Functional Analysis of HrpF, a Putative Type III Translocon Protein from *Xanthomonas campestris* pv. *vesicatoria*

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Type III secretion systems (TTSSs) are specialized protein transport systems in gram-negative bacteria which target effector proteins into the host cell. The TTSS of the plant pathogen *Xanthomonas campestris* pv. *vesicatoria*, encoded by the *hrp* (hypersensitive reaction and pathogenicity) gene cluster, is essential for the interaction with the plant. One of the secreted proteins is HrpF, which is required for pathogenicity but dispensable for type III secretion of effector proteins in vitro, suggesting a role in translocation. In this study, complementation analyses of an *hrpF* null mutant strain using various deletion derivatives revealed the functional importance of the C-terminal hydrophobic protein region. Deletion of the N terminus abolished type III secretion of HrpF. Employing the type III effector AvrBs3 as a reporter, we show that the N terminus of HrpF contains a signal for secretion but not a functional translocation signal. Experiments with lipid bilayers revealed a lipid-binding activity of HrpF as well as HrpF-dependent pore formation. These data indicate that HrpF presumably plays a role at the bacterial-plant interface as part of a bacterial translocon which mediates effector protein delivery across the host cell membrane.

The interaction of many gram-negative plant and animal pathogenic bacteria with their hosts depends on a highly conserved type III protein secretion system (TTSS), which transports proteins without cleavage of a classical N-terminal signal peptide into the extracellular milieu as well as into the host cell (13, 25). In plant-pathogenic bacteria, the TTSS is encoded by *hrp* (hypersensitive response and pathogenicity) genes. *hrp* mutants are no longer able to multiply and cause disease in susceptible plants and to induce defense responses such as the hypersensitive reaction (HR) in resistant host and nonhost plants (1). The HR is a rapid, localized cell death of infected plant tissue which halts bacterial ingress. At least nine *hrp* genes, designated *hrc* (for *hrp* conserved), are conserved between plant and animal bacterial pathogens (8, 22) and probably encode the core components of the type III secretion apparatus.

Analyses of nonpolar mutants revealed that an additional set of nonconserved proteins, encoded in the *hrp* gene clusters, were essential for secretion and/or translocation. Among the nonconserved proteins are secreted proteins such as the subunits of the Hrp pilus, which is associated with the TTSS of plant-pathogenic bacteria (48). Hrp pili have been described for *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Erwinia amylovora* (30, 47, 61) and probably mediate contact between the bacterial and plant cell surface. In addition, Hrp pili have been shown to be essential for type III secretion in vitro (47, 61) and were proposed to function as conduits for secreted proteins traversing the plant cell wall (29, 30). Other proteins traveling the TTSSs of plant-pathogenic bacteria include

harpins and effector proteins, the latter of which have been suggested to be translocated into the plant cell (32).

Intensive studies of *Yersinia* outer proteins (Yops) defined the N terminus of type III-secreted proteins as an important region which directs secretion (12, 37, 56). In addition, a secretion signal in the 5' region of the mRNA has been discussed (2, 3, 41). A translocation signal has been proposed to be located within the first 50 to 100 codons of genes encoding effector proteins of both plant and animal pathogens (41, 53, 56). Translocation of effector proteins into the host cell was first described for *Yersinia* spp. (19, 49, 57) and appears to be the key function of TTSSs.

Translocation is mediated by the translocon, a bacterial protein or protein complex which presumably forms channel-like structures in the host cell membrane. In plant-pathogenic bacteria, there is indirect evidence for translocation of effectors because expression of bacterial avirulence (*avr*) genes in the plant cell resulted in the induction of a resistance (*R*) gene-specific HR (11). *avr* genes are present in *Pseudomonas* and *Xanthomonas* spp. (69) and were originally defined based on their ability to trigger a host defense reaction, in most cases the HR. Plant defense induction depends on the specific recognition of an Avr protein by a plant expressing the corresponding *R* gene (31, 36). In the absence of the *avr* or the *R* gene or both, the interaction between pathogen and plant leads to disease.

Our laboratory studies type III secretion in *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot on pepper and tomato. The TTSS is encoded by the 23-kb chromosomal *hrp* gene cluster (9). Expression of the six operons, *hrpA* to *hrpF*, is induced in the plant (55) and in minimal medium XVM2 (66) and is regulated by the products of the regulatory genes *hrpX* and *hrpG*. HrpG belongs to the OmpR family of two-component response regulators and activates the expression of *hrpA* and *hrpX* (68). HrpX, an AraC-type transcriptional activator, controls the expression of operons *hrpB*

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to *hrpF* (66) as well as the expression of *avrXv3* (4) and a number of putative virulence factors (45).

The TTSS of *X. campestris* pv. *vesicatoria* secretes a number of Hrp and Avr proteins as well as Xops (*Xanthomonas* outer proteins) into the culture supernatant (6, 16, 41, 45, 50, 51). One of the proteins secreted by the TTSS is HrpF, an overall hydrophilic protein of 87 kDa (26). HrpF contains two N-terminal imperfect direct repeats and two C-terminal hydrophobic segments (26, 50) and shows 48% sequence identity with NolX, a type III-secreted protein from *Rhizobium fredii* (26, 63). Intriguingly, HrpF, which is dispensable for type III secretion in vitro, has been found to be essential for the interaction of *X. campestris* pv. *vesicatoria* with the plant. It has therefore been suggested that HrpF plays a role in type III translocation (50).

In this study, we report on the functional importance of different regions in the HrpF protein. Furthermore, we obtained indirect evidence that HrpF is not translocated into the host cell. Lipid-binding activity of secreted HrpF and the observation of HrpF-dependent pore formation in lipid bilayers support the hypothesis that HrpF acts as a translocon protein.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* cells were cultivated at 37°C in Luria-Bertani (LB) or Super medium (Qiagen, Hilden, Germany). *X. campestris* pv. *vesicatoria* strains were grown at 30°C in NYG broth (15) or in minimal medium A (5) supplemented with sucrose (10 mM) and Casamino Acids (0.3%), and *Agrobacterium tumefaciens* strains were grown at 30°C in yeast extract-beef (YEB) medium. Antibiotics were added to the media at following final concentrations: ampicillin, 100 µg/ml; kanamycin, 25 µg/ml; rifampin, 100 µg/ml; spectinomycin, 100 µg/ml; and tetracycline, 10 µg/ml.

Plasmids were introduced into *E. coli* by electroporation and into *X. campestris* pv. *vesicatoria* and *A. tumefaciens* by conjugation, using pRK2013 as a helper plasmid in triparental matings (17).

Plant material and plant inoculations. The near-isogenic pepper cultivars Early Cal Wonder (ECW), ECW-10R, and ECW-30R (40) were grown and inoculated with *X. campestris* pv. *vesicatoria* or *A. tumefaciens* as described previously (10, 60). Bacteria were hand-infiltrated into the intercellular spaces of fully expanded leaves at concentrations of 4×10^8 CFU/ml in 1 mM MgCl₂. Reactions were scored over a period of 3 days.

Agrobacterium-mediated transient expression. *Agrobacterium*-mediated transient expression assays were performed as described (60) with the following modifications: bacteria were incubated in induction medium for 4 to 6 h and infiltrated into the intercellular spaces of fully expanded leaves at concentrations of 5×10^8 CFU/ml in infiltration medium (10 mM MgCl₂, 5 mM MES [morpholineethanesulfonic acid, pH 5.3], 150 µM acetosyringone).

Generation of an *hrpF* null mutant. A 5.3-kb *EcoRV* fragment, derived from construct pBF (26), which contains the monocistronic *hrpF* locus, was subcloned in pUC119, giving pU5hrpF. A 2.9-kb fragment containing the *hrpF* coding region, 253 bp upstream, and 319 bp downstream region was deleted by *Clal* digestion and religation of the plasmid. This mutation should not have a polar effect on other operons. The remaining 2-kb insert was cloned into the suicide plasmid pOK1 (27), giving pOKΔhrpF. *X. campestris* pv. *vesicatoria* strains 85–10ΔhrpF and 85*ΔhrpF were generated by introduction of pOKΔhrpF into 85–10 and 85*, respectively, as described (27).

Construction of *hrpF* derivatives. For expression and purification of recombinant HrpF, plasmid pDhrpF was constructed. *hrpF* was amplified by PCR from genomic DNA of *X. campestris* pv. *vesicatoria* strain 85–10, using *Pfu* polymerase (Stratagene, Heidelberg, Germany) and primers HrpF-For (5'-TACTGAATTC GCCTCTATGTCGCTC-3'), containing an *EcoRI* site, and HrpF-Rev (5'-GTA AGCTTAGTGATGGTGATGCGCCGGTCTGCGACGGATCCG GAC-3'), introducing a *SmaI* site, the coding sequence for six histidine residues, and a *HindIII* site (restriction sites are underlined in primer sequences). The PCR product was cloned by partial *EcoRI* and *HindIII* digestion into pUC119, pBluescript II KS and the broad-host-range vectors pDSK602 and pLAFR3 for

expression in both *E. coli* and *X. campestris* pv. *vesicatoria*, giving constructs pUhrpF, pKhrpF, pDhrpF, and pLhrpF, respectively.

*hrpF*ΔI was generated by a 1,380-bp *SgrAI* deletion in construct pUhrpF which leads to the deletion of amino acids 118 to 575. The resulting fragment was cloned into pDSK602, giving pDhrpFΔI. Construct pDhrpFΔN is a derivative of pDhrpF in which 456 bp encoding the N terminus of HrpF were replaced by an adaptor (5'-AATTCGCCTCTATGTCTAGAC-3'). In construct pLhrpFΔC109, a nonsense mutation was introduced by PCR, resulting in a translational stop after amino acid 697. To construct a C-terminal deletion derivative of HrpF, a 600-bp fragment at the 3' end of *hrpF* in plasmid pLhrpF was deleted by digestion with *SacI* and *SmaI*, Klenow fill-in reaction, and religation. The resulting construct was designated pLhrpFΔC249.

For deletion of the C-terminal hydrophobic segments, the 600-bp *SacI/HindIII* fragment encoding the C terminus of HrpF was replaced by deletion derivatives. The *SacI/HindIII* cassette was amplified by PCR, introducing *XbaI* sites in the sequences flanking the hydrophobic segment-encoding region. The PCR products flanked by *SacI/XbaI* and *XbaI/HindIII* sites were fused at the *XbaI* site and introduced into the *SacI* and *HindIII* sites of constructs pLhrpF and pDhrpF. This resulted in deletion of amino acids 610 to 647 (pDhrpFΔH1), 647 to 692 (pLhrpFΔH2), and 610 to 692 (pDhrpFΔH12) in the corresponding gene products.

For *Agrobacterium*-mediated transient expression, *hrpF* derived from construct pKhrpF was cloned into the *EcoRI* and *XhoI* sites of pBI1.4t, giving construct pBIhrpF. For construct pBISPhrpF, a PCR product containing the signal peptide sequence of the tobacco PR1a gene (14) was fused to the *hrpF* open reading frame. For technical reasons, the signal peptide sequence contained 1 bp exchange, resulting in a conserved amino acid exchange at position 17 of the signal peptide. For generation of plasmids expressing the HrpF-AvrBs3 fusion protein, the 1.1-kb *EcoRI* fragment of construct pKhrpF encoding the N terminus of HrpF was cloned into the *EcoRI* site of constructs pDS356F and pBI356F, generating constructs pDhrpFN356 and pBIhrpFN356, respectively.

HrpF expression, purification, and antibody production. For the production of a polyclonal anti-HrpF antiserum and for TRANSIL experiments, HrpF was expressed from pDhrpF and purified from *E. coli* BL21. Bacteria were grown in Super medium at 37°C. Expression was induced at an optical density at 600 nm (OD₆₀₀) of 0.7 with IPTG (isopropylthiogalactopyranoside, 2 mM final concentration) for 2 h at 30°C. Cells were harvested, resuspended in 8 M urea–0.1 M NaH₂PO₄–0.01 M Tris-HCl, pH 8.0, and broken with a French press. After removal of cell debris, HrpF was purified from the supernatant using Ni-nitrilotriacetic acid-agarose (Qiagen). After washing with 0.1 M NaH₂PO₄ and 0.01 M Tris-HCl, pH 6.3, the protein was eluted with 0.1 M NaH₂PO₄–0.01 M Tris-HCl (pH 8.0)–150 mM histidine.

For antibody production, rabbits were immunized with the purified HrpF protein (Eurogentec, Herstal, Belgium). The serum after the third booster injection was used for immunoblot analyses.

For expression of glutathione *S*-transferase (GST) fusion proteins, *hrpF* derivatives from pUhrpF and pLhrpFΔC249 were cloned into the *EcoRI* and *XhoI* sites of the GST fusion vector pGEX-2TKM, generating pGhrpF and pGhrpFΔC249. In addition, *hrpE2* was PCR amplified and cloned into the *EcoRI* and *XhoI* sites of pGEX-2TKM, giving pGhrpE2.

For the analysis of recombinant proteins in planar lipid bilayer experiments, *E. coli* BL21 carrying pGhrpF, pGhrpFΔC249, or pGhrpE2 was grown as described above. All GST fusion proteins were purified from inclusion bodies. Cells were broken with a French press, and inclusion bodies were pelleted by centrifugation. After extensive washing with phosphate-buffered saline (PBS), the inclusion bodies were broken in 8 M urea–0.1 M NaH₂PO₄–0.01 M Tris-HCl, pH 8.0, with a French press. Insoluble debris was removed by centrifugation, and the samples were dialyzed for 16 h against PBS at 4°C. Then 200 µl of dialysate was incubated with 1 U of thrombin protease for 2 h at room temperature, and cleaved GST was removed by incubation with glutathione-Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) for 30 min at room temperature with shaking. After centrifugation, protein amounts in the supernatant fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining.

Secretion experiments and protein analysis. Bacteria were cultivated in minimal medium A overnight and resuspended to a concentration of 10^8 CFU/ml in minimal medium A at pH 5.4 (acidified by the addition of HCl and containing 100 µg of bovine serum albumin [BSA] per ml). After 3 h of cultivation, 0.5 ml of total cultures was pelleted by centrifugation ($10,000 \times g$ for 10 min at 4°C) and resuspended in 1/10th volume of Laemmli buffer (35). Two milliliters of culture supernatants was filtered with a low-protein-binding filter (HT Tuffryn; 0.45 µm; PALL Gelman Laboratory, Ann Arbor, Mich.), precipitated with 10% trichloroacetic acid, and resuspended in 1/100th volume of Laemmli buffer, and 10-µl

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
<i>X. campestris</i> pv. <i>vesicatoria</i>		
85-10	Pepper race 2; wild type; Rif ^r	10
85*	Derivative of 85-10 carrying <i>hrpG</i> *	67
82-8	Pepper race 1; wild type; Rif ^r	40
82*	Derivative of 82-8 carrying <i>hrpG</i> *	67
85-10Δ <i>hrpF</i>	<i>hrpF</i> deletion mutant of 85-10	This study
85*Δ <i>hrpF</i>	<i>hrpF</i> deletion mutant of 85*	This study
85*Δ <i>hrcV</i>	<i>hrcV</i> deletion mutant of 85*	50
82*Δ <i>hrpF</i>	<i>hrpF</i> deletion mutant of 82*	This study
82*Δ <i>hrcV</i>	<i>hrcV</i> deletion mutant of 82*	51
<i>E. coli</i>		
DH5α	F ⁻ <i>recA hsdR17</i> (r _K ⁻ m _K ⁺) φ80d <i>lacZ</i> Δ <i>M15</i>	Bethesda Research Laboratories, Bethesda, Md.
DH5α λpir	F ⁻ <i>recA hsdR17</i> (r _K ⁻ m _K ⁺) φ80d <i>lacZ</i> Δ <i>M15</i> (λpir)	38
BL21(DE3)	F ⁻ <i>ompT hsdS20</i> (r _B ⁻ m _B ⁻) <i>gal</i>	Stratagene
<i>A. tumefaciens</i> GV3101	Cured of Ti plasmid, Rif ^r	24
Plasmids		
pBI1.4t	Binary vector, derivative of pBI121 (Clontech), p35S* with bacterial transcription terminator; Km ^r	39
pBluescript (II) KS	Phagemid, pUC derivative; Ap ^r	Stratagene
pDSK602	Contains triple <i>lacUV5</i> promoter; Sm ^r	43
pGEX-2TKM	p _{lac} GST <i>lacI</i> ^q pBR322 <i>ori</i> ; Ap ^r , derivative of pGEX-2TK, polylinker of pDSK604	Stratagene (16)
pLAFR3	RK2 replicon, Mob ⁺ Tra ⁻ ; contains p _{lac} ; Tc ^r	58
pUC119	ColE1 replicon; Ap ^r	62
pOK1	<i>sacB sacQ mobRK2 oriRK6</i> ; Sm ^r	27
pRK2013	ColE1 replicon, TraRK ⁺ Mob ⁺ ; Km ^r	17
pDS300F	pDSK602 expressing AvrBs3, Flag tag	60
pVS300F	Binary vector pVB60 expressing AvrBs3, Flag tag	60
pDS356F	pDSK602 expressing AvrBs3 deleted in N-terminal 152 aa	O. Rossier and U. Bonas, unpublished data
pBI356F	Binary vector pBI1.4t carrying AvrBs3 deleted in N-terminal 152 aa	B. Szurek and U. Bonas, unpublished data
pOKΔ <i>hrpF</i>	2-kb fragment containing the <i>hrpF</i> flanking regions in pOK1	This study
pD <i>hrpF</i>	pDSK602 expressing HrpF, His ₆ tag	This study
pL <i>hrpF</i>	pLAFR3 expressing HrpF, His ₆ tag	This study
pD <i>hrpF</i> ΔI	pDSK602 expressing HrpF deleted in internal 460 aa, His ₆ tag	This study
pD <i>hrpF</i> ΔN	pDSK602 expressing HrpF deleted in N-terminal 152 aa, His ₆ tag	This study
pL <i>hrpF</i> ΔC109	pLAFR3 expressing HrpF deleted in C-terminal 109 aa	This study
pL <i>hrpF</i> ΔC249	pLAFR3 expressing HrpF deleted in C-terminal 249 aa	This study
pD <i>hrpF</i> ΔH1	pDSK602 expressing HrpF deleted in H1, His ₆ tag	This study
pL <i>hrpF</i> ΔH2	pLAFR3 expressing HrpF deleted in H2, His ₆ tag	This study
pD <i>hrpF</i> ΔH12	pDSK602 expressing HrpF deleted in H1 and H2, His ₆ tag	This study
pBI <i>hrpF</i>	pBI1.4t carrying HrpF, His ₆ tag	This study
pBI <i>S</i> Phr <i>F</i>	pBI1.4t carrying HrpF containing the PR1a signal peptide, His ₆ tag	This study
pD <i>hrpF</i> N356,	pDSK602 and pBI1.4t expressing the fusion protein between the N-terminal 387 aa of HrpF and AvrBs3Δ2	This study
pBI <i>hrpF</i> N356		
pG <i>hrpF</i> , pG <i>hrpF</i> ΔC249	pGEX-2TKM expressing HrpF and HrpFΔC249	This study
pG <i>hrpE</i> 2	pGEX-2TKM expressing HrpE2	This study

^a aa, amino acids; Ap, ampicillin; Km, kanamycin; Rif, rifampin; Sm, spectinomycin; Tc, tetracycline; r, resistant.

aliquots of cell extracts and 15-μl aliquots of supernatants, adjusted for equal protein loading, were separated by SDS-PAGE and transferred to nitrocellulose. Secretion experiments were performed at least three times.

Protein extracts from infected plant leaves were prepared by grinding leaf disks in 20 mM Tris-HCl (pH 8.0)–150 mM NaCl–1 mM EDTA–1% Triton–0.1% SDS. Laemmli buffer was added, and samples containing approximately 70 μg of proteins were analyzed by SDS-PAGE and immunoblotting.

Immunoblots were incubated with polyclonal antisera against HrpF, AvrBs3 (33), and the intracellular HrcN protein (50) to ensure that no cell lysis had occurred.

Horse radish peroxidase-labeled goat anti-rabbit immunoglobulin antibodies (Amersham Pharmacia Biotech) were used as secondary antibodies. Reactions were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Lipid binding and planar lipid bilayer experiments. Lipid binding experiments were performed with silica beads (TRANSIL; Nimbus Biotechnology, Leipzig,

Germany) coated with POPC [1-hexadecanoyl-2-(cis-9-octadecenoyl)-sn-glycero-3-phosphocholine]–POPS [1-hexadecanoyl-2-(cis-9-octadecenoyl)-sn-glycero-3-phosphoserine, 90:10]. From 1 to 3 μg of recombinant protein or 1 ml of *X. campestris* pv. *vesicatoria* culture supernatant was incubated with 2 to 5 μl of TRANSIL beads under shaking at room temperature for 1 h in 10 mM Tris (pH 7.4)–150 mM NaCl–0.5% Tween 20 (final concentration). In control samples, proteins and culture supernatants were incubated without TRANSIL beads. The beads were precipitated by centrifugation (10,000 × g for 30 s at room temperature), and the unbound material in the supernatant was collected. For secreted proteins, unbound material was precipitated on ice with 10% trichloroacetic acid and resuspended in 1/50th volume of Laemmli buffer. TRANSIL beads were washed three times with 1 ml of 10 mM Tris (pH 7.4)–1 M NaCl and resuspended in 20 μl of Laemmli buffer. Total proteins and bound and unbound material were analyzed by SDS-PAGE and Coomassie staining and/or immunoblotting.

Planar lipid bilayers (42) were prepared from a solution of 80 parts (wt/wt) 1-palmitoyl-2-oleoyl-glycero-3-phosphatidylcholine and 20 parts (wt/wt) 1,2-dio-

FIG. 1. Immunoblot analysis of wild-type and *hrpF* null mutant strains. (A) Detection of HrpF with a polyclonal antiserum. *X. campestris* pv. vesicatoria strains 85* (lane 1), 85* Δ *hrpF* (lane 2), and 85* Δ *hrcV* (lane 3), which contains a nonpolar deletion in a conserved TTSS gene, were grown in secretion medium. Equal protein amounts of total protein extracts (TE) and culture supernatants (SN) were analyzed by SDS-PAGE and immunoblotting with the HrpF-specific antiserum (see Materials and Methods). (B) The *hrpF* null mutant secretes AvrBs3. *X. campestris* pv. vesicatoria strains 82* (lane 4), 82* Δ *hrpF* (lane 5), and 82* Δ *hrcV* (lane 6) were grown in secretion medium as above. Equal protein amounts of total protein extracts (TE) and culture supernatants (SN) were analyzed by SDS-PAGE and immunoblotting with the AvrBs3-specific antiserum. The same blot was reacted with an HrcN-specific antibody to rule out that bacterial lysis had occurred (data not shown).

leoyl-glycero-3-phosphatidylethanolamine (Avanti Polar Lipids Inc., Alabaster, Ala.) dissolved in *n*-decane (15 mg/ml). Proteins were added to the *cis*-aqueous solution of the bilayer cuvette. Electrolyte solutions contained 100 mM KCl and 10 mM HEPES, pH 7.0. Current signals were filtered at corner frequencies between 3 and 10 kHz and recorded continuously on a digital tape recorder. As a membrane amplifier, we used a BLM-120 (Bio-Logic, Claix, France) with a low-pass linearized five-pole Tchebicheff filter.

RESULTS

Specific antiserum detects HrpF in the culture supernatant.

Recently, we showed type III-mediated secretion of HrpF, using a Flag-tagged version of the protein (50). For the analysis of native HrpF, a polyclonal antiserum was generated. In Western blot analyses, the antiserum reacted specifically with two proteins of approximately 100 and 70 kDa (Fig. 1A) in total extracts of *X. campestris* pv. vesicatoria strain 85* (85–10 carrying the *hrpG** mutation, which renders *hrp* gene expression constitutive [67]). Since both proteins were absent in extracts of an *hrpF* null mutant (Fig. 1A), they were *hrpF* specific, indicating that the smaller protein corresponds to a processed form of HrpF, which was designated HrpF'. The processing probably occurs at the C terminus, since an anti-His₆ antibody failed to detect HrpF' in protein extracts of strain 85*(pDhrpF), which expresses a C-terminally His₆-tagged HrpF protein (data not shown). In secretion experiments, HrpF and HrpF' were detected in the supernatant of strain 85* but not of 85* Δ *hrcV*, a TTSS mutant (Fig. 1A). Depending on the experiment and protein amounts loaded, the antiserum also detected two additional proteins which are probably minor degradation prod-

ucts of HrpF. Similar results were obtained for strain 82* and derivatives (data not shown).

Complementation analyses of *hrpF* deletion derivatives.

Previous genetic analysis of *hrpF*, which is the only gene in the operon, was based on transposon insertion mutants (26, 50). For functional studies of the HrpF protein, we deleted *hrpF* from the genome of *X. campestris* pv. vesicatoria strains 85–10, 85*, and 82*. As expected, *hrpF* null mutants displayed a typical *hrp* phenotype. When inoculated into the plant, strains 85–10 Δ *hrpF* and 85* Δ *hrpF* failed to grow in planta, and they were unable to cause disease symptoms in susceptible pepper ECW plants or to induce the HR in resistant ECW-10R plants, which carry the *BsI* gene.

The *BsI* resistance gene determines recognition of AvrBs1, which is expressed in strains 85–10 and 85* (40). Similar results were obtained when strain 82* Δ *hrpF* was inoculated into susceptible and resistant plants. *X. campestris* pv. vesicatoria strain 82* expresses the effector protein AvrBs3 (10) and induces the HR on ECW-30R plants, which carry the *Bs3* resistance gene. As shown in Fig. 1B, AvrBs3 was detected in Western blot analysis of culture supernatants of strain 82* Δ *hrpF*. Thus, in contrast to the *hrcV* TTSS mutant, *hrpF* null mutants were not impaired in type III secretion.

In order to identify functionally important regions in HrpF, several deletion derivatives (Fig. 2) were constructed and expressed in strains 85–10 Δ *hrpF* and 85* Δ *hrpF* from broad-host-range plasmids. In plant infection tests, the mutant phenotype of both strains could be complemented by constructs expressing full-length HrpF or HrpF Δ C109, but not by constructs expressing HrpF Δ N and deletion derivatives lacking hydrophobic region H1 or H2 or both (Fig. 2). Interestingly, expression of *hrpF* Δ I, which is deleted in the internal region, resulted in partial complementation of strain 85* Δ *hrpF*, i.e., delayed disease symptoms in pepper cultivar ECW and a partial hypersensitive reaction in ECW-10R plants (Fig. 2). In immunoblot analyses, HrpF Δ I was not recognized by the HrpF-specific antiserum and could only be detected by an anti-His₆ antibody in protein extracts of high-cell-density cultures (data not shown), indicating that the protein is unstable. In contrast, N- and C-terminal deletion derivatives were detected by the polyclonal HrpF-specific antibody, albeit in different amounts (Fig. 3).

The inability of *hrpF* deletion derivatives to complement an *hrpF* null mutant could be due to the lack of secretion of the corresponding gene products. However, all C-terminal HrpF deletion derivatives were secreted by the TTSS *in vitro* (Fig. 3). In contrast, the N-terminal deletion derivative HrpF Δ N was not detected in the culture supernatant.

The N terminus of HrpF does not target the AvrBs3 protein into the host cell. We then addressed the question of whether HrpF is not only secreted *in vitro* but could possibly be translocated into the plant cell, as has been observed for putative translocon proteins of animal-pathogenic bacteria (13). For this, we used an N-terminal deletion derivative (AvrBs3 Δ 2) of the type III effector AvrBs3 as a reporter. AvrBs3 Δ 2 is not secreted by *X. campestris* pv. vesicatoria but induces a specific hypersensitive reaction when fused with a functional translocation domain and when the gene is expressed in the resistant plant (E. Huguet, O. Rossier, and U. Bonas, unpublished data). Here, we fused the first 386 amino acids of HrpF to

FIG. 2. Analysis of HrpF and different HrpF deletion constructs. The HrpF protein (806 amino acids) contains two repeats (R1 and R2) and two hydrophobic segments (H1 and H2). HrpF deletion derivatives were generated as described in Materials and Methods. Construct numbers refer to the numbers of deleted amino acids. Except for HrpFΔC109 and HrpFΔC249, all HrpF derivatives contain a C-terminal His₆ tag (for details see Materials and Methods and Table 1). The ability of HrpF derivatives to complement an *X. campestris* pv. *vesicatoria* *hrpF* null mutant for disease symptom formation and HR induction is indicated on the right. Strains 85-10Δ*hrpF* and 85*Δ*hrpF* harboring the different deletion constructs were inoculated at a bacterial density of 4 × 10⁸ CFU/ml into the intercellular spaces of leaves of pepper plants ECW-10R and ECW. Plant reactions were scored over a period of 1 to 3 days. +, disease in susceptible plants and HR in resistant plants; −, no disease symptoms, no HR; +/-, intermediate phenotype: delayed disease symptoms, partial HR.

AvrBs3Δ2 and introduced the fusion construct (pDhrpFN356) into strain 85*.

In total protein extracts, the HrpF-AvrBs3Δ2 protein could be detected by both the AvrBs3-specific (Fig. 4) and the HrpF-specific (data not shown) antiserum. The presence of smaller proteins which are detected by the AvrBs3-specific antibody might be due to protein instability (Fig. 4). In secretion experiments, the HrpF-AvrBs3Δ2 construct was detected in culture supernatants of strain 85* but not of the TTSS mutant 85*Δ*hrcV*, indicating the presence of a type III secretion signal in the N terminus of HrpF. This is in agreement with the finding that deletion of the N terminus abolished type III secretion of HrpF (Fig. 3). In plant inoculation experiments,

strain 85*(pDhrpFN356) did not induce the HR in pepper genotype ECW-30R (Table 2).

To test whether the fusion of the HrpF-N terminus to AvrBs3Δ2 had a deleterious effect on recognition by *Bs3*, we expressed the fusion protein in ECW-30R, using pBIhrpFN356 and *Agrobacterium*-mediated gene transfer. In this case, the HrpF-AvrBs3Δ2 construct induced the HR in ECW-30R, as did full-length AvrBs3 and AvrBs3Δ2 (Table 2), demonstrating that the fusion protein is recognized by *Bs3* inside the plant cell. Taken together, these data show that the HrpF-AvrBs3Δ2 fusion protein was not delivered into the plant cell when expressed in *X. campestris* pv. *vesicatoria*, although it was secreted by the TTSS in vitro.

FIG. 3. Secretion analysis of HrpF deletion constructs. *X. campestris* pv. *vesicatoria* strain 85*Δ*hrpF* expressing HrpF or a mutant derivative was grown in secretion medium. Equal protein amounts of total protein extracts (TE) and culture supernatants (SN) were analyzed by SDS-PAGE and immunoblotting with antibodies directed against HrpF and the intracellular HrcN protein, respectively.

FIG. 4. The N terminus of HrpF contains a type III secretion signal. *X. campestris* pv. *vesicatoria* strains (lane 1) 85*(pDhrpFN356), (lane 2) 85* Δ hrpV(pDhrpFN356), and (lane 3) 85*(pDS356F), expressing the HrpF-AvrBs3 Δ 2 fusion protein (lanes 1 and 2) and AvrBs3 Δ 2 (lane 3), respectively, were grown in secretion medium. Equal amounts of total protein extracts (TE) and culture supernatants (SN) were analyzed by SDS-PAGE and immunoblotting using the AvrBs3-specific antiserum.

In additional experiments, we investigated a putative function of HrpF inside the plant cell by transient expression of HrpF in planta. When *A. tumefaciens* strain GV3101(pBIhrpF) was inoculated into ECW-30R plants, no obvious plant reaction was observed (Table 2), although the protein was expressed, as shown by immunoblot analyses (data not shown). Furthermore, transiently expressed HrpF could not transcomplement an *X. campestris* pv. *vesicatoria* *hrpF* null mutant strain for disease and HR induction in ECW and ECW-10R, respectively. Since fusion of HrpF to the signal peptide sequence of the tobacco PR1a gene (14) rendered the protein unstable (data not shown), transcomplementation experiments with in planta-expressed HrpF targeted to the apoplast could not be performed.

Lipid-binding activity of HrpF. Most putative type III translocon proteins identified so far contain predicted transmembrane regions and have been shown to associate with membranes (7, 20, 21, 23, 28, 52, 59, 64). Considering the presence of two putative transmembrane segments in HrpF as well as its proposed role during effector protein translocation, we investigated the lipid-binding activity of the protein. For this, we used a lipid bilayer system (TRANSIL beads; Nimbus Biotechnology), which consists of silica particles coated with a single phospholipid bilayer, noncovalently bound to the matrix. The bilayer contained negatively charged phospholipids which are also present in plant plasma membranes. Beads were incubated with purified recombinant HrpF protein (see Materials and Methods), and the lipid-bound and unbound material was analyzed by SDS-PAGE and Coomassie staining or immunoblotting. To remove proteins that were associated with the lipid

bilayer via ionic interactions, beads were washed under high-salt conditions (18).

As shown in Fig. 5A, recombinant HrpF associated with the lipid matrix. In contrast, BSA, used as a negative control, was only present in the supernatant. Furthermore, HrpF was not detected in the pellet when proteins were incubated without beads, ruling out that detection was due to protein precipitation. The lipid-binding activity of HrpF could be confirmed for the native protein expressed in *X. campestris* pv. *vesicatoria*. For this, TRANSIL beads were incubated with culture supernatants of strain 82*. While secreted HrpF bound to the lipid matrix, the truncated HrpF' protein (see above) was only detected in the unbound material (Fig. 5B). The comparison of HrpF present in total supernatants and unbound and bound material indicated that approximately 40% of secreted HrpF was bound to the TRANSIL beads (Fig. 5C).

In order to identify regions in HrpF which are important for membrane association, we tested HrpF deletion derivatives for their binding to TRANSIL beads. Deletion of the N terminus and of both hydrophobic segments did not affect the lipid-binding ability of HrpF (HrpF Δ H12 and HrpF Δ N, Fig. 5B). In contrast, HrpF Δ C249, lacking a large C-terminal protein region, was not detected in the lipid-bound material (Fig. 5B). This is in agreement with the finding that HrpF', which is similar in size to HrpF Δ C249 (see Fig. 3), did not bind to the lipid matrix.

HrpF-dependent pore formation. The lipid-binding activity of HrpF prompted us to investigate whether HrpF would form ion-conducting pores in a planar lipid bilayer system. When recombinant HrpF (see Materials and Methods) was added to the *cis* compartment of the artificial membrane, the conductivity across the bilayer increased when voltage was applied. We observed changes of open and closed channel states as well as prolonged phases of both states (Fig. 6A). HrpF-dependent pore formation did not alter significantly when different membrane potentials were applied (from -100 to $+100$ mV; data not shown). Surprisingly, the C-terminally truncated form of HrpF, HrpF Δ C249, induced channels with similar properties (data not shown). To rule out that the channel formation was due to *E. coli* proteins present in the HrpF preparation, we used HrpE2 as a control, which was isolated similarly to HrpF (see Materials and Methods). HrpE2 is an 18.4-kDa acidic protein from *X. campestris* pv. *vesicatoria* and is predicted to be a soluble protein (U. Bonas, unpublished data). When applied to the artificial membrane, recombinant HrpE2 did not induce any current fluctuations (Fig. 6B).

DISCUSSION

In this study, we investigated functionally important regions and membrane-binding activity of the HrpF protein from the plant pathogen *X. campestris* pv. *vesicatoria*. Our data strongly support the hypothesis that HrpF plays a role in protein translocation. So far, HrpF is the only known putative type III translocon protein of a plant-pathogenic bacterium.

Analyses of *hrpF* null mutant strains revealed that HrpF is essential for pathogenicity but dispensable for type III-mediated protein secretion in vitro. This is in agreement with results based on the analyses of *hrpF* transposon insertion mutants (50). An interesting feature of the HrpF protein is the presence

TABLE 2. Plant reactions to AvrBs3 and HrpF derivatives^a

Protein expressed	Phenotype on pepper ECW-30R	
	Delivery by <i>X. campestris</i> pv. <i>vesicatoria</i> 85*	Expression in planta via <i>A. tumefaciens</i> GV3101
AvrBs3	HR	HR
AvrBs3 Δ 2	+	HR
HrpF-AvrBs3 fusion	+	HR
HrpF	+	—

^a Symptoms observed 3 days after inoculation at a bacterial density of 4×10^8 CFU/ml (delivery) or 5×10^8 CFU/ml (in planta). HR, hypersensitive response. +, disease symptoms; —, no phenotypic reaction.

FIG. 5. HrpF binds to lipid bilayers. (A) TRANSIL beads (2 μ l) coated with POPC/POPS (90:10) were incubated with 3 μ g of recombinant HrpF and BSA. After centrifugation and stringent washing of the beads, 20% of total proteins (T) and unbound (UB) and lipid-bound (B) material were analyzed by SDS-PAGE and Coomassie staining. As a negative control (C), proteins were incubated without TRANSIL beads, and after centrifugation and stringent washing, samples corresponding to the lipid-bound material were analyzed as above. (B) *X. campestris* pv. *vesicatoria* strains 82* (top) and 82* Δ hrpF carrying plasmids pDhrpFΔH12 and pLhrpFΔC249 were grown in secretion medium. Culture supernatants were incubated with 4 μ l of TRANSIL beads as above. Total culture supernatants (T) as well as unbound (UB) and lipid-bound (B) material were analyzed by SDS-PAGE and immunoblotting with the HrpF-specific antiserum. HrpFΔN (bottom), which is not secreted in vitro, was analyzed as a recombinant protein; 0.5 μ g of recombinant HrpFΔN was incubated with TRANSIL as described for A, and 20% of total protein and unbound and lipid-bound material was analyzed by immunoblotting with the anti-HrpF antibody. As a negative control, culture supernatants as well as recombinant HrpFΔN were incubated without TRANSIL (C) and analyzed as above. (C) Quantification of membrane-bound HrpF. Culture supernatants of *X. campestris* pv. *vesicatoria* strain 82* were incubated with 4 μ l of TRANSIL beads and analyzed as described for B. Unbound (UB) and bound (B) material as well as samples incubated without TRANSIL (C) were analyzed by SDS-PAGE and immunoblotting with the HrpF-specific antiserum. As reference samples, 100, 80, 60, and 40% of total supernatant (T) were loaded.

of two imperfect repeats in the N terminus. Deletion studies showed that the presence of the second repeat is sufficient for protein function (26). Here, complementation experiments demonstrated that the region C-terminal to the hydrophobic segments is also not required. In contrast, the hydrophobic

FIG. 6. HrpF-dependent pore formation in planar lipid bilayers. (A) HrpF induces current fluctuations in planar lipid bilayers. Recombinant HrpF (0.5 μ g/ml, purified as described in Materials and Methods) was added to the *cis*-aqueous solution of the lipid bilayer cuvette, and current traces were recorded. c, closed state. (B) HrpE2 shows no pore-forming properties in lipid bilayer experiments. HrpE2 was tested as a control protein. The experimental conditions were the same as in A.

segments are essential for HrpF function. Interestingly, the internal protein region appears to be less important, since the *hrpF* null mutant could be partially complemented by HrpFΔI. However, partial complementation was only observed in an *hrpG** background, which leads to a more efficient expression of the TTSS. Lack of complementation of the *hrpF* null mutant by HrpFΔN is probably due to the inability of the bacteria to secrete this derivative. This indicates that HrpF is targeted for type III secretion by its N terminus and that secretion is essential for HrpF function.

The hypothesis of a secretion signal in the N terminus is supported by the finding that a fusion protein between the N-terminal 386 amino acids of HrpF and AvrBs3Δ2 is secreted in vitro. AvrBs3, like other type III effectors, is probably translocated into the plant cell, because in planta expression of the gene triggers the HR (60). However, bacteria expressing the HrpF-AvrBs3 fusion protein did not induce the AvrBs3-specific HR, indicating that the N terminus of HrpF does not contain a translocation signal. HrpF might have to be delivered from outside of the plant cell, a hypothesis which is supported by the inability of in planta-expressed HrpF to transcomplement the phenotype of an *hrpF* null mutant.

Taken together, these data support the hypothesis that HrpF acts at the bacterial-plant interface as a translocon protein. So far, putative components of the predicted type III translocon have only been identified in animal-pathogenic bacteria, where they presumably form a transmembrane channel. In *Yersinia*, *Shigella*, and enteropathogenic *E. coli* spp., the existence of a translocation channel has been proposed based on their ability to induce the lysis of erythrocytes and macrophages (7, 20, 44, 65). Furthermore, fusion of planar lipid bilayers with liposomes which had been incubated with secreted Yops induced

current fluxes, indicating the formation of transmembrane pores (59). Lytic activity of *Yersinia* requires at least three proteins, YopB, YopD, and LcrV (20, 23, 44). Recent demonstration of a pore-forming activity of purified LcrV suggested that LcrV is the channel size-determining component of the type III translocation channel, which is stabilized by YopB and YopD (23).

The putative translocon proteins from animal-pathogenic bacteria are not conserved among different species, but some of them have structural similarities, such as putative transmembrane regions. The presence of two predicted transmembrane segments in HrpF (26) prompted us to investigate its lipid-binding activity. A tendency of HrpF to bind to membranes had already been indicated by fractionation studies (26). From 30 to 40% of a Flag-tagged HrpF protein was localized to the inner membrane of *X. campestris* pv. *vesicatoria* under nonsecreting conditions. Here, using the TRANSIL system which provides a highly mobile artificial lipid bilayer, mimicking diffusion and other surface properties of natural lipid bilayers (54), we could demonstrate lipid-binding activity of recombinant as well as native secreted HrpF. The fact that high-salt washes did not remove HrpF from the lipid matrix is interpreted as membrane insertion rather than membrane binding.

Lipid-binding in vitro using the TRANSIL system was independent of the presence of the hydrophobic segments in HrpF, indicating that HrpF interacts with lipids via nonhydrophobic regions, presumably in the C terminus. This was surprising because the hydrophobic segments have been speculated to act as a transmembrane anchor. So far, except for IpaC from *Shigella flexneri* (46), it has not been investigated whether predicted transmembrane regions are essential for lipid binding in other putative translocon proteins. Interestingly, using the TM-PRED program (http://www.ch.embnet.org/software/TM-PRED_form.html), no transmembrane regions are predicted for LcrV from *Yersinia* spp. and PcrV from *Pseudomonas aeruginosa*, which have both been demonstrated to form transmembrane channels (23).

It is tempting to speculate that these proteins span the membrane via β -barrel structures. β -Barrel structures are typical of many bacterial outer membrane proteins (34) and are also found in several bacterial toxins which assemble to oligomeric channels in the eukaryotic host cell membrane. The presence of β -barrels in putative translocon proteins of the TTSS has not been investigated yet.

To address the question of whether HrpF forms transmembrane channels, we used a planar lipid bilayer system. Indeed, recombinant HrpF induced altered current fluxes, indicative of pore formation. Unexpectedly, this was also observed for an HrpF derivative lacking the C terminus, which was shown to be essential for lipid binding in the TRANSIL system. We speculate that this derivative has a residual in vitro lipid-binding activity which can only be detected by the very sensitive planar lipid bilayer assays. Similar findings have been reported for LcrV from *Yersinia enterocolitica*: deletion of a C-terminal protein region, which was demonstrated to be essential for Yop delivery as well as for induction of erythrocyte lysis, did not alter its pore-forming activity (23).

It has to be kept in mind that artificial lipid bilayers have smooth surfaces without any exposed structures, like lipid-bound polysaccharides or membrane proteins, which could

influence the insertion of proteins in natural membranes. Here, we show that the C terminus of HrpF from *X. campestris* pv. *vesicatoria*, which is dispensable for pore formation in vitro, is essential for its biological function. It is conceivable that this region is required for efficient protein translocation in vivo, stabilizing the pore or interacting with accessory proteins. Whether HrpF alone is sufficient to form the translocation channel in vivo remains to be investigated.

In animal-pathogenic bacteria, the observation of protein-protein interactions between putative translocon components suggests that the translocon is a heterogenic protein complex (23). Future experimental approaches will therefore focus on the identification of HrpF's interaction partners.

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