Domain Structure of HrpE, the Hrp Pilus Subunit of *Xanthomonas campestris* pv. *vesicatoria*

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The plant-pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* possesses a type III secretion (TTS) system necessary for pathogenicity in susceptible hosts and induction of the hypersensitive response in resistant plants. This specialized protein transport system is encoded by a 23-kb *hrp* (hypersensitive response and pathogenicity) gene cluster. *X. campestris* pv. *vesicatoria* produces filamentous structures, Hrp pili, at the cell surface under *hrp*-inducing conditions. The Hrp pilus acts as a cell surface appendage of the TTS system and serves as a conduit for the transfer of bacterial effector proteins into the plant cell cytosol. The major pilus component, the HrpE pilin, is unique to xanthomonads and is encoded within the *hrp* gene cluster. In this study, functional domains of HrpE were mapped by linker-scanning mutagenesis and by reporter protein fusions to an N-terminally truncated avirulence protein (AvrBs3 Δ 2). Thirteen five-amino-acid peptide insertion mutants were obtained and could be grouped into six phenotypic classes. Three permissive mutations were mapped in the N-terminal half of HrpE, which is weakly conserved within the HrpE protein family. Four dominant-negative peptide insertions in the strongly conserved C-terminal region suggest that this domain is critical for oligomerization of the pilus subunits. Reporter protein fusions revealed that the N-terminal 17 amino acid residues act as an efficient TTS signal. From these results, we postulate a three-domain structure of HrpE with an N-terminal secretion signal, a surface-exposed variable region of the N-terminal half, and a C-terminal polymerization domain. Comparisons with a mutant study of HrpA, the Hrp pilin from *Pseudomonas syringae* pv. *tomato* DC3000, and hydrophobicity plot analyses of several nonhomologous Hrp pilins suggest a common architecture of Hrp pilins of different plant-pathogenic bacteria.

Many gram-negative bacterial pathogens, including *Xanthomonas campestris* pv. *vesicatoria*, possess a unique protein transport system called the type III secretion (TTS) system which transfers so-called effector proteins directly into the host cell. In plant bacterial pathogens, genes encoding the TTS system are referred to as hypersensitive response and pathogenicity (*hrp*) genes because mutations in these genes abolish induction of the hypersensitive response in resistant host plants and pathogenicity in host plants (36).

In *X. campestris* pv. *vesicatoria*, the *hrp* gene cluster is located in a 23-kb chromosomal region and is organized into six operons, designated *hrpA* to *hrpF* (6, 17, 18, 24, 25, 48, 58). Twenty-two genes are encoded within the *hrp* gene cluster, 11 of which are highly conserved among plant and animal pathogens and therefore have been renamed *hrc* (*hrp* conserved) genes (5, 56). Eight *hrc* gene products are associated with bacterial membranes and build up a transenvelope multiprotein complex (21). The remaining three proteins localize in the cytoplasm and are probably involved in energizing the transport process (20, 44). The role of the nonconserved *hrp* and *hpa* (*hrp*-associated) genes is less well understood. Two proteins, HrpE and HrpF, are substrates of the TTS system and serve in the delivery of type III effector proteins to and across the plant cell plasma membrane (8, 48, 56). Since these proteins are not translocated into the plant cell, they are referred to as noneffectors (9). Translocation of noneffectors is presum-

ably inhibited by HpaB, an export control protein. While in the wild type noneffectors, such as HrpF and XopA, are not translocated into the plant cell, they are translocated in an *hpaB* mutant, as indicated by reporter protein fusions (9).

hrp gene expression is activated in planta by two regulatory genes, *hrpG* and *hrpX* (50). HrpG is a member of the OmpR family of two-component response regulators and controls, in most cases via the AraC-type regulator HrpX, a large regulon including genes for type III effector proteins (42, 57, 59).

These effector proteins presumably interfere with the metabolic pathways of the host, providing an advantage for the pathogen, or suppress the plant defense reaction (54). Some of these effectors are recognized in resistant plants by corresponding *R* gene products, triggering a defense response called the hypersensitive response, which ultimately restricts bacterial growth. They were therefore designated avirulence (Avr) proteins (60). AvrBs3 is the best-characterized avirulence protein of *X. campestris* pv. *vesicatoria* and serves as a well-established reporter for both secretion into the extracellular environment and translocation into plant cells (41).

In contrast to animal-pathogenic bacteria, plant-pathogenic bacteria have to overcome the plant cell wall for translocation of effector proteins. In order to fulfill this task, plant pathogens assemble a surface appendage, named the Hrp pilus. Hrp-dependent pili have been described in several plant-pathogenic or symbiotic bacteria that also contain a TTS system: *Pseudomonas syringae* pv. *tomato*, *Ralstonia solanacearum*, *Erwinia amylovora*, and *Sinorhizobium fredii* (28, 32, 46, 55). The Hrp pilus elongates by the addition of Hrp pilin subunits at the distal end, and also TTS system substrates are secreted only from the pilus tip (27, 35). These results indicate that the Hrp

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>X. campestris</i> pv. <i>vesicatoria</i>		
85E*	<i>eps</i> ::Tn3- <i>gus</i> insertion mutant of 85-10; <i>hrp</i> ⁺ Rif ^r Km ^r ; carrying <i>hrpG</i> * leading to constitutive <i>hrp</i> gene expression	59
85E*Δ <i>hrpF</i>	<i>hrpF</i> deletion mutant of 85E*; Rif ^r Km ^r	56
85E*Δ <i>hrcV</i>	<i>hrcV</i> deletion mutant of 85E*; Rif ^r Km ^r	56
85E*Δ <i>hrpE</i> Δ9-93	<i>hrpE</i> deletion mutant of 85E*; Rif ^r Km ^r	56
85E*Δ <i>hrpF</i> <i>hrpE</i> (Q56)	85E*Δ <i>hrpF</i> ; derivative carries <i>hrpE</i> (Q56)	This study
85E*Δ <i>hrcV</i> <i>hrpE</i> (Q56)	85E*Δ <i>hrcV</i> ; derivative carries <i>hrpE</i> (Q56)	This study
<i>E. coli</i>		
DH10b	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> ΔM15 Δ <i>lacX74</i> <i>endA1 recA1 deoR araΔ139</i> Δ(<i>ara leu</i>)7697 <i>galU galK</i> λ ⁻ <i>nupG rpsL</i> (Sm ^r)	Invitrogen, Carlsbad, CA
DH5α λ <i>pir</i>	F ⁻ φ80 <i>dlacZ</i> ΔM15(λ <i>pir</i>) Δ(<i>lacZYA-argF</i>)U169 <i>endA1 recA1 deoR hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44 thi-1 gyrA96 relA1</i> Nal ^r ; host for propagation of pOK derivatives	38
Plasmids		
pOK1	Suicide vector; <i>sacB sacQ mobRK2 oriR6K</i> ; Spc ^r	25
pXV9	pLAFR3 <i>hrcC</i> to <i>hrpE</i> clone from <i>X. campestris</i> pv. <i>vesicatoria</i> 75-3; Tc ^r	56
pRK2013	Helper plasmid for triparental matings; <i>traRK</i> ⁺ Mob ⁺ Km ^r	19
pDSM110	pDSK604 expressing a c-myc-tagged version of AvrBs1 from its own promoter; Spc ^r	16
pOK- <i>hrpE</i>	Wild-type <i>hrpE</i> in pOK1	56
pENTR/D-TOPO	Cloning vector; <i>attL</i> recombination sites; pUC- <i>ori</i> ; Km ^r	Invitrogen, Carlsbad, CA
pL6GW356	pLAFR6 derivative containing Gateway (Invitrogen, Carlsbad, CA) <i>attR</i> reading frame B cassette; Cm ^r Spc ^r	41
pGPS5	Transprimer donor; Km ^r Ap ^r	New England Biolabs, Beverly, MA
pL6HrpE _n -AvrBs3Δ2	pLAFR6 expressing a fusion protein between the N-terminal n amino acids of HrpE and AvrBs3Δ2 under control of the <i>hrpE</i> promoter	This study

pilus serves as a conduit through which substrates are transported. In all cases, the Hrp pilus subunits are small (6 to 11 kDa) and hypervariable, not only between different species, but also between pathovars. The best-characterized Hrp pilus subunit so far, the Hrp pilin HrpA from *P. syringae* pv. *tomato*, shares only 27% protein sequence identity with HrpA of *P. syringae* pv. *syringae* (14).

Based on preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoelectron microscopy, and mutational analyses, HrpE was identified as the structural component of the Hrp pilus from *X. campestris* pv. *vesicatoria* (56). *hrpE* is unique to the genus of *Xanthomonas* and shows no sequence similarity to other pilin genes. Bacteria carrying mutations in the *hrpE* gene neither cause disease in susceptible host plants nor elicit the hypersensitive response in resistant host plants. Additionally, HrpE has been shown to be essential for the type III-dependent secretion of proteins such as HrpF, AvrBs1, and AvrBs3. Hence, the Hrp pilus is an indispensable component of a functional TTS system. The 9-kDa HrpE protein forms a slender pilus 8 to 10 nm in diameter and up to 4 μm in length (56).

The present study reports on the domain structure of HrpE as revealed by linker-scanning mutagenesis and reporter protein fusions. The linker-scanning mutants contain pentapeptides inserted randomly within the polypeptide chain. Previous studies using this technique yielded significant structure-function information (3, 11, 53). Several phenotypic classes of mutants were obtained and characterized in detail. By means of

reporter protein fusions, we identify a signal for type III secretion but not for translocation into plant cells. From these results, a three-domain structure of HrpE is predicted. Hydrophobicity plot analyses of several Hrp pilin proteins, such as HrpE, HrpA, and HrpY from *R. solanacearum*, reveal a common domain organization. These findings strongly suggest that plant-pathogenic bacteria, challenged with the task of overcoming the barrier of a plant cell wall, independently evolved structurally similar proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Since the wild-type strain *X. campestris* pv. *vesicatoria* 85-10 produces large amounts of exopolysaccharides, which were suspected to affect electron microscopic (EM) analyses, the exopolysaccharide-negative strain 85E was used for most analyses. Plasmids were introduced into *Escherichia coli* by electroporation and into *X. campestris* pv. *vesicatoria* by conjugation, using pRK2013 as a helper plasmid in triparental matings (15, 19). *E. coli* cells were cultivated at 37°C in LB medium, and *X. campestris* pv. *vesicatoria* strains were grown at 30°C in NYG broth (13), on NYG 1.5% agar, in XVM2 minimal medium (57), or in MA secretion medium (49). Antibiotics were added to the media at the following final concentrations: kanamycin, 25 μg/ml; tetracycline, 10 μg/ml; rifampin, 100 μg/ml; spectinomycin, 100 μg/ml.

Plant material and plant inoculations. Pepper cultivar ECW and the near-isogenic lines ECW-10R and ECW-30R, carrying the resistance genes *Bs1* and *Bs3*, respectively, have been described previously (39). Inoculation of and reisolation of bacteria from plant tissue were performed as previously described (6). If not indicated otherwise, 8-week-old pepper plants were used.

Construction of the *hrpE* linker-scanning library. The GPS-LS mutagenesis kit was used to perform linker-scanning mutagenesis of the *hrpE* gene according

to the manufacturer's instructions (New England Biolabs, Beverly, MA). Briefly, the mobilizable suicide vector pOK-*hrpE* (containing *hrcD* to *hpaE*) was used as the target DNA for mutagenesis. In an in vitro reaction including a transposon donor plasmid, the target DNA, and the transposase, the transposase excises the transposon from the donor and inserts it into the target DNA. Five base pairs of the target sequence are duplicated by the process. The insertions are expected to occur randomly. *E. coli* DH5 α *lpir* cells were transformed with 2 μ l of the in vitro transposition reaction mixture. Transformants were selected for spectinomycin resistance of the vector and kanamycin resistance of the pGPS5 transposon. Insertions in *hrpE* were mapped by colony PCR using a transposon border primer contributed by the manufacturer and the vector-specific primer *hrpE*(-200) (5'-G CATGAGCTCGAAATCCCAAGCACATGACATCCCTGC). To determine the exact insertion site, positive clones were sequenced using the transposon border primer. Transposon sequences were removed from selected clones by restriction digestion with *PmeI* and religation, resulting in a 15-bp insertion, leading to either an insertion of five amino acids (in two of three reading frames) or a TAA stop codon (in the remaining frame).

Generation of chromosomal *hrpE* peptide insertion mutants. The chromosomal wild-type copy of *hrpE* was replaced with mutant versions by two consecutive crossover events as described previously (29). For all strains, the replacement was confirmed by colony PCR spanning the *hrpE* region, followed by *PmeI* restriction analysis.

Construction of HrpE-AvrBs3 Δ 2 fusion proteins. To create protein fusions of HrpE with the reporter protein AvrBs3 Δ 2, plasmid pL6GW356 (41) containing the Gateway *attR* reading frame B cassette (Invitrogen, Carlsbad, CA) in front of *avrBs3 Δ 2* was used. DNA regions starting 2.5 kb upstream of *hrpE* and containing different N-terminal regions of *hrpE* were PCR amplified from genomic DNA of *X. campestris* pv. vesicatoria strain 85E* and cloned into the entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA). Primer sequences are available upon request. The entry clones were then recombined with the destination vector pL6GW356, thus creating the expression clones pL6HrpE₁₀AvrBs3 Δ 2, pL6HrpE₁₇AvrBs3 Δ 2, pL6HrpE₁₉AvrBs3 Δ 2, pL6HrpE₂₃AvrBs3 Δ 2, pL6HrpE₄₂AvrBs3 Δ 2, pL6HrpE₅₀AvrBs3 Δ 2, and pL6HrpE₉₃AvrBs3 Δ 2. Constructs were conjugated into strains 85E*, 85E* Δ *hpaB*, and 85E* Δ *hrcV* by triparental matings as previously described (15).

Protein secretion experiments and immunoblot analyses. In vitro secretion experiments were performed as described previously (49). Western blots were incubated with polyclonal antiserum against HrpF (diluted 1:50,000) (10), a polyclonal antiserum against AvrBs3 (diluted 1:1,000) (30), or a monoclonal anti *c-myc* antiserum (diluted 1:1,000) (Roche Diagnostics GmbH, Mannheim, Germany) and with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Uppsala, Sweden), which was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). The membranes were reprobed with an antibody against the intracellular protein HrcN to ensure that no bacterial lysis had occurred (49).

EM techniques. For electron microscopy analysis, bacteria were incubated on EM grids according to a slightly modified protocol (7). Bacterial cultures were grown for 24 h in NYG medium at 30°C. Bacteria were washed twice with 1 mM MgCl₂ and resuspended in minimal medium. The bacteria were adjusted to an optical density at 600 nm of 0.02, and a 15- μ l droplet was applied to a 300-mesh gold EM grid (Sigma, Taufkirchen, Germany) coated with 1% Formvar (polyvinyl 1595E; Serva, Heidelberg, Germany). To avoid evaporation, the grids were placed in a 9-cm petri dish containing wet filter paper which was sealed with Parafilm. Grids were incubated for 6 h in a growth chamber at 30°C before bacteria and pili were fixed by transferring the grid twice into a 20- μ l drop of 2% formaldehyde and 0.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 20 min. Specimens were then negatively stained for 10 s in 2% phosphotungstic acid and air dried before examination on an EM 900 electron microscope (Zeiss, Oberkochen, Germany) at an operating voltage of 80 kV.

Bioinformatic analyses. Pairwise BLAST comparisons were performed at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/bl2.html>). Hydrophobicity profiles and aliphatic and instability indices were calculated with the ProtScale and the ProtParam tool, respectively, available on the ExPASy proteomics server of the Swiss Institute of Bioinformatics (<http://www.expasy.org/>).

RESULTS

Peptide insertion mutagenesis and initial screening of the mutants. To elucidate the domain structure of HrpE, we created a mutant collection containing pentapeptide insertions. A 2.3-kb region harboring *hrcD* to *hpaE*, including *hrpE*, was

cloned into the vector pOK. In vitro transposition mutagenesis was accomplished with the GPS-LS kit, which employs a Tn7-derived minitransposon carrying a selectable marker with modified inverted repeats at the ends harboring *PmeI* restriction sites (4). As a result, 5 bp of the target sequence are duplicated. Insertions were expected to occur randomly. After transformation of the transposition reaction mixture into *E. coli*, transformants were selected for the resistance markers of the target DNA and the transposon. The location of the transposon insertion was mapped by PCR and DNA sequencing. Restriction digestion of appropriate derivatives and recircularization led to insertion of 15 bp into the target DNA, either coding for five amino acids residues or creating a stop codon.

The *hrpE*-containing plasmid pOK-*hrpE* was mutagenized in two independent transposition reactions. In total, 5,000 clones were initially screened by PCR and 59 of those had an insertion in the *hrpE* region. DNA sequencing revealed that 34 insertions (including 10 siblings) were located in the coding sequence of *hrpE*. A premature stop codon was identified in 11 clones. In-frame insertions were found at 13 different positions in the *hrpE* gene. To avoid multicopy effects of these variants, they were introduced into the genomes of *X. campestris* pv. vesicatoria strains 85E* and 85-10, thus replacing the wild-type copy of *hrpE*. The nomenclature of the insertion mutants is based on the location of the five additional amino acids. For instance, *hrpE*(W10) stands for an insertion after tryptophan at position 10. The variant with the earliest stop codon was used as a nonpolar *hrpE* mutant (*hrpE* Δ 9-93) (56), while the other truncated variants were not further analyzed. A list of all mutants and their relevant phenotypes is given in Table 2.

The N terminus of HrpE is permissive for pentapeptide insertions. The mutant collection was screened for the ability to translocate the effector proteins into plant cells. As an assay, we used the avirulence protein AvrBs1, which leads to a hypersensitive response in resistant *BsI* pepper plants (47). Insertion mutant 85E**hrpE*(W10) showed a slightly reduced hypersensitive response when inoculated into 4-week-old resistant pepper plants. 85E**hrpE*(M18) and 85E**hrpE*(F21) reacted similarly to the wild type; however, on 12-week-old plants they showed strongly reduced responses. 85E**hrpE*(D53) elicited a strongly reduced and delayed reaction. All insertions between glutamine 56 and the C terminus of the protein exhibited an Hrp-negative phenotype, and even 5 days after inoculation, no hypersensitive response was observed (Table 2). These results show that the N-terminal half of HrpE tolerates pentapeptide insertions, whereas the C-terminal half does not.

The mutant 85EhrpE*(Q56) is able to secrete proteins although it does not elicit a hypersensitive response.** We wondered whether the inability to elicit a hypersensitive response is caused by a general defect in *hrp*-dependent secretion from the bacterium or by a defective translocation of avirulence proteins into the plant cell. Therefore we followed the in vitro secretion of two type III secreted proteins HrpF and AvrBs1. When wild-type bacteria were incubated in secretion medium, both proteins could be detected in total cell extracts by Western blot analysis (Fig. 1 and Table 2). In concordance with the hypersensitive-response assay, analysis of *X. campestris* pv. vesicatoria culture supernatants revealed that only strains 85E**hrpE*(W10) to 85E**hrpE*(D53) secreted HrpF and

TABLE 2. Phenotypes of HrpE insertion mutants used in this study

Mutation/ strain	Pentapeptide insertion ^a	HR ^b	Complementation ^c	Secretion of AvrBs1	Secretion of HrpF	<i>in vitro</i> Hrp pili ^{d,e}
wt				+	+	+
<i>hrpE</i> Δ9–93				-	-	-
<i>hrpE</i> (W10)	CLNTW			+	+	+
<i>hrpE</i> (M18)	VFKHM	*		+	+	600 nm
<i>hrpE</i> (F21)	CLNSF	*		+	+	120 nm
<i>hrpE</i> (D53)	VFKQD			+	+	-
<i>hrpE</i> (Q56)	MFKHQ			-	+	-
<i>hrpE</i> (K62)	CLNNK			-	-	n.d.
<i>hrpE</i> (T64)	MFKHT			-	-	-
<i>hrpE</i> (K67)	MFKHK			-	-	n.d.
<i>hrpE</i> (N68)	CLNKN			-	-	-
<i>hrpE</i> (N73)	CLNIN			-	-	-
<i>hrpE</i> (N77)	CLNIN			-	-	-
<i>hrpE</i> (K82)	VFKHK			-	-	-
<i>hrpE</i> (K88)	CLNTK			-	-	-

^a Pentapeptide insertion encoded by the 15-bp insertion.
^b In most cases, strains were inoculated into 12-week-old ECW-10R plants and hypersensitive-response (HR) induction was scored after 2 days. In the cases of *hrpE*(M18) and *hrpE*(F21), 4-week-old (*) and 12-week-old plants were inoculated, respectively. *hrpE*(D53) was scored 5 days postinoculation.
^c Mutants were complemented with pXV9 carrying *hrpE*. The hypersensitive response was scored after 2 days.
^d n.d., not determined.
^e Average length is given.

FIG. 1. Effects of *hrpE* insertion mutations on in vitro type III secretion. Immunoblot analyses of total protein extracts (TE) and culture supernatants (SN) of an *hrpE* wild-type (wt) strain (85E*), a TTS-deficient mutant (85E* $\Delta hrpV$), an *hrpF* deletion mutant (85E* $\Delta hrpF$), an *hrpE* Δ 9-93 mutant, and insertion mutants 85E**hrpE*(W10), 85E**hrpE*(M18), 85E**hrpE*(D53), 85E**hrpE*(Q56), and 85E**hrpE*(K88) grown in secretion medium are shown. The blots were reacted with a polyclonal antiserum directed against HrpF.

AvrBs1. Mutants with insertions downstream of lysine 62 failed to secrete HrpF and AvrBs1. Intriguingly, mutant 85E**hrpE*(Q56), which did not show any reaction in planta, was able to secrete HrpF, although in slightly reduced amounts. However, we could not detect AvrBs1 in supernatants of 85E**hrpE*(Q56).

Hypersensitive-response reduction in resistant plants is mirrored by growth defects of bacteria in susceptible plants. In contrast to the hypersensitive response, which is based on the translocation of one specific Avr protein, growth in susceptible plants reflects the ability to initiate a compatible interaction with the host plant, requiring the coordinated translocation of a set of effector proteins. Therefore, the insertion mutants were inoculated into susceptible ECW pepper leaves. The resulting growth curves of strains 85E*, 85E**hrpE*(W10), 85E**hrpE*(D53), 85E**hrpE*(Q56), and 85E**hrpE* Δ 9-93 are shown in Fig. 2. Compared to the 85E* wild-type strain, the mutants showed a gradual decrease in growth, which correlated well with the different capacities to elicit a hypersensitive response in resistant plants (Table 2). The growth of 85E**hrpE*(M18) and 85E**hrpE*(F21) was intermediate between those of 85E**hrpE*(W10) and 85E**hrpE*(D53) (data not shown). One representative variant with an Hrp-negative phenotype, 85E**hrpE*(K88), behaved like the negative control 85E**hrpE* Δ 9-93. A remarkable exception is the mutant 85E**hrpE*(Q56), which was not detectable in the plant tissue after 48 h, whereas *hrp* mutants, such as 85E**hrpE* Δ 9-93, grew slightly during this period of time. This is a novel phenotype which has not been observed before. We suspect that either the mutant protein itself had a toxic effect on *X. campestris* pv. vesicatoria or the plant was able to respond to tiny amounts of an effector protein by an active defense reaction. This hypothesis was based on the in vitro secretion of reduced amounts of HrpF. Since AvrBs1 secretion was not detectable, it appeared conceivable that effector proteins were translocated in significantly reduced amounts, which may not be sufficient to suppress a plant defense reaction. To study this effect in more detail, *hrpE*(Q56) was introduced into secretion-defective ($\Delta hrpV$) and translocation-defective ($\Delta hrpF$) mutant backgrounds. Strain 85E* $\Delta hrpV$ *hrpE*(Q56) behaved like a typical *hrp* mutant, whereas the number of viable 85E* $\Delta hrpF$ *hrpE*

FIG. 2. Analysis of *hrpE* insertion mutants for growth in planta. (A) Bacterial growth of strains 85E*, 85E**hrpE*(W10), 85E**hrpE*(D53), 85E**hrpE*(Q56), and 85E**hrpE* Δ 9-93 in the susceptible pepper line ECW is shown. (B) Bacterial growth of strains 85E*, 85E* $\Delta hrpF$, 85E* $\Delta hrpF$ *hrpE*(Q56), 85E* $\Delta hrpV$ *hrpE*(Q56), and 85E**hrpE*(Q56). Bacteria were inoculated into leaves at 10^4 CFU/ml. Values are the means of four samples of two different plants taken at each time point, and the error bars indicate the standard deviations. Each graph is based on data from one representative experiment.

(Q56) cells was reduced in comparison to the *hrp* mutant (data not shown). Hence, HrpF is not required for this effect.

Peptide insertion mutants in the C-terminal region inhibit Hrp pilus assembly. To answer the question of whether some of the mutants can interfere with the function of wild-type HrpE, we introduced a low-copy-number cosmid with an extra copy of the entire *hrp* gene cluster, except for *hrpF*, into the mutant strains. Under these conditions, the wild-type protein was assumed to be expressed at a higher level than the mutant protein. To test for complementation, the strains were inoculated into resistant pepper plants and the AvrBs1-dependent hypersensitive response was monitored. Mutants which showed slightly reduced responses, 85E**hrpE*(W10) to 85E**hrpE*

FIG. 3. Morphological characterization of 85E**hrpE*(M18) cells and pili by electron microscopy. Bacteria were incubated on coated EM gold grids for 6 h at 30°C. Transmission electron micrographs of negatively stained specimen are shown. (A) 85E* forms Hrp pili with a diameter of 8 to 10 nm and a length of up to 4 μm . (B to D) Hrp pili of 85E**hrpE*(M18) are not altered in diameter but are significantly shorter (B). Broken Hrp pili are in close contact with the bacterial cell (C) or being detached (D). Broken Hrp pilus fragments show a predominant length of 0.5 to 1 μm . Bars: 0.25 μm . (E) Length distribution of Hrp pili and pilus fragments of strain 85E**hrpE*(M18). The number of Hrp pili connected to the bacterial cell (gray columns) or of broken pilus fragments (white columns) found with a given length was recorded. For comparison, the length distribution of Hrp pili formed by wild-type 85E* is illustrated by black columns.

(D53), as well as 85E**hrpE*(K62), 85E**hrpE*(K67), 85E**hrpE*(K82), and 85E**hrpE*(K88), could be complemented.

In four cases, 85E**hrpE*(T64), 85E**hrpE*(N68), 85E**hrpE*(N73), and 85E**hrpE*(N77), the ability to trigger a hypersensitive response could not be restored by the presence of wild-type *hrpE*. This dominant-negative effect suggests that the mutant variant may interfere with the assembly of the Hrp pilus.

Morphology studies reveal insertion mutants with shortened Hrp pili. Next we were interested in determining whether morphological peculiarities of the Hrp pili might be the reason for the observed phenotypes. Therefore, we investigated the *hrpE* mutants by electron microscopy. The pili of mutant 85E**hrpE*(W10) showed no significant alteration in morphology or the number of pili per cell (see Fig. 4A). Insertion mutants 85E**hrpE*(M18) and 85E**hrpE*(F21), which led to a reduced hypersensitive response after inoculation of 12-week-

old plants, had significantly shorter pili (Fig. 3 and data not shown). The length of Hrp pili ranged from 0.25 μm to 1 μm , whereas wild-type pili reached more than 4 μm in length. Interestingly, numerous detached pilus fragments were observed (Fig. 3C and D). Both the short pili and the pilus fragments had a diameter of 8 to 10 nm, like the wild type. The length of the detached fragments varied in a remarkably small range, between 0.5 and 1 μm . There was a clear correlation between fragment length and the maximal length of the attached pili (Fig. 3E). The number of pili was similar to that of the wild type.

For 85E**hrpE*(D53) and 85E**hrpE*(Q56), which were still able to secrete proteins, no Hrp pili could be detected (Fig. 4B and C). Possibly, the Hrp pilus already starts within and is not only attached on top of the TTS apparatus. We also examined two of the dominant-negative mutants. As expected,

FIG. 4. Effects of insertion mutations on Hrp pilus assembly. Electron micrographs of 85E*-derived bacteria incubated in XVM2 medium are shown. Insertion mutant *hrpE*(W10) (A) produced pili, whereas *hrpE*(D53), *hrpE*(Q56), and *hrpE*(N73) did not show Hrp pili (B to D). Bars: 200 nm.

85E**hrpE*(N73) and 85E**hrpE*(N77) failed to assemble Hrp pili (Fig. 4D).

The TTS signal is located in the first 17 codons. In order to define the TTS signal of HrpE, protein fusions between N-terminal fragments of HrpE and an N-terminally truncated AvrBs3 reporter protein (AvrBs3 Δ 2) were generated. This truncated AvrBs3 variant, which is devoid of its own secretion signal, is able to cause a hypersensitive response when transiently expressed in planta using *Agrobacterium tumefaciens* (52). The resulting constructs (HrpE₁₀-, HrpE₁₇-, HrpE₁₉-, HrpE₂₃-, HrpE₄₂-, HrpE₅₀-, and HrpE₉₃-AvrBs3 Δ 2) were introduced into strain 85E*. Western blot analysis of total protein extracts demonstrated that all fusion proteins were expressed. After incubation in secretion medium, all fusions, except for HrpE₁₀-AvrBs3 Δ 2, were well detected in the culture supernatants (Fig. 5A). The HrpE₁₀-AvrBs3 Δ 2 fusion protein could be detected in very small amounts, demonstrating that even this short peptide is able to permit secretion, albeit at a reduced rate. To confirm that secretion follows the TTS pathway, construct HrpE₅₀-AvrBs3 Δ 2 was introduced into 85E* Δ *hrcV*. In this genetic background, no in vitro secretion of the reporter protein could be detected (Fig. 5A). These results show that a minimal TTS signal is encoded within the first 10 codons, but full secretion efficiency was only observed with 17 or more codons.

HrpE is not translocated into plant cells by strains 85E* and 85E* Δ *hpaB*. To test whether HrpE-AvrBs3 Δ 2 fusions are translocated into plant cells, 85E* strains expressing HrpE₁₀-, HrpE₁₇-, HrpE₁₉-, HrpE₂₃-, HrpE₄₂-, HrpE₅₀-, and HrpE₉₃-AvrBs3 Δ 2 were inoculated into ECW-30R pepper leaves ex-

pressing the *Bs3* resistance gene. As shown in Fig. 5B, none of the HrpE-AvrBs3 Δ 2 fusions elicited a hypersensitive response, indicating that the HrpE-AvrBs3 Δ 2 fusions were not translocated into the plant cell. Instead, water-soaked lesions were formed, indicating that the fusion proteins had no inhibitory effect on the translocation of other effector proteins.

Recently it was shown that the N termini of noneffectors, such as XopA and HrpF, target the AvrBs3 Δ 2 reporter into plant cells in a Δ *hpaB* mutant but not in the wild type. This finding suggested that these noneffectors contain not only a secretion signal but also a translocation signal but that their translocation is inhibited by HpaB (9). Therefore, translocation of HrpE_n-AvrBs3 Δ 2 fusions was also tested in an 85E* Δ *hpaB* background. Like 85E*, 85E* Δ *hpaB* strains did not elicit a hypersensitive response, whereas the noneffector fusion HrpF₂₀₀-AvrBs3 Δ 2 did (Fig. 5B). These results indicate that the pilin HrpE is not delivered across the plant plasma membrane regardless of whether or not *hpaB* was present. Hence, HrpE does not possess a translocation signal controlled by HpaB.

Hrp pilins share similar physicochemical profiles. The major subunits of Hrp pili of *P. syringae* (HrpA), *R. solanacearum* (HrpY), and *X. campestris* (HrpE) assemble into pilus structures which are nearly identical in diameter and length (46, 55, 56). Surprisingly, their sequences are not conserved (Fig. 6A), which implies the existence of other common properties necessary for their function. To elucidate structural homology among Hrp pilins despite low sequence conservation, we analyzed the physicochemical features of the three proteins. This approach has already been described for components of the

FIG. 5. Secretion and translocation of HrpE_n-AvrBs3ΔN fusions. (A) Analysis of the secretion signal of HrpE. Immunoblot analysis of total protein extracts (TE) and culture supernatants (SN) of *X. campestris* pv. *vesicatoria* cells expressing HrpE_n-AvrBs3Δ2 fusion proteins, grown under secretion conditions, is shown. The blot was probed with a polyclonal antiserum directed against AvrBs3. (B) Translocation assay in pepper plant ECW-30R. The translocation of HrpE_n-AvrBs3Δ2 fusions was analyzed in the wild type (85E*) and in an isogenic *hpaB* deletion mutant (85E*Δ*hpaB*). All 85E* derivatives led to water-soaked lesions 2 days after inoculation. In contrast, no reaction occurred when 85E*Δ*hpaB* derivatives were used. The reporter fusion HrpF₂₀₀-AvrBs3Δ2 served as a control, leading to water-soaked lesions when expressed from 85E* or a hypersensitive response when expressed from 85E*Δ*hpaB*. *X. campestris* pv. *vesicatoria* strains were inoculated at 5×10^8 CFU/ml. Two days after inoculation, the leaves were bleached with ethanol.

flagellar system (1). As shown in Fig. 6B, all three pilins have very similar hydrophobicity profiles with three rapidly alternating hydrophilic and hydrophobic stretches in the C-terminal half, indicating that this part of the protein contains exposed and embedded regions. Also, the N termini show similar hydrophobicity patterns and a pronounced hydrophilic drop at the end of the presumed secretion signal (Fig. 6B). Also, the instability indices (HrpE, 25.4; HrpA, 18.3; HrpY, 18.3) and aliphatic indices (HrpE, 72.5; HrpA, 85.7; HrpY, 57.4) were similar in all three cases. These findings suggest a common fold of the Hrp pilus subunits of all three plant-pathogenic bacteria.

DISCUSSION

In this work, we studied the domain structure of HrpE, the major pilus subunit of *X. campestris* pv. *vesicatoria*. Intriguingly,

homologs of this protein are only found in other xanthomonads and comparison of the five available sequences shows a variable N-terminal half, in contrast to the highly conserved C-terminal half, where the last 24 amino acids are identical (56). This small 9-kDa protein is fascinating because of its functional versatility. First, as an outer component of the TTS system it has to be secreted and therefore needs a secretion signal. Second, it has to assemble into an extracellular polymeric structure which is stable and flexible enough to pass the plant cell wall (23). Third, an inner channel has to be formed which allows the passage of the Hrp pilus subunit itself, of the HrpF translocon protein, and of more than 20 different type III effectors (F. Thieme and U. Bonas, unpublished data). To fulfill all these tasks, interactions between HrpE and several components of the TTS system have to take place. HrpF, HrpE, HrpB2, and the HrcC

secretin are candidate partners, but up to now there is no experimental proof for any of these interactions (48). In this study, functional domains of HrpE were mapped by linker-scanning mutagenesis and by reporter protein fusions.

We mapped the TTS signal of HrpE by constructing protein fusions between HrpE with the reporter AvrBs3Δ2. HrpE_n-AvrBs3Δ2 fusions containing the first 17, 19, 23, 42, or 50 amino acids of HrpE or the full-length HrpE protein were expressed and secreted into the culture supernatant, whereas the first 10 amino acids allowed the secretion of only small amounts of the fusion protein. Hence, the signal sufficient for efficient secretion of HrpE is localized between the codons for amino acids 1 to 17. This finding is consistent with other studies of the TTS signals of many effector proteins (2, 22, 40, 51).

We also analyzed the competence of the HrpE N terminus to serve as a translocation signal. The inoculation of the 85E* strains expressing HrpE_n-AvrBs3Δ2 fusion proteins into pepper ECW-30R plants revealed that none of the variants was translocated. Interestingly, the noneffectors HrpF and XopA are translocated in a *ΔhpaB* mutant, showing that HpaB prevents them from being translocated in the wild type (9). In contrast to HrpF and XopA, HrpE is not translocated in a *ΔhpaB* mutant, indicating that there is no translocation signal in HrpE or that another negative regulator prevents HrpE from translocation. Of special interest is the full-length HrpE fusion to AvrBs3Δ2. This variant was readily detected in culture supernatants in an in vitro secretion assay and did not interfere with pilus assembly of wild-type HrpE, as indicated by the development of disease symptoms on ECW-30R plants (Fig. 5B) or by development of an AvrBs1-induced hypersensitive response in ECW-10R plants (data not shown). This finding indicates that C-terminal extensions to HrpE may prevent incorporation into the growing pilus.

To define the domain structure of HrpE further, linker-scanning mutagenesis was performed. The mutants were characterized with respect to the in vitro secretion of TTS substrates and the elicitation of a hypersensitive response in resistant plants or growth in susceptible plants. Furthermore, the pilus morphology was analyzed by electron microscopy and complementation studies were performed. Based on these assays, we grouped the insertion mutants into six classes, which are summarized in Table 3.

Class I mutant 85E**hrpE*(W10) behaved like the wild type except for slightly reduced growth in planta. This is a very sensitive assay and therefore may also reveal subtle distortions of the pilus which were not observable by electron microscopy. Surprisingly, the N-terminal secretion signal of HrpE was not disturbed by the five extra amino acids. We took advantage of this permissive site by inserting two epitope tags. While the hexahistidine epitope did not interfere with HrpE function,

FIG. 6. Hrp pilins show no sequence homology but similar hydrophobicity profiles. (A) Amino acid identities and similarities of Hrp pilin proteins. Pairwise BLAST analyses were performed using the BLOSUM62 scoring matrix. (B) Hydrophobicity plots of Hrp pilins, calculated over a sliding window of five amino acid residues, using the Kyte and Doolittle hydrophobicity scale (33). Conserved peaks of low hydrophobicity are indicated.

TABLE 3. Classes of HrpE insertion mutants

Class	Insertion mutant(s)	Relevant characteristics
I	<i>hrpE</i> (W10)	Pili same as wild type; slightly reduced growth in planta
II	<i>hrpE</i> (M18), <i>hrpE</i> (F21)	Short Hrp pili; secretion and translocation competent; reduced growth in planta, delayed hypersensitive response in old plants
III	<i>hrpE</i> (D53)	No Hrp pili; secretion and translocation competent; strongly reduced growth in planta, delayed hypersensitive response in young and old plants
IV	<i>hrpE</i> (Q56)	No Hrp pili; secretion of HrpF; no secretion of AvrBs1; dying in planta after 48 h
V	<i>hrpE</i> (K62), <i>hrpE</i> (K67), <i>hrpE</i> (K82), <i>hrpE</i> (K88)	No Hrp pili; Hrp phenotype
VI	<i>hrpE</i> (T64), <i>hrpE</i> (N68), <i>hrpE</i> (N73), <i>hrpE</i> (N77)	No Hrp pili; Hrp phenotype, dominant-negative phenotype

introducing the strongly charged FLAG epitope led to a non-functional HrpE variant (E. Weber, unpublished data).

Class II mutations *hrpE*(M18) and *hrpE*(F21) also allowed efficient *in vitro* secretion of HrpF and AvrBs1. However, with respect to virulence these mutants grew significantly less than the wild type in susceptible pepper plants. This finding may be explained by a reduced stability of the Hrp pili or by a slightly altered effector translocation. EM analyses revealed that these mutants form short and unstable Hrp pili. Two facts are remarkable. First, the uniformity of the Hrp pilus fragments shows that the breaking point is not distributed randomly. Second, the length of Hrp pilus fragments and the maximal length of the attached pili are similar. We assume that the Hrp pili are destabilized at their connection with the base and break if the structure reaches a certain length. Hence, the N terminus of HrpE contributes to the anchorage and stability of the pilus. A candidate interaction partner for the connection to the base is the outer membrane-localized secretin HrcC. In line with the shortened pili, we observed that only in 4-week-old plants did a hypersensitive response occur while no reaction occurred in 12-week-old plants. The continuous hardening process of plant cell walls by an increase in wall cross-linking or an alteration in the structure and composition of cell wall components may explain these findings (12).

Class III mutation *hrpE*(D53) and class IV mutation *hrpE*(Q56) are located at the boundary between the variable N terminus and the highly conserved C terminus. This transition is also reflected by their phenotypes. 85E**hrpE*(D53) was still able to elicit a hypersensitive response in resistant plants and to secrete HrpF and AvrBs1 (Table 2). In contrast, 85E**hrpE*(Q56) was able to secrete HrpF, but no longer AvrBs1. Accordingly, this mutant also failed to elicit a hypersensitive response in ECW-10R pepper plants. Despite their ability to secrete proteins, both mutants did not form Hrp pili *in vitro*. Probably, HrpE(D53) could form pili that are too fragile to be detected by the method used. In the case of HrpE(Q56), the mutant proteins are probably disturbed in their interaction with each other but can still form a “minimal pilus” *in vivo* inside the TTS apparatus which is stabilized by the surrounding TTS components. This hypothetical structure would be able to connect the cytoplasm with the outer environment and therefore to perform secretion. The degree of distortion may be larger in *hrpE*(Q56) than in *hrpE*(D53), thus explaining their different behaviors in protein secretion.

Surprisingly, the 85E**hrpE*(Q56) variant died in planta 48 h postinoculation. Its growth in liquid rich medium and in min-

imal secretion medium was not altered, indicating that the toxic effect is not an intrinsic feature of the mutant protein (data not shown). The toxic effect in planta depends on a functional TTS system, since the Δ *hrcV* *hrpE*(Q56) double mutant survived. However, translocation of effector proteins—even in very small amounts—was not required for the toxic effect since also a Δ *hrpF* Δ *hrpE*(Q56) double mutant died. We do not know if pathogen-associated molecular patterns are involved in this process or if even the HrpE(Q56) variant acts as a pathogen-associated molecular pattern (43). We consider this possibility unlikely, since no reaction occurred after inoculation of 85E**hrpE*(Q56) in susceptible ECW plants (data not shown). Alternatively, different gating behaviors of the TTS system under *in vitro* and *in planta* conditions may be the reason for this phenotype. An “open”-state TTS system may lead to leakage of protons into the environment.

In the highly conserved C terminus, all insertion mutations (class V and class VI) show much more drastic effects. The mutants did not elicit any reaction in plants, neither a hypersensitive response in resistant plants nor water-soaked lesions in susceptible ones. They also failed to secrete proteins and were not able to form pili. This complete loss of function shows the key importance of the C-terminal domain for function, which is also reflected by its sequence conservation among the five HrpE proteins. The four C-terminal mutants of class VI are of special interest since they could not be complemented. Such dominant-negative effects have also been described for other proteins where polymerization is indispensable for function (26). Therefore, it is most likely that the highly conserved C terminus acts as the polymerization domain of HrpE. For the HrpA pilin from *P. syringae* pv. tomato, it was shown that purified pilins polymerized *in vitro* (45). From this point of view, a polymerization-deficient but folding-competent protein variant may be instrumental for nuclear magnetic resonance spectroscopy or X-ray crystallography of HrpE.

Taken together, our results suggest a three-domain organization of HrpE (Fig. 7). The N-terminal 17 codons account for the TTS signal, as demonstrated by our reporter protein fusions. We found, however, that this signal is tolerant of mutations since two of three insertions behind tryptophan 10 did not disturb the TTS signal. A FLAG epitope was not tolerated, which may be due to its highly charged character. Other work with synthetic TTS signals has shown that an amphipathic character is required for function as a TTS signal (37). The second domain encloses all insertions (class I to III) up to aspartic acid 53, which were at least partially tolerated (Fig. 2;

FIG. 7. Proposed domain structure of HrpE. Insertion points are marked as lollipops with mutant numbers above. The six mutant classes are indicated as follows: class I, open circle; class II, gray circles; class III, black circle; class IV, open square; class V, gray squares; class VI, black squares.

Table 2). Therefore, this region cannot be responsible for the polymerization of the HrpE pilin. Instead, we suggest that this region is largely exposed to the exterior of the pilus. This suggestion is supported by the high variability of this region. Nevertheless, this region also contributes to the stability of the Hrp pilus since two of three insertions led to drastically shortened pili. The insertions behind aspartic acid 53 (class IV to VI), which did not allow productive interaction with host plants or production of Hrp pili (Table 2), define the third domain. This proposal is consistent with the high conservation of this region at the level of the primary and secondary structures (56). Interestingly, four mutations were dominant negative. This effect shows that this part is involved in the polymerization process.

Besides HrpE, only the pilus protein HrpA from *P. syringae* pv. tomato has been characterized in detail. Similar to our finding, the first 15 residues are sufficient for secretion of a reporter protein and almost all insertions in the C-terminal half prevent pilus formation (22, 53). Interestingly, the two proteins, and also the HrpY pilin from *R. solanacearum*, do not share any significant sequence homology (Fig. 6A). However, all these proteins share a number of physicochemical features. They are small (HrpE, 9.7 kDa; HrpA, 11.3 kDa; HrpY, 8.7 kDa) and predicted to consist almost exclusively of α -helices (31, 56). Additionally, they show very similar hydrophobicity profiles (Fig. 6B) and resemble each other in their instability and aliphatic indices. It seems as if three bacterial species, all faced with the challenge of overcoming the extraordinary barricade of a plant cell wall, evolved functionally and structurally similar proteins. In future work, the epitope-tagged and dominant-negative HrpE variants will be used for high-resolution structural analyses.

After completing this report, we became aware of a study from the laboratory of Sheng Yang He describing dominant-negative *Pseudomonas hrpA* mutants (34). In addition to our mutant classes, they identified mutants (class IB) which in the presence of the wild-type allele did not interfere with in vitro secretion of two tested type III effectors (AvrPto and HopPtoM) although they did not form Hrp pili. This phenotype is reminiscent of that of our *hrpE*(D53) and *hrpE*(Q56) mutants which, however, are not dominant negative. We believe that in all cases unstable Hrp pili [HrpA hybrid pili, HrpE(D53) pili, or HrpE(Q56) pili] are formed which are

stabilized within the TTS apparatus, thus allowing in vitro secretion. In contrast to the HrpA and HrpE(Q56) pili, HrpE(D53) pili appear to be slightly more stable since residual hypersensitive-response induction was observed. Alternatively, the differences could be due to the different plant assays used in the two studies.

ACKNOWLEDGMENTS

Generous support of this study by Ulla Bonas is highly appreciated. We thank Hannelore Espenhahn for excellent technical assistance and Bianca Rosinsky for greenhouse work. We are grateful to J. Boch, D. Büttner, S. Kay, D. Kühn, D. Philip, and U. Wahrmond for critical reading of the manuscript.

This work was funded by grant KO 1686/3-2 from the Deutsche Forschungsgemeinschaft (R.K.) and Landesstipendium Sachsen-Anhalt (E.W.).

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