hrpF of *Xanthomonas campestris* pv. *vesicatoria* Encodes an 87-kDa Protein with Homology to NolX of *Rhizobium fredii*

Elisabeth Huguet and Ulla Bonas

Institut des Sciences Végétales, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette, France.

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The gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* is the causal agent of bacterial spot disease on pepper and tomato plants. The main *hrp* (hypersensitive reaction and pathogenicity) gene cluster in *X. campestris* pv. *vesicatoria* spans a 23-kb chromosomal region, comprising six complementation groups designated *hrpA* to *hrpF*. Analysis of the *hrpF* locus revealed a single open reading frame encoding HrpF (86.4 kDa). HrpF is predominantly hydrophilic, and contains two hydrophobic domains in the C terminus. An interesting feature is the presence of two imperfect direct repeats in the N-terminal region. Deletion studies showed that one repeat is sufficient for function. Epitope tagging of HrpF allowed detection of the protein in *X. campestris* pv. *vesicatoria*. Subcellular localization studies suggest that HrpF is both in the soluble fraction and in the inner membrane. Interestingly, HrpF is 48% identical (67% similar) to the *Rhizobium fredii* NolX protein that is part of the host specificity locus. Since several Hrp proteins are believed to be components of the type III Hrp protein secretion apparatus, allowing export of proteins essential for the interaction with the plant, the possible role of *hrpF* and *nolX* in secretion is discussed.

Additional keywords: FLAG epitope.

The molecular mechanisms by which plant-pathogenic bacteria successfully infect plants and multiply in host tissues are still poorly understood. Certain bacteria secrete an arsenal of degradative enzymes (pectinases, cellulases, proteases), exopolysaccharides, and toxins (for review, see Beattie and Lindow 1994; Collmer and Bauer 1994; Dow and Daniels 1994) that often have an influence on the severity of disease symptoms, and act in synergy to cause disease. In addition, genes involved in basic pathogenicity, designated *hrp* genes (for hypersensitive reaction and pathogenicity), have been isolated from all major gram-negative bacteria except *Agrobacterium* (Lindgren et al. 1986; for a review, see Bonas 1994). *hrp* mutants have a pleiotropic phenotype: not only are

they unable to multiply and cause disease in a susceptible plant, but they are also unable to induce the hypersensitive reaction (HR) in resistant host or non-host plants. The HR is a rapid, local defense response, characterized by necrosis of the infected tissue (Klement 1982).

Xanthomonas campestris pv. *vesicatoria* is the causal agent of bacterial spot disease on pepper and tomato and enters the plant through natural openings (e.g., stomata) or wounds. In a susceptible plant the bacteria multiply in the intercellular space and cause water-soaked lesions, which later become necrotic. In a resistant plant, however, the HR is induced and bacterial growth is blocked (Minsavage et al. 1990; Brown et al. 1993; Stall and Cook 1979). Both types of interaction require the presence of *hrp* genes in the bacterium. Most *hrp* genes in *X. campestris* pv. *vesicatoria* have been mapped to a 23-kb chromosomal region, where they are organized in six complementation groups designated *hrpA* to *hrpF* (Bonas et al. 1991). This *hrp* gene cluster is predicted to contain 21 genes, based on sequence analysis (U. Bonas, *unpublished*; Fenselau et al. 1992; Fenselau and Bonas 1995). Expression of *hrp* genes was found to be suppressed in complex medium, but induced in planta, in tomato conditioned medium and in a synthetic medium, XVM2 (Schulte and Bonas 1992a, 1992b; Wengelnik et al. 1996a). The regulatory genes *hrpX* and *hrpG* are situated outside of the cluster (Wengelnik and Bonas 1996; Wengelnik et al. 1996b). HrpG, which encodes a response regulator of two-component systems, is at the top of the cascade regulating the expression of *hrpA* and *hrpX*, which in turn activates the *hrpB* to *hrpF* loci (Wengelnik and Bonas 1996; Wengelnik et al. 1996a, 1996b).

Clues to the biochemical function of 10 Hrp proteins (now renamed Hrc, for “common Hrp proteins”) (Bogdanove et al. 1996) encoded in the cluster were uncovered after striking sequence similarities were found to proteins from bacterial pathogens of mammals, such as *Yersinia* spp. and *Shigella flexneri* (U. Bonas, *unpublished*; Fenselau et al. 1992; Fenselau and Bonas 1995). In these organisms, the corresponding proteins are components of a type III secretion system, required for the secretion of proteinaceous pathogenicity factors (for a review, see Van Gijsegem et al. 1993). Although Hrp-dependent secretion has yet to be shown in *X. campestris* pv. *vesicatoria*, we postulate that the Hrc proteins are members of a secretion machinery that allows the secretion of pathogenicity factors and elicitors (Van den Ackerveken et al. 1996).

Corresponding author: Ulla Bonas; Tele.: 33 1 69823617; Fax: 33 1 69823695; E-mail: bonas@cactus.isv.cnrs-gif.fr

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HrpA1 (HrcC in the new nomenclature), for example, encoded at the left end of the cluster, has been shown to be an outer-membrane, possibly pore-forming protein that belongs to the PulD superfamily of proteins involved in both type II and type III secretion (Wengelnik et al. 1996a). HrpB3 (HrcJ) is predicted to be a lipoprotein, while HrpB6 (HrcN) could be the energizer of the secretion machinery as it shows homology with ATPases (Fenselau and Bonas 1995). HrpC2 (HrcV) contains eight putative transmembrane domains, and is predicted to be an inner-membrane protein (Fenselau et al. 1992). HrpD1 to HrpD3 (HrcQ, R, S) also show conservation with type III secretion systems (E. Huguët and U. Bonas, *unpublished*); however, beyond this point homology ceases. The analysis of other Hrp proteins that are not conserved in the bacterial animal pathogens will enable us to assess whether they also play a role in the formation of the secretion apparatus or whether they are secreted or act as chaperones.

The region immediately downstream of *hrpE* does not seem to contain *hrp* genes; however, Tn3-*gus* insertions revealed the presence of yet another *hrp* locus, *hrpF*, 3.6 kb downstream of *hrpE* (see Figure 1) (Bonas et al. 1991). In this paper, we report on the molecular characterization of the *hrpF* locus, and the detection and localization of its protein product.

RESULTS

DNA sequence analysis of the *hrpF* region.

The *hrpF* locus, spanning 3 kb, is located at the right border of the *hrp* cluster (Fig. 1A). The maximal extent of *hrpF* was defined by transposon Tn3-*gus* insertions no. 311 and no. 315, which were reported as having no effect on *hrp* function (Fig. 1B) (Bonas et al. 1991). Expression studies showed that *hrpF* is transcribed from left to right (Schulte and Bonas 1992a). The 5.3-kb *EcoRV* fragment containing *hrpF* was sequenced. One long open reading frame (ORF) was identified based on the CodonPreference program (Fig. 1). The first ATG codon is located at position 201 (in Figure 2); however, the ATG at position 282, which is preceded by a putative ribosome binding site, is most probably used as translation start according to primer extension data (see below). A TAA stop codon is at position 2700, thus predicting a coding sequence of 2,418 bp. Tn3-*gus* insertions no. 311 and no. 315 are inserted 94 bp upstream of the predicted translation start site, and 22 bp downstream of the stop codon, respectively. Insertions no. 440 and no. 312, which abolish *hrpF* function, interrupt the coding sequence after nucleotide positions 479 and 2138, respectively. As mutants containing transposon insertions in *hrpF* can be complemented by pLFF and pLF143F (Fig. 1), it is probable

Fig. 1. Genomic organization of the right border of the *Xanthomonas campestris* pv. *vesicatoria* *hrp* cluster. **A**, Restriction map of the region comprising *hrpE* and *hrpF*. Location and orientation of the *hrp* loci are indicated by arrows. Only relevant restriction sites are shown. **B**, Restriction map of the genomic *EcoRV* fragment in plasmid pBF, and subcloned fragments. Open box indicates the *hrpF* open reading frame. Positions and orientations of Tn3-*gus* insertions are indicated by triangles. Open triangles indicate insertions resulting in a *hrp* mutant phenotype. Filled-in triangles refer to a wild-type reaction of the insertion mutant on pepper. Insertion no. 311 has an intermediate phenotype. Positions of insertions no. 314 and no. 462 were determined by restriction analysis. pLFF contains the *hrpF* gene to which has been fused the "FLAG sequence" (indicated by a checkered box) encoding the FLAG epitope, and a stop codon, in pLAFR3. pLF143 contains a translational fusion of *hrpF* to β -galactosidase that is under the control of the *lacZ* promoter in pLAFR3. pLF143F is derived from pLF143 and contains the FLAG at the 3' end. B: *Bam*HI; C: *Cla*I; E: *Eco*RI; V: *Eco*RV; H: *Hind*III; S: *Sac*I; Sp: *Sph*I.

that *hrpF* is the only *hrp* gene present in this locus. Furthermore, *X. campestris* pv. *vesicatoria* 85-10 strains carrying Tn3-*gus* insertions no. 462 or no. 534, which have been mapped to the region downstream of *hrpF* (no. 534 is situated 1,106 bp from the *hrpF* stop codon) have a wild-type phenotype, suggesting that no *hrp* locus is present immediately downstream of *hrpF*.

The *hrpF* locus is separated from the *hrpE* operon by a 3.6-kb region in which Tn3-*gus* insertions had no detectable phenotypic effect (Bonas et al. 1991). To test whether this region contains *hrp* genes, a deletion was created by replacing the 2.9-kb *Clal* fragment by the omega cassette, which confers resistance to spectinomycin and which contains translation stop codons in all three reading frames (Prentki and Krisch 1984) (see Figure 1A). This deletion was introduced into the *X. campestris* pv. *vesicatoria* wild-type strain 85-10 by marker

exchange mutagenesis. The resulting mutant, 85-10Δ2, was able to cause disease on pepper ECW and to induce the HR on the resistant pepper line ECW-10R, in a similar way as strain 85-10, indicating that no *hrp* genes are present between *hrpE* and *hrpF*.

Features of the HrpF protein.

The HrpF protein is predicted to be 806 amino acids (aa) long, and to have a molecular mass of 86.4 kDa. HrpF is predominantly hydrophilic, lacks a cleavable signal peptide, and contains two hydrophobic domains (H1 and H2) in its C-terminal region (Fig. 3). These domains contain, respectively, 26 aa and 22 aa with a majority of hydrophobic residues, and a maximum of two charged residues per domain. According to the Chou-Fasman and Garnier-Osguthorpe-Robson predictions on secondary structure, 16 out of 26 aa

Fig. 2. Nucleotide sequence of the promoter region and the first 1,345 nucleotides of the *hrpF* open reading frame. Sequence from the first nucleotide of deletion clone pSF53 to the *EcoRI* site is shown. Thick arrows indicate the beginning of deletion derivatives obtained from pBF: “53,” “49,” and “143.” The small arrow (+1) indicates the transcription start site; SD (Shine Dalgarno) refers to a possible ribosome binding site. Putative ATG or GTG start codons are shaded in gray. Oligonucleotides 109 and 138 were used to determine the transcription initiation start site in primer extension experiments. Position of Tn3-*gus* insertion no. 311, which has an intermediate phenotype, is indicated by a shaded triangle. An imperfect PIP-box is underlined. Amino acid (aa) sequence of the deduced translation product is given in single letter code below the nucleotide sequence. Large arrows refer to the first nucleotide of deletion subclones. Boxed sequences represent two 110-aa imperfect direct repeats R1, and R2. Nucleotide sequences in bold refer to the small 25-bp imperfect direct repeat flanking R1 and R2. The *BstEII* site indicated and a new *BstEII* site created in position 656 were used to delete the end of R1 and the beginning of R2.

direct repeat of 25 bp (68% identical) is flanking these longer direct repeats (Fig. 2).

HrpF shares homology with the NolX protein of *Rhizobium fredii*.

The amino acid sequence of HrpF was compared with sequences in the data bases and was found to be overall 48% identical (67% similar) to NolX from *R. fredii* (Meinhardt et al. 1993). When the HrpF protein from the second repeat onward is compared with NolX, the proteins are 51% identical, 69% similar (Fig. 4). NolX is a 596 aa protein with a predicted molecular mass of 64.1 kDa (Kovacs et al. 1995). The N termini of the two proteins (i.e., the first 100 aa), are less well conserved (29% identity, 44% similarity). Interestingly, the NolX protein also contains 2 hydrophobic domains in the C terminus, but has only one copy of the 110-aa HrpF direct repeat (Fig. 3). This sequence motif of the NolX protein is most closely related to R1 in HrpF (66% identity, 88% similarity).

Mapping of the *hrpF* promoter region.

In order to define the *hrpF* promoter, primer extension analysis was performed. RNA was isolated from *X. campestris* pv. *vesicatoria* cells grown either in NYG or in *hrp*-gene-inducing medium XVM2 (Wengelnik et al. 1996a), or for 3 days in pepper ECW. To increase the level of *hrpF*-specific transcripts strain 85E(pXV74) was used. This strain carries two to three additional copies of the entire *hrp* cluster (Wengelnik et al. 1996a). A strong signal was obtained with oligonucleotide 109 (refer to Figure 2 for position), with RNA isolated from bacteria grown in XVM2 (Fig. 5) and in planta

Fig. 3. Schematic representation of HrpF, and NolX from *Rhizobium fredii*. HrpF and NolX are 48% identical (67% similar). Both proteins contain two hydrophobic domains in their C terminus (H1 and H2). R1 and R2 (shaded boxes) are imperfect direct repeats of approximately 100 amino acids, in the N-terminal region of HrpF. NolX contains only one such motif (R). R1 and R2 are 70% identical (81% similar), while R1 and R are 66% identical (80% similar), and R2 and R are 62% identical (79% similar).

Fig. 4. Amino acid sequence comparison between HrpF and NolX. HrpF of *Xanthomonas campestris* pv. *vesicatoria* (from second repeat to end of protein) was compared with entire NolX protein of *Rhizobium fredii* with the GAP program. Proteins share 51% identity (69% similarity).

Fig. 5. Mapping of transcription start site of *hrpF* by primer extension analysis. RNA was extracted from strain 85E(pXV74) grown in XVM2 (lane 1) or NYG (lane 2), and used as template for reverse transcriptase with oligonucleotide 109. Sequence is the reverse complement of the coding strand and was obtained with plasmid pBF as template and oligonucleotide 109 as primer. Boxed nucleotide corresponds to the transcription start, indicated by an arrow.

(data not shown). An identical but very weak signal was obtained with RNA from NYG-grown bacteria (not visible in Figure 5). Similar results were obtained with oligonucleotide 138 (data not shown). Mapping of this signal indicated that transcription of *hrpF* starts at the thymidine nucleotide at position 193 in Figure 2. A second weaker signal (Fig. 5) maps to position 195 (Fig. 2).

To better determine the minimal region required for *hrpF* function, deletion clones generated for sequence analysis were subcloned into the broad-host-range vector pLAFR6, which contains a multiple cloning site flanked by transcription termination signals. Plasmids pSF53 and pSF49, containing sequences 192 and 136 bp upstream of the major transcription site, respectively (Fig. 2), were able to complement the Tn3-*gus* insertion strains 85-10::*hrpF*314, and 85-10::*hrpF*440. This result indicates that a region of 136 bp upstream of the transcription start site contains a functional *hrpF* promoter. Plasmids pSF143 and pSF136, with inserts starting 28 and 445 bp, respectively, downstream of the putative translation start site (Fig. 2), could not complement the *hrpF* mutants.

Inspection of the *hrpF* promoter did not reveal any homology to the *E. coli* canonical sequence elements of sigma 70 transcription factor regulated promoters. No plant-inducible promoter (PIP) box was identified in the region upstream of the *hrpF* transcription start site, contrary to what has previously been reported (Fenselau and Bonas 1995). The PIP-box is a sequence motif that contains two direct repeats with the exact spacing of 15 nucleotides. The PIP-box is present in the promoters of *hrpB*, *hrpC*, and *hrpD* (Fenselau and Bonas 1995) and is thought to be involved in *hrp*-gene activation by the positive regulator *hrpXv* (Wengelnik and Bonas 1996). A sequence resembling a PIP-box is present 56 bp upstream of the *hrpF* transcription start site: TTCGC-N8-TTCGT (see Figure 2). As *hrpF* is also dependent on *hrpXv* for activation of transcription, this could indicate that this imperfect PIP-box is sufficient, or that other as yet unidentified sequences are also necessary for activation by *hrpXv*.

The Tn3-*gus* insertion no. 311 results in an intermediate phenotype on plants.

The results of the promoter mapping were in contradiction to the phenotype observed with insertion mutant 85-10::311. In this mutant the transposon is inserted 6 bp upstream of the *hrpF* transcription start site (Fig. 2), but has previously been reported to have a *hrp*⁺ phenotype (Bonas et al. 1991). Sequencing of the genomic mutant confirmed the position of the transposon insertion. Plant inoculations were repeated with 85-10::311, at 10⁸ CFU/ml, and showed that in fact this strain induced only a partial HR (60 to 70% of the inoculated area) on the resistant line ECW-10R; this reaction was delayed by 1 day compared with the wild-type strain. Water-soaking symptoms on the susceptible pepper line ECW were also partial with strain 85-10::311, but the strain did grow to levels comparable to those of the wild-type strain 85-10. One possible explanation why insertion no. 311 does not yield a true *hrp* phenotype is that there is the accidental creation of promoter sequences at the transposon-target junction. Similar findings have been reported in *R. fredii*, in which three mini-*Mu* insertions did not give the expected phenotype (Meinhardt et al. 1993). However, primer extension analysis on RNA from strain 85-10::311, using oligonucleotides 109 and 138, gave

no signals, ruling out this possibility. A second possibility is the presence of an alternative promoter further downstream with a different transcription start site, which leads to the production of a smaller, less active HrpF protein. In *E. coli*, for example, the *glmU* gene that encodes a protein necessary for the formation of UDP-N-acetyl-D-glucosamine is expressed from two independent promoters that have two different transcription start sites (Plumbridge 1995). Since plasmid pSF143, containing an insert starting 28 bp downstream of the translation start site, is unable to complement the *hrpF* mutant 85-10::*hrpF*314, the alternative promoter is predicted to reside in the region between position 187 and 310 (Fig. 2).

Deletion of one 110-aa repeat does not abolish *hrpF* function.

The presence of two direct repeats in the N-terminal region of HrpF and the fact that NolX contains only one copy could indicate that one repeat is sufficient for *hrpF* function. To test this idea, a 354-bp fragment from the beginning of R1 to the beginning of R2 was deleted (pSΔ49) (see Materials and Methods). Transconjugants of the mutants 85-10::*hrpF*314 and 85-10::*hrpF*440 containing pSΔ49 induced water soaking on ECW and the HR on ECW-10R in the same fashion as the wild-type strain 85-10, indicating that one repeat is sufficient for *hrpF* function.

Epitope tagging and detection of the HrpF protein.

To study protein expression, a small oligonucleotide encoding the 8-aa FLAG epitope was fused in-frame to the 3' end of the *hrpF* coding sequence. Two *hrpF*-FLAG constructs were made: pLFF, in which the expression of the *hrpF*-FLAG gene is under the control of its own promoter in pLAFR3; and pLF143F, in which *hrpF* lacks the first 13 aa and is translationally fused to the N-terminal 17 aa of β-galactosidase, which is expressed from the *lacZ* promoter (Fig. 1). Both constructs were able to complement the *hrpF* insertion mutants 85-10::*hrpF*314 and 85-10::*hrpF*440, indicating that the introduction of the FLAG epitope at the C terminus and modifications at the N terminus did not interfere with the function of the HrpF protein.

Western blot (immunoblot) analysis with the anti-FLAG M2 monoclonal antibody revealed the presence of a 100-kDa protein in *E. coli* and *X. campestris* pv. *vesicatoria* strains harboring pLF143F (Fig. 6, lanes 2 and 4). Only a very weak signal was visible when *hrpF* was expressed from its own promoter (data not shown). The 100-kDa protein was absent in extracts from bacteria not containing the *hrpF*-FLAG construct (Fig. 6, lanes 1 and 3), indicating that the protein seen in lanes 2 and 4 is HrpF (Fig. 6). A smaller protein of ca. 60 kDa detected by the antibody in total protein extracts of *X. campestris* pv. *vesicatoria* is independent of the presence of the HrpF-FLAG protein.

Cross-reaction of HrpF with anti-NolX antibody.

Polyclonal anti-NolX antibody was tested for cross-reactivity with HrpF. As shown in Figure 6, lane 6, the anti-NolX antibody recognizes a 100-kDa protein in *E. coli* containing pLF143F, which expresses *hrpF*-FLAG. This signal is absent in DH5α containing the vector alone, indicating that the 100-kDa protein corresponds to HrpF (Fig. 6, lane 5). In total protein extract from *E. coli* harboring pDRfX, a clone

expressing *nolX* under the control of *placZ*, the antibody revealed the presence of a ca. 66-kDa protein that corresponds to the predicted size of NolX (Kovacs et al. 1995) (Fig. 6, lane 8).

To test whether the *nolX* gene from *R. fredii* could complement a *X. campestris* pv. *vesicatoria* *hrpF* mutant, pDRfX (plasmid expressing *nolX* under the control of *placZ*), was introduced into 85-10::*hrpF*314. Transconjugants were unable to cause water soaking on ECW and to induce the HR on ECW-10R, although Western blot analysis clearly showed the presence of the expected NolX protein in the transconjugants. When introduced into *R. fredii*, pDRfX also expressed NolX (S. Pueppke and H. Krishnan, *personal communication*). However, because NolX is a negatively acting host-range determinant the plasmid is unstable, making it difficult to test for functionality of the protein. Therefore, although *X. campestris* pv. *vesicatoria* transconjugants harboring pDRfX are capable of expressing the NolX protein, heterologous complementation of a *hrpF* mutant does not seem to be possible with NolX, indicating that the proteins are not functionally interchangeable.

Localization of the HrpF protein.

To study the subcellular localization of HrpF in *X. campestris* pv. *vesicatoria*, total protein extracts of *hrp*-induced bacteria were used for cell fractionation experiments and analyzed by Western blotting. As HrpF could not be detected in strains expressing the *hrpF*-FLAG gene from its own promoter, the overexpressing, and phenotypically wild-type, strain 85-10::*hrpF*314 (pLF143F) was used. HrpF was mainly found in the soluble fraction, but also in the membrane fraction (Fig. 7). Total membranes were further fractionated with

a sucrose step gradient. Fractions were analyzed for their content in protein and sucrose, and for NADH oxidase activity and the presence of OmpA protein as markers for inner and outer membranes, respectively. Most NADH oxidase activity was found in fractions 3, 4, 5, and 6. The OmpA protein of *X. campestris* pv. *vesicatoria* was detected with an antibody specific for the *E. coli* OmpA protein. A 34-kDa protein that we assume corresponds to the OmpA protein of *X. campestris* pv. *vesicatoria* (Wengelnik et al. 1996a) was detected in fractions 9, 10, and 11. The distribution of the NADH oxidase activity and OmpA indicated a good separation of inner and outer membranes. Figure 7 (lanes 3, 4, 5, and 6) shows that HrpF localized to the inner membrane. It was estimated by a series of dilutions and comparison of signal strength that 60 to 70% of the total amount of HrpF in *X. campestris* pv. *vesicatoria* localized to the soluble fraction; the remaining 30 to 40% co-fractionated with the inner membrane.

To study the nature of the interaction between HrpF and the inner membrane, total membranes were treated either with 1 M NaCl, 6 M urea, 1% Triton X-100, or with buffer only. Treatment of membranes with 1 M NaCl or 6 M urea strips off peripheral membrane proteins by disrupting ionic interactions involved in membrane associations (Finberg et al. 1995; Penefsky and Tzagoloff 1971). Treatment with 1% Triton X-100 solubilizes membranes, and results in the release of most integral membrane proteins (Schnaitman 1971). Results showed that HrpF could only be partially removed from the inner membrane after treatment with NaCl and urea, but was totally released after treatment with Triton X-100 (data not shown). These results could suggest that HrpF is an integral inner membrane protein. Identical HrpF localization was obtained with an *hrpG* mutant strain containing pLF143F. HrpF localization is therefore not affected by other Hrp proteins that are absent in the *hrpG* mutant strain.

Fig. 6. Detection of the HrpF protein. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% polyacrylamide), and transferred to nitrocellulose. Blot was reacted with anti-FLAG monoclonal antibody (lanes 1 to 4), or with anti-NolX polyclonal antibody (lanes 5 to 8). Bound antibody was visualized with secondary anti-mouse or anti-rabbit antibodies conjugated to alkaline phosphatase. Total extracts of *Escherichia coli* DH5 α lane 1 (pLF143); lanes 2 and 6 (pLF143F); lane 5 (pLAFR3); lane 7 (pDSK600); lane 8 (pDRfX); grown in Luria-Bertani, and induced for 3 h with 1 mM IPTG (isopropyl- β -thiogalactopyranoside). Total extracts of *Xanthomonas campestris* pv. *vesicatoria* 85-10 lane 3 (pLF143); lane 4 (pLF143F), grown in XVM2.

DISCUSSION

The *hrpF* gene, located at the right border of the *X. campestris* pv. *vesicatoria* 23-kb *hrp* cluster, encodes the largest Hrp protein in this cluster. *hrpF* is in a unique position regarding other *hrp* clusters both in a literal and figurative sense. First, the *hrpF* gene is separated from the other *hrp* loci in the cluster by a 3.6-kb region that lacks *hrp* genes. In other plant-pathogenic bacteria the *hrp* cluster is flanked by avirulence (*avr*) genes, as in the case of *Pseudomonas syringae* (Lorang and Keen 1995; Mansfield et al. 1994), genes encoding non-specific elicitors such as PopA in *Ralstonia solanacearum* (Arlat et al. 1994), or genes involved in pathogenicity such as the *dsp* region in *Erwinia* (Barny et al. 1990). Secondly, analysis of the sequence databases revealed that so far no HrpF homologues have been identified in the other gram-negative phytopathogenic bacteria, or in the mammalian bacterial pathogens that contain conserved components of type III secretion systems (for a review, see Van Gijsegem et al. 1993). The only *hrp* gene to which *hrpF* might be slightly related is *hrpY* of *P. syringae* pv. *phaseolicola* (Mansfield et al. 1994), which is predicted to encode a predominantly hydrophilic protein of 80 kDa with a hydrophobic domain at the C terminus. Sequence comparison, however, revealed only 18% identity and 41% similarity between HrpF and HrpY, which is at the limit of statistical significance. Interestingly, HrpF

shares high similarity to the nodulation protein NolX from *R. fredii* (Meinhardt et al. 1993) (see below).

Epitope tagging of HrpF allowed detection and subcellular localization of the protein. Sixty to 70% of the total amount of the HrpF protein was found in the soluble fraction, while 30 to 40% co-fractionated with the inner membrane. Since overexpression of *hrpF* was necessary to obtain clear signals in Western blot analysis we tried to determine whether the membrane localization is an artifact. The finding that HrpF could only be partially solubilized from the membrane fraction by treatment with urea and NaCl and could be totally released from the inner membrane by Triton X-100 suggests that part of the protein is embedded in the lipid bilayer and that this association might be genuine. Similar findings were reported for the SecA protein, which has been described as being a "dynamic" constituent of the *E. coli* sec transport system since cytoplasmic, peripheral, and integral membrane forms of the protein have been detected (Cabelli et al. 1991; Kim et al. 1994). The membrane-bound SecA can be only partially extracted by urea, alkali, or high salt (Cabelli et al. 1991). To test whether the localization of HrpF in *X. campestris* pv. *vesicatoria* in the inner membrane could be due to interaction with other membrane-localized Hrp proteins, pLF143F was introduced into a *hrpG* mutant. This mutant no longer expresses the *hrp* cluster (Wengelnik et al. 1996b). Since no differences in localization of HrpF were observed, compared with the wild type, HrpF does not depend on other Hrp proteins identified so far for its association with the inner membrane. A clearer answer to the localization of HrpF will be

possible when a more sensitive detection assay is available so that a *hrpF* construct that expresses the protein from the native promoter can be used. Earlier data had suggested that the *hrpF* promoter had a relatively strong activity (Schulte and Bonas 1992a); however, as only small amounts of protein could be detected, this may indicate that *hrpF* is subject to post-transcriptional regulation, or that the protein has a high turn-over.

The only hint to the possible biochemical function of HrpF is its homology to the *R. fredii* NolX protein. The fact that the HrpF and NolX proteins are highly conserved is interesting, considering that the bacteria are not closely related and, at first glance, seem to have quite different effects on their host plant: *Rhizobium* establishes a symbiosis that will benefit both partners, while *Xanthomonas* multiplies at the host's expense. *nolX* belongs to a cluster of genes (the *nolXWBTUV* locus) in *R. fredii* that is involved in host specificity. A mutation in any one of the so-far-identified seven ORFs in this "cultivar specificity locus" leads to host-range extension of strain USDA257 to a genetically improved soybean cultivar that cannot be nodulated by the wild type (Meinhardt et al. 1993). Two additional Nol proteins encoded in this cluster, NolW and NolT (Meinhardt et al. 1993), also show homology to *Xanthomonas* Hrp proteins: HrpA (HrcC) and HrpB3 (HrcJ), respectively, which are thought to be components of the Hrp type III secretion system (Fenselau et al. 1992; Fenselau and Bonas 1995; Wengelnik et al. 1996a). Interestingly, *R. fredii* secretes a set of five proteins (designated Signal Responsive, SR) in response to certain flavonoids that are involved in the regulation

Fig. 7. Localization of the HrpF protein in *Xanthomonas campestris* pv. *vesicatoria*. Protein extracts from *X. campestris* pv. *vesicatoria* strain 85-10::hrpF314(pLF143F) grown in XVM2 were fractionated by a sucrose step gradient as previously described (Wengelnik et al. 1996a). Results shown are taken from a typical fractionation experiment. **A**, Proteins corresponding to total extracts (TE), total soluble (TS), total membranes (TM), and fractions 2 to 11 collected from a sucrose step gradient were separated on 7% polyacrylamide gels, transferred to nitrocellulose, and reacted with anti-FLAG monoclonal antibody. For TE, TS, and TM the amount of proteins loaded corresponds to equal amounts of cells. Three micrograms of protein of each fraction was loaded. **B**, Fractions 2 to 11 were separated on a 10% polyacrylamide gel, transferred to nitrocellulose, and reacted with anti-OmpA antibody. The OmpA protein is indicated by an arrow. **C**, Sucrose concentrations were measured by a refractometer (—). Protein concentrations were determined by the Bradford assay (——). NADH oxidase activity was measured in fractions 2 to 11 (---).

of the *noIXWBTUV* locus. Furthermore, preliminary data on SR3 indicate that it does not seem to have undergone N-terminal processing (Krishnan and Pueppke 1993). The cultivar specificity locus in *R. fredii* could therefore encode a type III secretion apparatus through which host range restricting proteins are transported. This is reminiscent of the proposed transport of avirulence (Avr) proteins via the Hrp secretion system (see below). In plant-pathogenic bacteria the presence of *avr* genes determines the “recognition” of the bacteria by a plant harboring the corresponding resistance gene. This recognition then leads to activation of plant defense reactions and restriction of pathogen growth (for review, see Leach and White 1996)

Is HrpF a basic constituent of the *Xanthomonas* Hrp secretion apparatus, and what is the link to recognition of avirulent bacteria by resistant plants? Recently, it has emerged that several Avr proteins from pathovars of *P. syringae* (AvrB, AvrPto, AvrRpt2) and from *Xanthomonas* (AvrBs3) are recognized inside the plant cell (Gopalan et al. 1996; Leister et al. 1996; Scofield et al. 1996; Tang et al. 1996; Van den Ackerveken et al. 1996). Although *hrp* genes are not required for the recognition of these Avr proteins once inside the host cell, they are

essential for the recognition of the bacterium by the resistant plant. We and others therefore propose that the Hrp proteins form a translocation apparatus allowing delivery of bacterial proteins (e.g., Avr proteins) into the plant cell. As the HrpF protein is clearly needed for AvrBs3 recognition (Knoop et al. 1991), and it is partially localized in the inner membrane, we propose that HrpF is a component of the Hrp translocation apparatus.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

The bacterial strains and plasmids used are described in Table 1. Plasmids were introduced into *Escherichia coli* by electroporation and into *Xanthomonas* by conjugation, using pRK2013 as a helper plasmid in triparental matings (Ditta et al. 1980; Figurski and Helinski 1979). *E. coli* cells were cultivated at 37°C in Luria-Bertani medium and *Xanthomonas* strains at 28°C in NYG broth (Daniels et al. 1984) or on NYG 1.5% agar, or in XVM2 medium (Wengelnik et al. 1996a). Antibiotics were added to the media at the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 25 µg/ml;

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>		
85-10	Pepper race 2; wild type; Rif ^r	Bonas et al. 1989
85E	<i>gumG</i> -Tn3- <i>gus</i> insertion mutant of 85-10 <i>hrp</i> ⁺ , EPS ⁻ ; Rif ^r , Km ^r	Wengelnik et al. 1996a
85-10::311; 315; 534; 462	Tn3- <i>gus</i> insertion mutant of 85-10; Rif ^r , Km ^r	Schulte and Bonas 1992b; Wengelnik and Bonas 1996
85-10:: <i>hrpF</i> 312; <i>hrpF</i> 314; <i>hrpF</i> 440	Tn3- <i>gus</i> insertion mutant of 85-10; <i>hrpF</i> ; Rif ^r , Km ^r	
85-10Δ2	85-10 derivative: a 2.9-kb <i>Cla</i> I fragment between <i>hrpE</i> and <i>hrpF</i> was replaced by the omega cassette; Rif ^r , Spec ^r	This study
<i>Escherichia coli</i>		
DH5α	F ⁻ <i>recA</i> φ80 <i>dlacZ</i> Δ <i>M15</i>	BRL, Bethesda, MD
Plasmids		
pBF, pBFI	5.3-kb <i>Eco</i> RV fragment from pXV2 in pBluescript in both orientations; contains <i>hrpF</i> ; Ap ^r	This study
pBluescript-KS II	phagemid, pUC-derivative; Ap ^r	Stratagene, La Jolla, CA
pDSK600	Contains triple <i>lacZ</i> UV5 promoter; Spec ^r , Sm ^r	Murillo et al. 1994
pDRfX	3.4-kb <i>Nhe</i> I- <i>Eco</i> RI fragment from pRfDH412 in pDSK600; contains <i>noIX</i> under the control of triple <i>lacZ</i> UV5 promoter; Spec ^r , Sm ^r	This study
pLAFR3	RK2 replicon Mob ⁺ Tra ⁻ ; Tc ^r ; contains <i>placZ</i>	Staskawicz et al. 1987
pLAFR6	pLAFR3 derivative; multiple cloning site flanked by transcriptional terminators; Tc ^r	Bonas et al. 1989
pLFF	3.3-kb <i>Sp</i> HI- <i>Hind</i> III fragment in pLAFR3; contains flagged <i>hrpF</i> under the control of its own promoter	This study
pLF143	4-kb deletion derivative from pBF in pLAFR3; lacks first 28 nucleotides of <i>hrpF</i> coding sequence, translational fusion with β-galactosidase (17 amino acids)	This study
pLF143F	Derivative from pLF143; contains flagged <i>hrpF</i>	This study
pRfDH412	8-kb <i>Eco</i> RI fragment containing <i>noIV</i> - <i>noIX</i> in pGEM-7Zf(-)	Meinhardt et al. 1993
pRK2013	ColE1 replicon TraRK ⁺ Mob ⁺ ; Km ^r	Figurski and Helinski 1979
pSF49; pSF53	4.38-kb and 4.33-kb deletion derivatives from pBF in pLAFR6	This study
pSΔ49	3.9-kb fragment from pS49 in pLAFR6; contains <i>hrpF</i> with only one repeat	This study
pUC118	ColE1 replicon; Ap ^r	Vieira and Messing 1987
pXV74	pLAFR3 clone from <i>X. campestris</i> pv. <i>vesicatoria</i> 75-3 genomic library, contains <i>hrpA</i> to <i>hrpF</i>	Wengelnik et al. 1996a

^a Ap: ampicillin; Km: kanamycin; Rif: rifampicin; Sm: spectinomycin; Sp: streptomycin; Tc: tetracycline; r: resistant.

tetracycline, 10 µg/ml; rifampicin, 100 µg/ml; spectinomycin, 100 µg/ml.

Plant material and plant inoculations.

Inoculation of the near-isogenic pepper lines ECW and ECW-10R and reisolation of bacteria from plant tissue 3 days after inoculation were performed as described (Bonas et al. 1991).

Sequence analysis.

For sequence analysis, the 5.3-kb *EcoRV* fragment spanning the *hrpF* locus was subcloned in both orientations into pBlue-scriptKSII, giving pBF and pBFI. Standard molecular techniques were used (Sambrook et al. 1989). A series of nested deletions was generated with DNaseI as previously described (Bonas et al. 1989). The DNA sequence of both strands of overlapping deletion subclones was determined with commercial primers (Stratagene, La Jolla, CA) or custom primers, and the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden). Tn3-*gus* insertion sites in pXV2 and pXV4 were sequenced with an oligonucleotide complementary to a sequence in the N terminus of the β-glucuronidase gene (5' GATTCACGGGTTGGGG 3'). Sequence data were analyzed with the University of Wisconsin GCG package (Version 8.0; 20), and TBLAST (Altschul et al. 1990). For sequence analysis of the genomic Tn3-*gus* insertion no. 311, total genomic DNA of 85-10::311 was digested with *EcoRI*, and fragments were ligated into pBluescriptKSII. Transformants were selected on ampicillin, and kanamycin containing medium, thereby screening for clones containing part of the transposon insertion, and the genomic target site. Sequence analysis was performed with oligonucleotide 138 (see Figure 2).

DNA constructs.

Deletion mutant 85Δ2 was constructed as follows. pU76 is a pUC118 derivative that contains a 7.6-kb *Bam*HI fragment (encompassing *hrpE* to *hrpF*) (Fig. 1). A partial *Cla*I digestion of pU76 deleted the 2.9-kb *Cla*I fragment, which was replaced by the omega cassette conferring spectinomycin resistance (Prentki and Krisch 1984), giving pUΔ2. The 4.6-kb fragment from pUΔ2 was cloned into pLAFR6, giving pSΔ2, and introduced into *X. campestris* pv. *vesicatoria* strain 85-10 to generate the mutant 85Δ2 by marker gene exchange. To generate the internal *hrpF* deletion pSΔ49, a *Bst*EII site was introduced, for technical reasons, by polymerase chain reaction (PCR) mutagenesis in the *hrpF* deletion subclone pBF49 at position 656 (GGTGACT became GGTAACC) (see Figure 2). By *Bst*EII digestion of pBF49 and religation, 354 bp were deleted, generating pBΔ49. pBΔ49 therefore contains *hrpF* with only one repeat (consisting of the beginning of R1, fused to the end of R2). The 4-kb *Xba*I-*Hind*III fragment from pBΔ49 was cloned into the broad-host-range vector pLAFR6, giving pSΔ49. In order to express *nolX* of *R. fredii* under the control of *placZ*, which is constitutive in *X. campestris* pv. *vesicatoria*, a 3.4-kb *Nhe*I-*Eco*RI fragment from the *nolX* containing plasmid pRfDH412 (Meinhardt et al. 1993) was cloned into the *Xba*I and *Eco*RI sites of the broad-host-range vector pDSK600, generating pDRfX. The *Nhe*I site is situated 123 bp upstream of the ATG start site, and 56 bp downstream of the RNA transcription start site (Kovacs et al. 1995).

Primer extension analysis.

Bacteria were grown for 16 h in NYG or XVM2 or recovered from susceptible pepper plants 3 days after whole plant infiltration. Bacterial RNA was extracted with hot phenol as described (Aiba et al. 1981). The following oligonucleotides were used (positions refer to Figure 2). #109: (5'-GGACGAA TCCGACCCGAGCAGC-3', position 362); #138: (5'-GGGG TACCAGCAGCAGGTAGATTGCTGAAAGATG-3', position 419); oligonucleotide 138 has 8 non-matching nucleotides in its 5' end for technical purposes. Primer extension was done as described (Ausubel et al. 1996).

Epitope tagging of the HrpF protein.

PCR was performed on a 950-bp *Sac*I fragment containing the 3' end of *hrpF*, and 185 bp downstream, in order to introduce the nucleotide sequence encoding the FLAG epitope (IBI/Kodak, New Haven, CT). Primers used were the T3 universal primer, and the FLAG primer (5'CCCAAGC TTCCTTGTGTCATCGTCGTCCTTGTAGTCCGATCTGCGA CGGATCCGG-3'). The FLAG primer contains 16 nucleotides that correspond to the 3' end of the *hrpF* gene, the remaining sequence encodes the FLAG peptide (DYKDDDDK), and contains a stop codon, followed by a *Hind*III site to facilitate further cloning. Template DNA was denatured for 3 min at 96°C, followed by 30 cycles of 15 s denaturation at 96°C, 30 s annealing at 40°C, and 1 min 30 s extension at 72°C; a final extension of 2 min at 72°C terminated the reaction. The PCR reaction generated a 936-bp fragment encoding the end of the HrpF protein with the FLAG epitope. The very end of *hrpF* with the FLAG was sequenced to ensure that the fusion was correct, and introduced into the *hrpF* gene cloned behind its own promoter in pLAFR3 (pLFF), and into the *hrpF* gene under the control of the *lacZ* promoter (pLF143F) (see Figure 1).

Protein expression in *E. coli* and in

X. campestris pv. *vesicatoria* and Western blot analysis.

pDRfX, pLFF, and pLF143F were introduced into *E. coli* DH5α and into *X. campestris* pv. *vesicatoria* 85-10::*hrpF*314 and 85-10::*hrpF*440. DH5α early log cultures containing pDRfX or pLF143F were induced for 1 or 3 h with IPTG (isopropyl-β-thiogalactopyranoside, 1 mM) at 37°C. *X. campestris* pv. *vesicatoria* strains harboring these constructs were grown for 16 h either in NYG or XVM2, at 28°C. Cells were harvested, and resuspended in 1× Laemmli buffer (Laemmli 1970). Western blot analysis was carried out as previously described (Wengelnik et al. 1996a); blots were reacted either with anti-NolX polyclonal antibody (kindly provided by S. Pueppke, University of Missouri, Columbia) or with anti-FLAG M2 monoclonal antibody according to the manufacturer's instructions (IBI FLAG Epitope/Kodak).

Subcellular fractionation and solubilization experiments.

Subcellular fractionation and marker protein determination were carried out as previously described (Wengelnik et al. 1996a). Membrane solubilization experiments were performed as described (Finberg et al. 1995). Total *X. campestris* pv. *vesicatoria* cell extracts were separated into soluble and insoluble material in the presence of 5 mM EDTA by centrifugation at 300,000 × g for 3 to 4 h at 4°C. Total membrane pellets obtained were extracted with 5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH

7.4), containing 20% sucrose, 0.2 M KCl, and either 1% TX-100, 1 M NaCl, or 6 M urea for 30 min at room temperature (urea), 30°C (TX-100), or on ice (buffer alone, or NaCl). Following the extraction, samples were centrifuged for 1 h at $130,000 \times g$ (4°C). Proteins in the soluble fraction were precipitated with 80% ethanol and pellets were resuspended in 1× Laemmli buffer.

Accession number.

The nucleotide sequence of the 3.8-kb *EcoRV-SphI* fragment containing *hrpF* can be retrieved from GenBank (accession no. U79116). Position 1 in Figure 2 corresponds to position 1070 in the sequence deposited.

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