kb DNA fragment 5′ of the *hrpA* start codon was amplified by PCR using the following oligonucleotide set: 5′-GGAAACAGCTATGACCATG-3′ (pBluescript SK reverse primer) and 5′-GGGGTACCCCTTAAGATTTACCAGCGTGATTGC-3′ (containing a *Kpn*I site at the 5′ end). A 3.8-kb fragment 3′ of the *hrpA* stop codon was amplified using the following primer set: 5′-CGGGATCCCGTTGCCCCCTCATCAGAGG-3′ (containing a *Bam*HI site at the 5′ end) and 5′-GTAAAACGACGGCCAGT-3′ (M13–20 primer). The 0.8-kb ORF of the *aph* gene (conferring kanamycin resistance) without its own promoter or terminator was amplified from mini-Tn*5 xylE* (33) using the following oligonucleotides: 5′-GGGGTACCCTGTTATGAGCCATATTCAACG-3′ (containing a *Kpn*I site at the 5′ end) and 5′-CGGGATCCCGTTAGAAAAACTCATCGAGCATC-3′ (containing a *Bam*HI site at the 5′ end). Through multiple cloning steps, the three PCR products were ligated (*hrpRS–aph–hrpZBCDEF*) and cloned into pBluescript II SK(−). The *hrpA* ORF was precisely replaced by the *aph* ORF without changing the promoter or Shine–Dalgarno sequences upstream of the *hrpA* ORF or the sequence and spacing between *hrpA* and *hrpZ*. For marker exchange mutagenesis, the *hrpRS–aph–hrpZBCDEF* fragment was cloned into pRK415 (34), which was then electroporated into DC3000. Marker exchange events were selected following a standard procedure (35), except that *hrp*-inducing medium was used for activating the expression of the *aph* gene. The junction sequences of the *aph* gene in the marker-exchanged *hrpA* mutant were cloned, sequenced, and found to be correct. For complementation, the *hrpA* ORF plus its native promoter were amplified using the following primers: 5′-TTGCAAAGACGCTGGAACCGTATCGC-3′ and 5′-GGGGTACCTCCTCAAGGTAGCGGCCCCCTC-3′. The PCR product was cloned into the *Sma*I site of pUCP18 (36), resulting in pHRPA. Construction of *hrpS* and *hrcC* mutants were described elsewhere (37).

Purification of Bacterial Surface Structures (Flagella and Hrp Pili). All purification steps were performed at 4°C. Bacterial surface structures and proteins were sheared off as described above and subjected to ultracentrifugation in a Beckman Ti45 rotor at 100,000 × *g* for 3 hr. The pellet was resuspended in 10 mM Tris·HCl (pH 7.5) to which sodium deoxycholate was added to a final concentration of 0.5% (wt/vol). After overnight incubation at 4°C, proteins were subjected to ultracentrifugation in a 10–60% (wt/wt) sucrose gradient in a Beckman SW27 rotor at 80,000 × *g* for 20 hr. Ten fractions were taken from the gradient and were dialyzed against 10 mM Tris·HCl (pH 7.5) and then against water. The pellet of the gradient was resuspended in 10 mM Tris·HCl (pH 7.5), repelleted again to remove the sucrose (in a Beckman Ti50 rotor at 125,000 × *g* for 3 hr), and finally resuspended in 10 mM Tris·HCl (pH 7.5). Dialyzed gradient fractions and the pellet were then used for transmission electron microscopy (TEM) and for SDS/PAGE analysis. For TEM observation, a drop of bacteria or flagellum plus pilus suspension was applied to a copper grid coated with pioloform and carbon, followed by staining with 1% potassium phosphotungstic acid adjusted to pH 6.5 with potassium hydroxide. The grids were then examined with a transmission electron microscope.

Pathogenesis Assays. Bacteria were grown in King's B broth to an OD_{600} of 0.6–0.8. Bacterial suspensions in distilled water were infiltrated into leaves of tomato (*Lycopersicon esculentum* cultivar Rio Grande-PtoR), *Arabidopsis thaliana* ecotype Columbia (Col), and tobacco (*Nicotiana tabacum* cultivar Samsun NN) using needleless syringes. The concentrations of bacteria used were 2×10^8 colony-forming units (cfu)/ml and 2×10^6 cfu/ml for HR and pathogenesis assays, respectively. Plant responses were recorded at 24 hr (for HR assay) or 4 days (for pathogenesis assay) postinfiltration. HR is indicated by rapid, localized tissue collapse in the infiltrated area within 24 hr. Disease symptoms caused by *P. syringae* pv. *tomato* DC3000 and complemented *hrpA* mutants in tomato and *Arabidopsis* leaves were characterized by slowly developing necrosis and spreading tissue chlorosis, usually observed 3 days after infiltration. Strain DC3000 and its *hrpA* and *hrpS* mutants contain *avrPto* in the chromosome (38). Plasmid pAVRB contains *P. syringae* pv. *glycinea avrB* in pDSK609 (kindly provided by N. T. Keen, University of California). Tobacco and tomato plants were grown in greenhouses. *Arabidopsis* plants were grown in growth chambers at 20°C with 70% relative humidity and a 12-hr photoperiod. HR assays with tobacco and tomato plants were performed at room temperature in the laboratory. Pathogenesis assays with *Arabidopsis* plants were performed at 20°C in growth chambers with 70% relative humidity and a 12-hr photoperiod.

RESULTS

Production of Multiple EXPs by DC3000 in a *hrp*-Inducing Medium. In an "*hrp*-inducing minimal broth" (10) that induces *hrp* genes of *P. syringae*, DC3000 produces at least seven major EXPs, two of which (EXP-50 and EXP-21) are also produced in the nutrient-rich, *hrp*-repressing King's medium B broth (30) (Fig. 1). EXP-36, which was not present in the experiment shown in Fig. 1 (lane 2), appears in many King's medium B

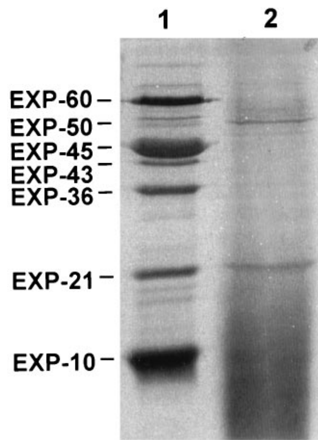

FIG. 1. EXPs produced by *P. syringae* pv. *tomato* DC3000. Lane 1, EXPs from an *hrp*-induced culture; lane 2, EXPs from a King's medium B culture. The gel was stained with 0.025% Coomassie blue R-250. Proteins are named according to their molecular masses (in kDa).

cultures, but the amount varies greatly. EXP-60, EXP-45, EXP-43, and EXP-10 were never observed in the King's B culture supernatant of DC3000. Thus, expression of *hrp* genes is correlated with the production of at least these four EXPs.

Formation of an *hrp* Gene-Dependent Pilus by DC3000 on Solid *hrp*-Inducing Medium. Examination of bacteria grown on solid *hrp*-inducing medium by transmission electron microscope revealed that DC3000 produces two to three polar flagella (15–18 nm in diameter) on both King's medium B (Fig. 2*A*) and *hrp*-inducing agar plates (Fig. 2*B*). In addition, DC3000 also produces many pilus-like appendages (6–8 nm in diameter) on solid *hrp*-inducing medium (Fig. 2*B*), but not on King's medium B plates (Fig. 2*A*). These pilus-like appendages were found both on the bacterial surface with no consistent distribution pattern, and as detached pilus clusters. The pilus-like appendages were easily fragmented during sample preparation, as evidenced by the presence of many short pieces of pili (Fig. 2*B*); therefore, the length of these pilus-like appendages could not be determined. The effect of temperature (from 16–28°C) on the formation of the pilus-like appendages was examined. The number of pili observed decreases dramatically when incubation temperature exceeds 25°C.

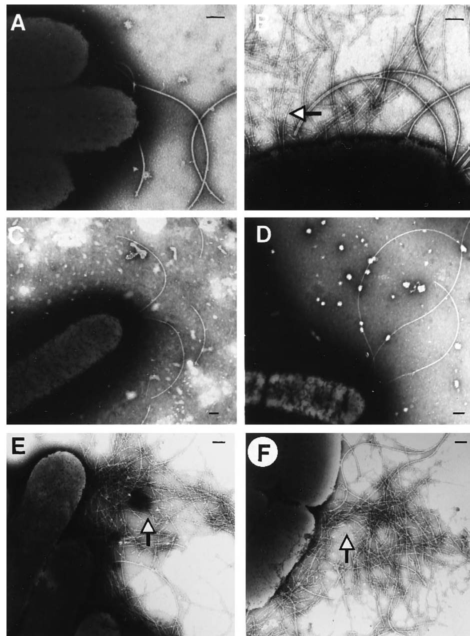

FIG. 2. Detection of the Hrp pilus on the surface of *P. syringae* pv. *tomato* DC3000. (*A*) DC3000 grown on King's medium B agar plates, and (*B*) DC3000, (*C*) *hrcC,* (*D*) *hrpS,* (*E*) *pilA,* and (*F*) *pilD* mutants grown on solid *hrp*-inducing medium were examined with a transmission electron microscope after staining with 1% potassium phosphotungstic acid (pH 6.5). One to three polar flagella of 15–18 nm in diameter are present on most rod-shaped bacteria (surrounded by dark shadows) in samples (*A–F*); in *B*, *E*, and *F*, many Hrp pili of 6–8 nm in diameter are also present (indicated by arrows). (Scale bars = 200 nm.) *pilA* and *pilD* mutants were kindly provided by D. Nunn (University of Illinois).

To determine whether the formation of pilus-like appendages is under the control of *hrp* genes, *hrcC* and *hrpS* mutants of DC3000 (37), grown on solid *hrp*-inducing medium, were examined by TEM. *hrcC* and *hrpS* genes are involved in the secretion of harpin$_{Pss}$ (16) and regulation of bacterial *hrp* and *avr* genes (3), respectively. Polar flagella, but not the pilus-like appendages, were seen on the surfaces of the *hrcC* and *hrpS* mutant bacteria (Fig. 2 *C* and *D*). These results demonstrate that the formation of the pilus-like appendages, but not flagella, is under the control of *hrp* genes. Based on its *hrp*-dependent property, we propose the name "Hrp pilus" for this pilus-like structure.

In King's medium B cultures, many strains of pseudomonads also produce type IV pili, whose assembly is dependent on the type II secretion apparatus (39, 40). However, DC3000 mutant strains that are defective in the type IV pilin structural gene *pilA* or in the *pilD* gene, which encodes a leader peptidase involved in the assembly of type IV pili (D. Nunn, personal communication), still produce Hrp pili (Fig. 2 *E* and *F*). This result shows that the Hrp pilus is distinct from type IV pili.

Identification of Proteins Associated with the DC3000 Flagellum and the Hrp Pilus. The presence of the Hrp pilus is specifically correlated with the appearance of a 10-kDa protein on the bacterial surface. Only DC3000 grown on solid *hrp*-inducing medium produces the 10-kDa protein (Fig. 3, lane 1). Neither DC3000 grown on King's B agar medium, nor the *hrcC* and *hrpS* mutant bacteria grown on solid *hrp*-inducing medium, produce the protein (Fig. 3, lanes 2–4).

To determine the identity of the Hrp pilus, a preparation of cell surface structures (flagella and Hrp pili) from DC3000 grown on solid M9 minimal medium was subjected to ultracentrifugation in a 10–60% sucrose gradient. Hrp pili together with flagella were found in the pellet (Fig. 4*A*). SDS/PAGE analysis of the pellet fraction revealed three major proteins of 50, 36, and 10 kDa in size, respectively, associated with these filamentous structures (Fig. 4*C*, lane 1). A minor, contaminating, 100-kDa protein is also present in some, but not all, preparations (Fig. 4*C*, lane 1). Another fraction from the middle of the sucrose gradient contains only flagella (Fig. 4*B*).

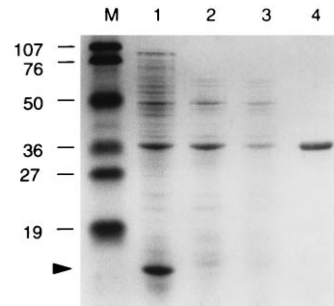

FIG. 3. SDS/PAGE analysis of bacterial surface proteins. An SDS/15% PAGE gel loaded with protein samples prepared from the surface of DC3000 (lane 1), *hrcC* mutant (lane 2), and *hrpS* mutant (lane 3) grown on solid *hrp*-inducing medium, or DC3000 grown on King's medium B agar plates (lane 4). The gel was stained with 0.025% Coomassie brilliant blue R-250. Lane M, molecular mass markers (Bio-Rad) in kDa. Arrowhead indicates the 10-kDa protein.

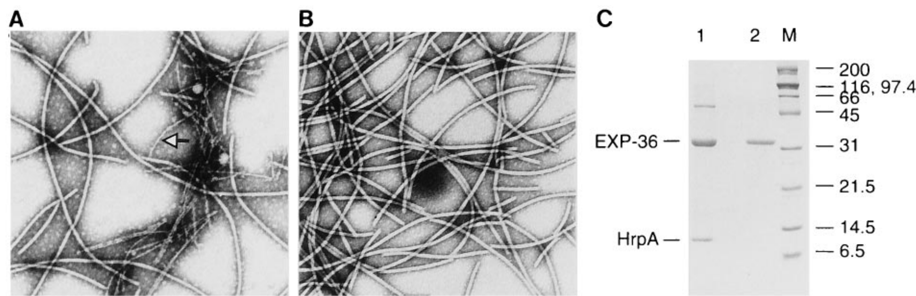

FIG. 4. Purification of *P. syringae* pv. *tomato* DC3000 extracellular appendages (flagella and Hrp pili). (*A*) Electron micrograph of the pellet fraction containing flagella and Hrp pili (indicated by an arrow). (*B*) Electron micrograph of a fraction from the middle of the gradient containing only flagella. (*C*) An SDS/15% PAGE gel of proteins from the pellet fraction (lane 1) or a fraction from the middle of the gradient containing only flagella (lane 2). The gel was stained with 0.025% Coomassie brilliant blue R-250. Lane M, molecular mass markers (Bio-Rad) in kDa.

When this fraction was analyzed by SDS/PAGE, only the 36-kDa protein, but not the 10- or 50-kDa protein, was found. This result suggests that the 36-kDa protein is flagellin that makes up flagella, and that the 10- and 50-kDa proteins are associated with the Hrp pilus.

The N-terminal sequences of the 36-, 10-, and 50-kDa proteins were determined. The N-terminal sequence (ALTVNTNVASLNVQKNLGRASDALST) of the 36-kDa protein, from both the pellet fraction containing flagella and Hrp pili (Fig. 4*C*, lane 1) and the flagella fraction (Fig. 4*C*, lane 2), is almost identical to those of flagellins of *Pseudomonas aeruginosa* and *Pseudomonas putida* (41, 42), confirming that the 36-kDa protein is the DC3000 flagellin. The first 35 amino acids (VAFAGLTSKLTNLGNSAVGGVGGALQGVNTVASNA) of the 10-kDa protein match exactly that of HrpA, encoded by *hrpA* (32). The sequence of the first 16 amino acids (ASPITSTTGLGSGLAI) of the N terminus of the 50-kDa protein does not show any significant similarity to any proteins in the current gene/protein databases.

To determine whether the three extracellular proteins (EXP-36, EXP-10, and EXP-50) detected in the experiment shown in Fig. 1 correspond to the 36-, 10-, and 50-kDa proteins associated with flagella and Hrp pili, we determined the N-terminal sequences of these EXPs. The first 15 amino acids of EXP-36 (ALTVNTNVASLNVQK) and EXP-10 (VAFAGLTSKLTNLGN) and the first 10 amino acids (ASPITSTTGL) of EXP-50 match exactly those of the 36-kDa flagellin protein, 10-kDa HrpA protein, and 50-kDa protein, respectively. This confirms that flagellin, HrpA, and the 50-kDa protein are secreted into the medium in shaking culture but remain attached to the bacterial surface on static plates.

Mutational Analysis of the *hrpA* Gene. To confirm that HrpA is a major component of the Hrp pilus, a nonpolar mutation in the *hrpA* gene was constructed by precisely replacing the *hrpA* ORF with the *aph* ORF conferring kanamycin resistance (33). Like *hrcC* and *hrpS* mutants (Figs. 2 *C* and *D* and 3), the *hrpA* mutant does not produce any Hrp pili in solid *hrp*-inducing medium (Fig. 5*A*) and does not produce the HrpA protein (Fig. 5*C*). The *hrpA* mutant still produced flagella (Fig. 5*A*) and the 50- and 36-kDa proteins (Fig. 5*C*). pHRPA (containing only the *hrpA* gene with its native promoter; see *MATERIALS AND METHODS*) enables the *hrpA* mutant to produce HrpA protein (Fig. 5*C*) and the Hrp pilus on the bacterial surface (Fig. 5*B*).

Pathogenesis Assay. The *hrpA* mutant is unable to elicit the HR in the leaves of the nonhost tobacco or to cause disease symptoms (tissue chlorosis and necrosis) in the leaves of the host *A. thaliana* (Table 1). The responses of tobacco and *A. thaliana* leaves to the *hrpA* mutant are very similar to the responses to the *hrpS* mutant, which is a typical *hrp* mutant unable to initiate any *hrp*-mediated plant responses. Plasmid pHRPA restored the ability of the *hrpA* mutant to elicit HR necrosis in tobacco leaves and to cause disease symptoms in *A. thaliana* leaves (Table 1). HrpA is also required for two well-characterized *P. syringae avr* genes, *avrB* and *avrPto*, to trigger genotype-specific HR. DC3000 contains *avrPto*, which mediates the elicitation of an HR on tomato cv. Rio Grande-PtoR containing the Pto resistance gene (38, 43). *avrB* was originally cloned from *P. syringae* pv. *glycinea* and later was found to mediate the elicitation of an *RPM1*-dependent HR and resistance in *A. thaliana* Col (44). Unlike DC3000, *hrpA* and *hrpS* mutants fail to elicit an HR in Rio-Grande PtoR (Table 1). Similarly, strains carrying *avrB* on a plasmid fail to elicit an HR in *A. thaliana* Columbia (Col) when expressed in *hrpA* and *hrpS* mutants (Table 1).

DISCUSSION

In this study, we have provided evidence that *P. syringae* pv. *tomato* strain DC3000 *hrp* genes are involved in the production

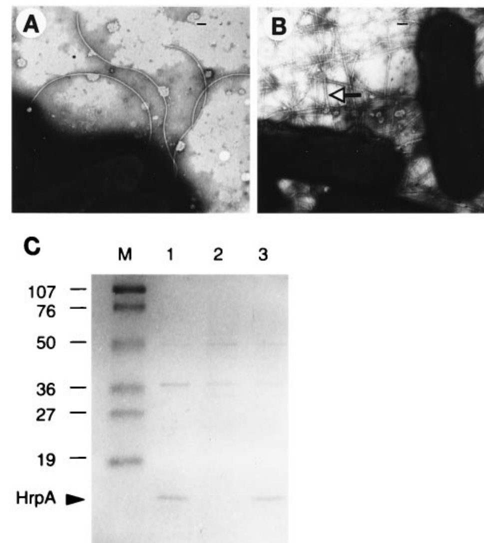

FIG. 5. Surface appendages and associated proteins on *P. syringae* pv. *tomato* DC3000 *hrpA* mutant and *hrpA* mutant containing pHRPA. (*A*) *hrpA* mutant and (*B*) *hrpA* mutant containing pHRPA grown on solid *hrp*-inducing medium were examined with a transmission electron microscope after staining with 1% potassium phosphotungstic acid (pH 6.5). Polar flagella of 15–18 nm in diameter are present on most cells of the *hrpA* mutant and the *hrpA* mutant containing pHRPA. Flagella were not seen in the field shown in *B*. In *B*, many Hrp pili of 6–8 nm in diameter are present (indicated by arrow). (Scale bars = 200 nm.) (*C*) An SDS/15% PAGE gel loaded with protein samples prepared from surface of DC3000 (lane 1), *hrpA* mutant (lane 2), and *hrpA* mutant containing pHRPA (lane 3) grown on solid *hrp*-inducing medium. Lane M, molecular weight markers (Bio-Rad) in kDa. The gel was stained with 0.025% Coomassie brilliant blue R-250.

Table 1. Plant reactions to DC3000 and its *hrpA* and *hrpS* mutants

Bacteria	Arabidopsis (Col)	Tobacco (Samsun NN)	Tomato (Rio Grande-PtoR)
DC3000, *avrPto*$^+$	D	HR	HR
hrpS, *avrPto*$^+$	Null	Null	Null
hrpA, *avrPto*$^+$	Null	Null	Null
hrpA/pHRPA, *avrPto*$^+$	D	HR	HR
DC3000/pAVRB	HR	HR	HR
hrpS/pAVRB	Null	Null	NA
hrpA/pAVRB	Null	Null	NA

HR, rapid, localized tissue collapse in the infiltrated area; D, tissue chlorosis and necrosis typical of disease symptoms caused by DC3000; Null, no visible plant reactions; NA, not assayed. DC3000 and its derivatives, *hrpA* and *hrpS* mutants, contain *avrPto* in the chromosome (38).

of a novel *hrp*-dependent pilus on the cell surface when bacteria are grown on solid *hrp*-inducing medium. Furthermore, we have demonstrated that HrpA is a structural protein of the Hrp pilus. Finally, we have shown that a nonpolar *hrpA* mutant strain does not produce HrpA or form the Hrp-pilus, and loses the ability to initiate pathogenesis or to elicit the HR in plants, a typical phenotype of all *hrp* mutant strains.

The nucleic acid sequence of the *P. syringae* pv. *tomato* DC3000 *hrpA* gene, the first gene of the *hrpZ* operon, was previously determined by Preston *et al.* (32). *hrpA* codes for a hydrophilic protein with a predicted molecular weight of 11 kDa (32). The primary amino acid sequence of HrpA protein does not show any significant homology to those of characterized pilin proteins. However, computer analysis using the PROPSEARCH program (45), which identifies structural similarities between proteins without looking for primary amino acid sequence homology, indicates that HrpA is structurally similar to several pilin proteins, especially to the AF/R1 pilus chain A precursor of *E. coli* (with 41% reliability; ref. 46). This analysis is in agreement with our results showing that HrpA is part of the Hrp pilus structure.

We do not know the role, if any, of the 50-kDa protein in the assembly of the Hrp pilus. The 50-kDa protein was produced by *P. syringae* pv. *tomato* DC3000 growing in King's B medium, which represses *hrp* gene expression. Furthermore, *hrpA, hrcC*, and *hrpS* mutants, which do not produce the Hrp pilus, still produce the 50-kDa protein (Figs. 3 and 5*C*). These two observations suggest that the production and secretion of this protein is independent of the Hrp secretion system. It is possible that the 50-kDa protein is involved in the formation of some surface structure independent of the Hrp pilus.

To our knowledge, this is the first study showing that the Hrp pathway is involved in the formation of a pilus structure and that the HrpA protein is a structural component of the pilus structure. Our finding is consistent with the observation that many Hrp proteins are structurally related to those that participate in the construction of bacterial flagella (23), suggesting an involvement of Hrp proteins in the assembly of an extracellular macromolecular structure. *Salmonella*, *Shigella*, and *Yersinia*, all of which contain type III secretion systems, secrete proteins required for pathogenesis (47–51). Both *Salmonella typhimurium* and *Shigella flexneri* are enteroinvasive pathogens. *S. typhimurium* transiently produces filamentous surface appendages of 60 nm in diameter upon contact with epithelial cells during its invasion of host cells (52). The structural components of these appendages have not been identified. *Yersinia* spp. are not intracellular pathogens, but during infection they secrete virulence proteins through contact zones between bacteria and host cells (50). The secreted proteins of *S. flexneri* and *Yersinia* spp. form various aggregates and protein complexes in liquid, stationary-phase cultures (48, 49). However, the relationship between these protein aggregates and possible formation of surface appendages in these bacteria remains to be determined.

Recently, *Agrobacterium tumefaciens* was shown to produce pili involved in T-DNA transfer (53). Pili of 3.5 nm in diameter were formed under *vir* gene-inducing conditions. These pili were proposed to function as conjugation pili in T-DNA transfer between bacteria and plant cells. The protein components of the T-DNA secretion pathway encoded by *virB* genes share sequence similarities with proteins involved in the assembly of conjugative pili, but not with protein components of the type III secretion system. The structural proteins of the *A. tumefaciens* pilus have yet to be identified.

Morphologically, the Hrp pilus of *P. syringae* pv. *tomato* strain DC3000 characterized in this study resembles most closely the pilus produced by *A. tumefaciens*. Both pili are much thinner than the surface appendages of *S. typhimurium*. This similarity may reflect an adaptation of the two bacteria in the infection of wall-bound plant cells. Conditions for pilus production by the two bacteria are also very similar. As for the Hrp pilus, far fewer *A. tumefaciens* pili are produced at higher temperatures (e.g., 28°C) than at lower temperatures (e.g., 19°C). Furthermore, formation of the Hrp pilus requires solid growth medium, the condition used also for growing *A. tumefaciens* for pilus production (53). This may reflect the requirement for contact between bacteria and plant cells for pilus formation *in planta*.

Recent results suggest that the action sites of *P. syringae* pv. *glycinea* AvrB and possibly *X. campestris* pv. *malvacearum* Avr/Pth proteins are inside the plant cell. A previous study showed that close bacterial contact is required for bacterial elicitation of the HR (54). The requirement of the Hrp pilus structural gene *hrpA* in the phenotypic expression of *avrB*, as demonstrated in this study, suggests that the Hrp pilus may be involved in the delivery of AvrB and possibly other virulence and avirulence proteins to the plant cell. Alternatively, it may be involved in mediating contact between bacterial and plant cells in the plant intercellular space. The exact function of the Hrp pilus in protein transfer or cell–cell contact remains to be determined.

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