Characterization of the *hrpF* Pathogenicity Peninsula of *Xanthomonas oryzae* pv. *oryzae*

Akiko Sugio, Bing Yang, and Frank F. White

Department of Plant Pathology, Throckmorton Hall, Kansas State University, Manhattan 66506-5502, U.S.A.

Submitted 10 December 2004. Accepted 14 February 2005.

The *hrp* gene cluster of *Xanthomonas* spp. contains genes for the assembly and function of a type III secretion system (TTSS). The *hrpF* genes reside in a region between *hpaB* and the right end of the *hrp* cluster. The region of the *hrpF* gene of *Xanthomonas oryzae* pv. *oryzae* is bounded by two IS elements and also contains a homolog of *hpaF* of *X. campestris* pv. *vesicatoria* and two newly identified genes, *hpa3* and *hpa4*. A comparison of the *hrp* gene clusters of different species of *Xanthomonas* revealed that the *hrpF* region is a constant yet more variable peninsula of the *hrp* pathogenicity island. Mutations in *hpaF*, *hpa3*, and *hpa4* had no effect on virulence, whereas *hrpF* mutants were severely reduced in virulence on susceptible rice cultivars. The *hrpF* genes from *X. campestris* pv. *vesicatoria*, *X. campestris* pv. *campestris*, and *X. axonopodis* pv. *citri* each were capable of restoring virulence to the *hrpF* mutant of *X. oryzae* pv. *oryzae*. Correspondingly, none of the *Xanthomonas* pathovars with *hrpF* from *X. oryzae* pv. *oryzae* elicited a hypersensitive reaction in their respective hosts. Therefore, no evidence was found for *hrpF* as a host-specialization factor. In contrast to the loss of *Bs3*-dependent reactions by *hrpF* mutants of *X. campestris* pv. *vesicatoria*, *hrpF* mutants of *X. oryzae* pv. *oryzae* with either *avrXa10* or *avrXa7* elicited hypersensitive reactions in rice cultivars with the corresponding *R* genes. A double *hrpF**xoo*-*hpa1* mutant also elicited an *Xa10*-dependent resistance reaction. Thus, loss of *hrpF*, *hpa1*, or both may reduce delivery or effectiveness of type III effectors. However, the mutations did not completely prevent the delivery of effectors from *X. oryzae* pv. *oryzae* into the host cells.

Many pathogenic species of gram-negative bacteria have type III secretion systems (TTSS) that are required for disease in their specific hosts. Hypersensitive reaction and pathogenicity (*hrp*) proteins are the main components of the TTSS in plant-pathogenic bacteria (Lindgren 1986) and are required for delivery of the secreted substrate proteins, which consist of virulence effectors and the so-called avirulence (*Avr*) proteins. Many TTSS effectors and *Avr* proteins enter the host cells via an unknown mechanism after delivery by the TTSS pathway (Buttner and Bonas 2002a). In the case of *Avr* proteins, internalization triggers a hypersensitive reaction (HR) in plants carrying corresponding resistance (*R*) genes. The HR is a localized plant cell death and part of the plant defense mechanism for prevention of further pathogen ingress. A set of nine *hrp* genes, which encode structural components of the TTSS, are well conserved between gram-negative plant and animal patho-

gens and are called *hrp*-conserved (*hrc*) genes. The *hrp*-associated (*hpa*) genes represent another set of genes from *Xanthomonas* spp. (Buttner and Bonas 2002a). The *hpa* genes may contribute to virulence, but strains with mutations in *hpa* genes generally do not exhibit the severe phenotypic changes in disease symptoms compared with *hrp* gene mutations. The *hrp*, *hrc*, and *hpa* genes are clustered in a chromosomal region known as the *hrp* gene cluster. The *hrp* gene clusters from the various species also have properties of pathogenicity islands, which are genes flanked by mobile genetic elements, tRNA-related sequences, or atypical GC composition for the particular genome (Alfano et al. 2000; Hacker and Kaper 2000). Pathogenicity island structures are construed as evidence of genetic exchange among bacteria and genetic rearrangements.

The *hrp* gene clusters of *Xanthomonas axonopodis* pv. *glycine* (Kim et al. 2003), *X. campestris* pv. *vesicatoria*, *X. campestris* pv. *campestris*, and *X. axonopodis* pv. *citri* (da Silva et al. 2002) have been sequenced, and the genetic organization of the left and right borders of the *hrp* gene clusters, though highly similar, have variations in terms of overall structure and gene content between the species. Whether any of the variations contribute to host specialization is unknown. One of the variable loci present in the right side of *hrp* gene cluster of *Xanthomonas* spp. is *hrpF*. *X. axonopodis* pv. *glycine* and *X. axonopodis* pv. *citri* both contain *hrpF* and a second locus known as *hpaF*, whereas *X. campestris* pv. *vesicatoria* has *hrpF*, *hpaF*, and a third gene named *hpaG* (Noel et al. 2002). Both *hpaFxcv* and *hpaGxcv* have high similarity with different portions of the *hpaF* genes of other species. *X. campestris* pv. *campestris* has *hrpFxc* in the opposite orientation relative to other *hrp* gene clusters and does not have an *hpaF* homolog at the right border. Instead, *X. campestris* pv. *campestris* has a homolog of *hrpW*, which originally was identified in *Erwinia* and *Pseudomonas* spp. (Charkowski et al. 1998; Kim and Beer 1998). *hpaF* encodes a leucine-rich repeat protein. However, no function for *hpaF* had been ascribed in virulence or avirulence until recently, when mutations in *hpaF* of *X. axonopodis* pv. *glycine* were reported to contribute to virulence (Kim et al. 2003). In addition to genes in the other *Xanthomonas* pathogens, the *hrpF* gene product (*HrpF*) shares amino acid sequence similarity with *NolX* from *Sinorhizobium fredii* USDA257 (Krishnan 2002), which is secreted during the early stage of nitrogen-fixing nodule development and required for efficient nodule formation (Bellato et al. 1997; Krishnan 2002; Meinhardt et al. 1993), and two predicted proteins, *PopF1* and *PopF2*, from *Ralstonia solanacearum* (Salanoubat et al. 2002).

The *hrpF* gene from *X. campestris* pv. *vesicatoria* has been studied in more detail (Buttner et al. 2002). *HrpFxcv* is secreted via the TTSS (Rossier et al. 2000) and has two N-terminal imperfect direct repeats and a C-terminal hydrophobic segment (Huguet and Bonas 1997). The protein also has lipid-

Corresponding author: Frank F. White; Telephone: (785) 532-6176; Fax: (785) 532-5692; E-mail fwhite@ksu.edu

binding activity and the ability to form ion-conducting pores in artificial lipid bilayers (Buttner et al. 2002). HrpFxcv was found not to be required for the secretion of TTSS substrate proteins but was required for the elicitation of a *Bs3*-dependent HR in pepper by AvrBs3 (Rossier et al. 2000). The behavior of HrpF provides evidence that the protein may be inserted in plant cell membrane and required for the type III effector proteins to enter the host cells (Buttner et al. 2002). Therefore, as a translocator, HrpF would contact the plant cell membrane directly and possibly be subjected to the plant surveillance mechanisms. As an essential and conserved component of the TTSS, HrpF and similar TTSS components represent attractive targets for genetic resistance strategies that may provide broad and durable resistance to some bacterial diseases. This study reports the characterization of *hrpF*_{xoo} and the surrounding region from *X. oryzae* pv. *oryzae* and the results of the exchange of the *hrpF* alleles from different species.

RESULTS

The *hrpF* region

of X. oryzae pv. *oryzae* is bounded by IS elements.

Cosmid pAS49 was retrieved from a genomic library of *X. oryzae* pv. *oryzae* strain PXO99^A by hybridization using an *hrpF* fragment probe designed from the related published sequence, and a 12.9-kb region was sequenced, revealing the organization of the right end of the *hrp* gene cluster (Fig. 1). The region included nine open reading frames (ORFs), including an *X. campestris* pv. *vesicatoria* *hrpF* homolog (*hrpF*_{xoo}) and two insertion elements. A second ORF, with similarity to both the *hpaF* and *hpaG* genes of *X. campestris* pv. *vesicatoria* and named *hpaF*_{xoo}, was located immediately downstream of *hrpF*_{xoo}. The 5.1 kb of DNA between *hrpF*_{xoo} and a homolog of the *hpaB* of *X. campestris* pv. *vesicatoria* included two ORFs, which were designated as *hpa3* and *hpa4*, and a portion of sequence related to the ISXo8 transposase. An insertion element was located immediately to the right of the conserved *hpaB* gene (Fig. 1, IS1). BLAST analysis of IS1 revealed 2 related copies in the region of the xanthomonadin biosynthesis export pathway in strain BXO1 of *X. oryzae* pv. *oryzae* (Goel

et al. 2002), 18 copies in *X. axonopodis* pv. *citri* 306, and 71 copies in *X. campestris* pv. *campestris* ATCC33913 (da Silva et al. 2002), although the related copies were not associated with the respective *hrp* gene clusters. A second insertion element was found immediately downstream of *hpaF* (Fig. 1, IS2). The region downstream of IS2 encoded three putative ORFs with highest similarity and colinear to three non-*hrp*-associated or linked hypothetical proteins from *X. axonopodis* pv. *citri* (ORFs XAC3981, XAC3982, and XAC3983 in accession number AE012047). The IS2 element was designated the right end of the *hrpF* region and the right end of *hrp* gene cluster of strain PXO99^A.

The regions of *hrpF* from different species of *Xanthomonas* were aligned and compared from the *hpaB* locus to the right end of the respective *hrp* gene clusters. *X. axonopodis* pv. *citri* and *X. axonopodis* pv. *glycines* are virtually colinear throughout the region, whereas the *X. campestris* pv. *vesicatoria* region is very similar to the corresponding region of PXO99^A and also ends in a tRNA sequence (Fig. 1). *X. campestris* pv. *vesicatoria* encoded sequences similar to *hpa3* and *hpa4*, but the sequence related to *hpa3* had a stop codon in the middle of the gene. *X. axonopodis* pv. *glycines* encoded sequences related to *X. oryzae* pv. *oryzae* *hpa3*, and only partial similarity with *hpa4*. Upstream of the *hpa3*-related sequences is an imperfect plant-inducible promoter (PIP) box (TTCGT...15nt... TTCGC). The *X. campestris* pv. *campestris* sequence was not related to *hpa3* but encoded a sequence related to a C-terminal coding portion of *hpa4* that was designated previously as XCC1218 (da Silva et al. 2002). Of five sequences, only *X. oryzae* pv. *oryzae* carried intact *hpa3* and *hpa4*. Therefore, the *hrpF* region of the respective *hrp* clusters of the different *Xanthomonas* species forms a peninsula of genetic variability connected to the main *hrp* pathogenicity island.

hrpF mutants have reduced virulence, whereas mutations in *hpa3*, *hpa4*, and *hpaF* did not affect the virulence.

The *hrpF* locus and surrounding region of *X. oryzae* pv. *oryzae* has not been characterized genetically; therefore, mutations in the individual ORFs were created. The *hrpF* mutant ASX41 was generated by an integration of a plasmid which

Fig. 1. Comparison of the *hrpF* peninsulas of five *Xanthomonas* spp. Dashed vertical lines indicate the region of the *hrpF* peninsula. The following sequences were used: AY536514 (*Xanthomonas oryzae* pv. *oryzae*; Xoo), AF056246 (*X. campestris* pv. *vesicatoria*; Xcv), AF499777 (*X. axonopodis* pv. *glycines*; Xag), AE011665 (*X. axonopodis* pv. *citri*; Xac), and AE012221 (*X. campestris* pv. *campestris*; Xcc). Arrows with crosses indicate open reading frames (ORFs) that are either disrupted or carrying a frame shift mutation.

contains a partial *hrpF* sequence by single homologous recombination. Virulence of ASX41 was determined by measuring lesion lengths and bacterial numbers in inoculated leaves, and the results were compared with similar measurements for the parental strain PXO99^A and the *hrpC1* mutant PXO99^AME7. PXO99^AME7 has a defective TTSS and is essentially non-pathogenic. ASX41 showed both reduced lesion lengths (Fig. 2A) and bacterial growth (Fig. 2B) in rice leaves compared with the parental strain PXO99^A but caused longer lesions and had more growth than the nonpathogenic strain PXO99^AME7. The difference between the virulence of ASX41 and PXO99^AME7 indicated that the *hrpF* mutant still was competent for secretion of virulence effectors into the plant cells. When the complete *hrpF* gene in pAS65 was introduced into *hrpF* mutant ASX41, the mutant was restored to full virulence (Fig. 2A). To rule out that the residual virulence of ASX41 was due to a low level of reversion during growth in the plant, a second *hrpF* mutant strain, ASX85, was generated by double recombination of a transposon into the *hrpF* locus. ASX85 showed the same loss of virulence as the single recombinant strain ASX41 and was more virulent than PXO99^AME7 (data not shown). The *hpa3*, *hpa4*, and *hpaF* also were inactivated individually by homologous recombination, and none of the respective mutants showed significant differences in lesion length compared with the parental strain PXO99^A (data not shown).

hrpF mutants are competent for race-specific reactions.

Mutations in *hrpF* of *X. campestris* pv. *vesicatoria* prevented the elicitation of resistance in pepper plants containing the resistance gene *Bs3* by bacteria harboring the *avrBs3* avirulence gene (Rossier et al. 2000). Strains of *X. oryzae* pv. *oryzae* contain homologs to *avrBs3*, including the genes *avrXa7* and *avrXa10* (Hopkins et al. 1992). Experiments were performed to determine whether the *hrpF*_{xo2} mutant was able to deliver AvrXa7 and AvrXa10 into plant cells based on an *R* gene-specific HR. The plasmids pZWavrXa10 and pZWavrXa7 contain the avirulence genes *avrXa10* and *avrXa7*, respectively, and strains of *X. oryzae* pv. *oryzae* con-

taining the genes elicit resistance when inoculated on rice plants with either *Xa10* or *Xa7*, respectively. When introduced into the *hrpF* mutant ASX41, both resulting strains caused typical water-soaked symptoms on IR24 and an HR on the respective plants containing either *Xa10* or *Xa7* (Fig. 3A; IRBB7, results not shown). However, the degree of the water soaking and HR were reduced compared with the wild-type responses. *POX22.3* is a rice peroxidase gene that is induced specifically during the resistance reactions caused by PXO99^A harboring either *avrXa10* or *avrXa7*, whereas *TFIIAγ1* is a rice gene that is specifically induced by PXO99^A in susceptible interactions. (Chittoor et al. 1997; unpublished results). Both genes are not induced by bacteria lacking a functional TTSS. However, *POX22.3* was induced upon inoculation of plants with ASX41 harboring pZWavrXa10, and *TFIIAγ1* was induced by ASX41 (Fig. 3B and C). *TFIIAγ1* induction was reduced in the level of induction based on the intensity of the bands.

The results indicated that the *hrpF* mutant of *X. oryzae* pv. *oryzae* retained at least a partial ability to translocate AvrXa10 and virulence effectors into plant cells. HrpF was reported to be required for *Bs3*-dependent resistance in pepper (Rossier et al. 2000); therefore, *avrBs3* and *avrBs1* were introduced into PXO99^A, which is not pathogenic and does not elicit a nonhost HR on pepper, and the *hrpF* mutant ASX41, respectively. Each strain was inoculated on pepper plants 30R or 10R that carried either *Bs3* or *Bs1*, respectively. Although PXO99^A harboring *avrBs3* or *avrBs1* induced the appropriate gene-specific resistance reactions on pepper plants with 30R or 10R, respectively, ASX41 harboring *avrBs3* or *avrBs1* did not induce visible resistance reactions with either *avr/R* gene combination (data not shown).

Hpa1 is a harpin-like protein encoded by the *hpa1* locus of *X. oryzae* pv. *oryzae* (Zhu et al. 2000). Harpin-like proteins also have been postulated to function as translocons in the TTSS and are known to form pores on lipid bilayers (Lee et al. 2001). Therefore, *hpa1* was hypothesized to represent a second gene controlling the uptake of effectors by the plant cells and responsible for the residual virulence and elicitor activity of the *hrpF* mutants. To investigate this hypothesis, an *hpa1 hrpF*

Fig. 2. *hrpF* mutants are reduced in virulence but remain pathogenic. **A**, Lesion-length measurements of *hrpF* mutants. The third leaf of IR24 seedlings was inoculated individually with PXO99^A (wild type) harboring pHM1 (empty plasmid), ASX41 (*hrpF* mutant) harboring pAS65 (*hrpF*_{xo2}), ASX41 harboring pHM1, ME7 (*hrpC* mutant) harboring pHM1, and water. The lesion lengths after 12 days are shown. **B**, Bacterial leaf populations of *hrpF* mutants. The third leaf of 14-day-old IR24 seedlings were clip inoculated with PXO99^A (pHM1), ASX41 (pAS65), ASX41 (pHM1), and PXO99^AME7 (pHM1), and a 20-cm portion of three leaves was harvested on the indicated days. Statistically different data groups ($P < 0.05$) are indicated by different letters.

double mutant, ASX153, was generated along with a single *hpa1* mutant, ASX155. The virulence of *hrpF hpa1* double mutant ASX153 was weaker compared with the reactions of the *hpa1* mutant ASX155, *hrpF* mutant ASX85, and wild-type strain PXO99^A and weakly virulent compared with the non-pathogenic *hrpC* mutant PXO99^AME7 (Fig. 4). The plasmid pZWavrXa10 was introduced into *hrpF hpa1* double mutant ASX153 and the transformant was inoculated on rice lines IR24 and IRBB10. Although weaker, ASX153 harboring pZWavrXa10 triggered an *Xa10*-dependent HR in IRBB10 (data not shown) and concomitantly elicited the induction of *POX22.3* in IRBB10. PXO99^AME7 with pZWavrXa10, in comparison, neither triggered an HR (data not shown) nor elicited the induction of *POX22.3* above levels observed for the control infiltration with water (Fig. 5).

Fig. 3. *hrpF* mutants are competent for *R* gene-specific reactions. **A**, The second leaf of 14-day-old seedlings of IRBB10 was inoculated by needleless syringe with PXO99^A (pHM1), PXO99^A (pZWavrXa10), *hrpF* mutant ASX41 (pHM1), and ASX41 (pZWavrXa10). Both PXO99^A and ASX41 harboring pZWavrXa10 triggered a hypersensitive response (HR) on IRBB10, whereas both strains without *avrXa10* caused water soaking on IRBB10. **B**, Total RNA was prepared from 14-day-old IRBB10 leaves 12 h after inoculated with either water, PXO99^A (pHM1), PXO99^A (pZWavrXa10), *hrpF* mutant ASX41 (pHM1), or ASX41 (pZWavrXa10). The RNA was fixed on a membrane and hybridized with a probe for *POX22.3*, which is induced during HR. ASX41 (pZWavrXa10) induced *POX22.3*. **C**, The second leaf of 14-day-old rice IR24 was inoculated with PXO99^A, ASX41, PXO99^AME7 (*hrpC* mutant), and water. The leaves were harvested 24 h after inoculation and subjected to Northern analysis with a probe for *TFIIAγ1*. PXO99^A induced *TFIIAγ1* fully, and *hrpF* mutant ASX41 partially induced *TFIIAγ1*, whereas *hrpC* mutant PXO99^AME7 did not induce *TFIIAγ1*.

hrpF alleles complement loss of *hrpF*Exoo.

The *hrpF* alleles of the various species and pathovars of *Xanthomonas* have a greater degree of sequence divergence relative to the conserved core *hrp* genes. Overall HrpF_{Exoo} similarity with HrpF from *X. campestris* pv. *vesicatoria* is 94%, *X. axonopodis* pv. *glycine* 84%, *X. axonopodis* pv. *citri* 80%, and *X. campestris* pv. *campestris* 59% at the protein level. The alleles, with the exception of HrpF_{Excc}, have the two N-terminal repeat sequences and two C-terminal hydrophobic sequences which previously were identified in HrpF_{Excv} (Huguet and Bonas 1997). HrpF_{Excc} has three N-terminal repeat sequences. The differences may represent adaptations of the respective strains to the respective host plants. To examine this possibility, the genes from *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, and *X. axonopodis* pv. *citri* were cloned and tested for complementation of *hrpF*Exoo of PXO99^A. Each gene was cloned with the original promoter and introduced into *hrpF*Exoo mutant ASX41, and the transformants were inoculated on rice. None of the transformants triggered an HR on rice (data not shown), and all three *hrpF* genes complemented the *hrpF*Exoo2 mutation as determined by measurement of lesion lengths on inoculated leaves (Fig. 6). Conversely, pAS65 containing *hrpF*Exoo was introduced into *X. campestris* pv. *campestris* 33913, *X. axonopodis* pv. *citri* 306, and *X. campestris* pv. *vesicatoria* XV157, and the resulting strains were inoculated on their respective host plants. *X. campestris* pv. *campestris* 33913 and *X. axonopodis* pv. *citri*

Fig. 4. *hrpF hpa1* double mutant is weakly virulent. The third leaf of 14-day-old rice plants of IR24 was inoculated with the following strains: 1, PXO99^A; 2, ASX155 (*hrpA* mutant); 3, ASX85 (*hrpF* mutant); 4, ASX153 (*hrpF* and *hrpA* double mutant); 5, PXO99^AME7 (*hrpC* mutant); and 6, water. The lesion lengths were measured each day after inoculation. The results after the 14th day are shown. Statistically different data groups ($P < 0.05$) are indicated by different letters.

Fig. 5. *hrpF hpa1* double mutant is competent for *R* gene-specific reactions. Total RNA was prepared from IRBB10 intact leaves or leaves densely inoculated with water, *hrpC* mutant PXO99^AME7 (pHM1), PXO99^AME7 (pZWavrXa10), *hrpF hpaA* double mutant ASX153 (pHM1), ASX153 (pZWavrXa10), wild-type PXO99^A (pHM1), or PXO99^A (pZWavrXa10) 12 h after inoculation and subjected to Northern analysis using a *POX22.3*-specific probe. ASX153 harboring pZWavrXa10 induced *POX22.3* weakly.

306 were tested on cabbage and grapefruit, respectively. *X. campestris* pv. *vesicatoria* XV157 was tested on tomato and pepper. PXO99^A itself caused an HR on cabbage and tomato and did not show any phenotype on citrus. *X. campestris* pv. *campestris* 33913, *X. axonopodis* pv. *citri* 306, and *X. campestris* pv. *vesicatoria* XV157 with pAS65 did not trigger HR on any of the respective host plants (data not shown).

DISCUSSION

The HrpF peninsula, which is defined here as the region of the *hrp* pathogenicity island lying between *hpaB* and the right end of the *hrp* gene cluster, is a region of relatively high nucleotide and gene variability within the *hrp* gene clusters of different *Xanthomonas* spp.. In *X. oryzae* pv. *oryzae*, the region is bounded by two IS elements and encompasses four ORFs and a degenerate coding region with similarity to a gene for transposase. The right end in *X. oryzae* pv. *oryzae* is defined by an insertion element, which occurs between the homolog of *hpaF* and three ORFs that are colinear with a region of the *X. axonopodis* pv. *citri* genome. However, the three ORFs in *X. axonopodis* pv. *citri* are not linked to the *hrp* gene cluster, indicating that a rearrangement of the region occurred or that different insertion events of *hrp* clusters occurred in the two species.

The peninsula of *X. oryzae* pv. *oryzae*, as represented in PXO99^A, has two novel complete ORFs, designated *hpa3* and *hpa4* that are only partially represented in the *hrpF* peninsula of other species and pathovars. The ORFs appear to be organized within an operon and are preceded by a PIP motif located 121 bp upstream of the putative operon. The genes also were expressed in an HrpX-dependent manner (A. Sugio and F. F. White, unpublished data). The organization of the genes and the small, acidic, and leucine-rich nature of the predicted protein for *hpa3* suggested that the *hpa3* product may be a chaperone for the product of *hpa4* for secretion by the type III system. However, these predictions remain to be verified by functional analyses. In any event, mutations in either ORF did not affect the virulence as measured by the lengths of lesion on plants. Recently, a genome-wide screening of TTSS effectors of *X. campestris* pv. *vesicatoria* identified a homolog of Hpa4 called XopF1, providing further evidence that Hpa4 represents a type III effector (Roden et al. 2004). Similar to the results with

hpa4, mutants in the gene corresponding to XopF1 did not result in an appreciable loss of virulence by *X. campestris* pv. *vesicatoria* (Roden et al. 2004). The presence of intact *hpa3* and *hpa4* in *X. oryzae* pv. *oryzae* and related but partial or degenerate sequences in the related pathogens provides clues that the *hrpF* peninsula of *X. oryzae* pv. *oryzae* may reflect the founding *hrp* gene cluster of the common ancestor.

Mutations in *hrpF* were the only mutations in the genes of the peninsula that affected the ability of the bacterium to cause disease. Previous evidence has shown that secretion of the protein occurs via the TTSS system, and alleles of HrpF have variations in their amino acid sequence similar to the substrate effectors. At the same time, *hrpF* alleles are found in all of the species and pathovars of *Xanthomonas* that have been examined, and related genes are found in strains of *R. solanacearum* and several *Rhizobium* species. HrpF also is generally required for pathogenicity. Previous studies with *hrpF* alleles of *X. campestris* pv. *vesicatoria* and *X. axonopodis* pv. *glycines* indicated that mutations in *hrpF* resulted in strains that were nonpathogenic and unable to elicit race-specific HRs (Kim et al. 2003; Rossier et al. 2000). Mutants of *hrpF* in *X. oryzae* pv. *oryzae* were not devoid of pathogenicity. The mutant strains displayed both a reduced ability to grow within the host and reduced ability to cause lesions. However, the mutants were more virulent compared with a mutant for the secretion apparatus.

The proposed HrpF function as a translocon and as a secreted protein places the protein at the plant-bacterium interface (Rossier et al. 2000). Like the effector proteins, HrpF possibly would be subject to the diversifying selective pressure to avoid detection from the plant defense mechanisms or to meet special structural requirements for function in different host species. Differences are found among the encoded proteins of the alleles. The most striking is the number of N-terminal imperfect repeat sequences. HrpF_{xcc} has three repeats, whereas proteins from other *Xanthomonas* strains have two repeat sequences. NolX from *S. fredii* has only one. A series of studies on HrpF_{xcc} showed that one repeat was sufficient for *hrpF* function in *Xanthomonas* spp., but *hrpF_{xcc}* and *nolX* were not functionally interchangeable (Huguët and Bonas 1997). The results further indicated that the repeat in some manner stabilized the structure of HrpF (Buttner et al. 2002). No evidence for a role of HrpF in the adaptation of *Xanthomonas* spp. to the different

Fig. 6. Alleles of *hrpF* from different *Xanthomonas* strains complement the mutation in *hrpF_{xoo2}*. The third leaf of 21-day-old seedling of IR24 was inoculated with PXO99^A (pHM1), *hrpF_{xoo2}* mutant ASX41 (pAS65:*hrpF_{xoo2}*), ASX41 (pAS74:*hrpF_{xac}*), ASX41 (pAS76:*hrpF_{xcv}*), ASX41 (pAS109:*hrpF_{xcc}*), ASX41 (pHM1), and water. The lesion lengths were measured each day after the inoculation. The results, which represent the mean of three leaves, after the 12th day are shown. Statistically different data groups ($P < 0.05$) are indicated by different letters. The graph shows the results of two experiments.

host species was apparent from these studies. All of the homologous genes that were tested could complement the loss of *hrpF_{Xoo2}* to the extent revealed by lesion-length assays. Similarly, an analysis of the synonymous and nonsynonymous substitutions did not reveal whether the genes were subject to diversifying selection (data not shown). Therefore, the *hrpF* alleles of *Xanthomonas* spp. were not critical host determinants in the pathogen–host combination used in this study or under the experimental conditions. The results do not eliminate the possibility that specific *hrpF* alleles may function more efficiently in effector transfer in the respective hosts, which could have important ecological impacts on the persistence of the bacteria in the environment.

The mechanism of protein translocation across the host cell wall remains unclear. Gram-negative bacteria secrete virulence factors through the extracellular needle-like structure (pilus) into host cells. Plant-pathogenic bacteria have longer TTSS than animal-pathogenic bacteria, which may reflect the need to penetrate thick plant cell walls (Roine et al. 1997). Some evidence showed that pili of plant-pathogenic bacteria penetrate the wall (Brown et al. 2001). However, whether pili penetrate the cellular plasmalemma is subject to debate. The translocation of the effectors into the host cells may be mediated by a protein or protein complex called a translocon (Buttner and Bonas 2002b). In the case of the animal pathogen *Yersinia*, the protein LcrV forms channel-like pores in eukaryotic cell membranes by itself (Holmstrom et al. 2001), and YopB and YopD also are required for protein translocation. Both proteins were found to be inserted in liposomes (Neyt and Cornelis 1999). YopB and YopD are hypothesized to stabilize the pore formed by LcrV and constitute the translocon protein complex, and all three are required to form functional translocon complexes (Broms et al. 2003).

HrpF, harpins, and harpin-like proteins have been proposed to function in facilitating effector transfer in plant-pathogenic bacteria. HrpF_{Xcv} formed pores in an artificial lipid bilayer. The protein also was required for race-specific reactions using the *avrBs3/Bs3* combination despite the fact that AvrBs3 was delivered to the extracellular milieu, suggesting an absolute requirement for HrpF in the delivery of effectors into the host cell (Rossier et al. 2000). In contrast to *X. campestris* pv. *vesicatoria*, *hrpF* mutants of *X. oryzae* pv. *oryzae* delivered AvrXa10 or AvrXa7, which require internalization for activity (Zhu et al. 1998), into rice cells on the basis of respective *R* gene-specific resistance reactions and the specific induction of a gene for peroxidase. The mutants also were able to induce genes for peroxidase and *TFIIAγ1*, which are rice genes induced in a TTSS-dependent manner. The residual virulence and avirulence activities could not be explained by reversion of the mutants. The results indicate that HrpF, although a possible facilitator of transport, is not absolutely required for the translocation of TTSS proteins into plant cells. A similar observation was observed for the *hrpK* gene, which is a possible homolog of HrpF in *Pseudomonas syringae* pv. *tomato*. Mutations in *hrpK*, although reducing activity, did not eliminate virulence and the ability of the bacteria to elicit an *R* gene-dependent HR (Petnicki-Ocwieja et al. 2005).

The results concerning *Xa10*-dependent elicitation by *hrpF* mutants may be explained by several alternative hypotheses. First, additional pore-forming proteins in addition to HrpF may exist. Harpins also have been postulated to create pores in plant cell membranes (Lee et al. 2001). Hpa1 is the product of the *hpa1* gene and a hairpin-like protein present in all species of *Xanthomonas* examined (Kim et al. 2003; Noel et al. 2002; Zhu et al. 2000). However, the double *hpa1 hrpF_{Xoo2}* mutant still elicited *Xa10*-dependent resistance, indicating that Hpa1 and HrpF are not solely responsible for effector translocation.

A second possibility is that plant cells themselves have the ability to take up proteins delivered to the cell membrane by endocytosis (Horn et al. 1989). In the absence of HrpF (and Hpa1), AvrXa10 and some other virulence factors may be delivered to the plant membrane in quantities sufficient for uptake by the plant cells resulting in subsequent elicitation. A third explanation is that the TTSS needle-like structure may actually puncture some of the plant cells and secrete the effector proteins directly into the plant cells (Hoiczyk and Blobel 2001). Finally, HrpF may affect effector activity in some unknown manner, which facilitates effector uptake. In any event, *X. oryzae* pv. *oryzae* *hrpF* mutants also were unable to trigger *Bs3*-dependent reactions in pepper plants. If *X. oryzae* pv. *oryzae* and *X. campestris* pv. *vesicatoria* are assumed to deliver the effectors with equal efficiencies, differences may exist in the sensitivity of the AvrB3/Bs3 interaction or ability of rice and pepper cells to take up the effectors.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial strains and plasmids used in the experiments are listed in Table 1. *Escherichia coli* strains were grown in terrific broth or on Luria agar plates at 37°C with the appropriate antibiotics. *Xanthomonas* strains were cultured on tryptone sucrose agar at 28°C (Zhu et al. 2000). The genomic library of PXO99^A in pHC79 was constructed before (Yang and White 2004). Carbenicillin, spectinomycin, gentamicin, and kanamycin were used at 100 µg/ml.

Recombinant DNA techniques.

DNA manipulations were performed by standard procedures (Sambrook et al. 1987). Ligase, SuperScript II reverse transcriptase, and Accuprime *Taq* DNA polymerase were purchased from Invitrogen (Carlsbad, CA, U.S.A.). An EZ::TN <KAN-2> insertion kit (Epicentre Technologies, Madison, WI, U.S.A.) was used for random insertion of transposon into plasmids and cosmids. Radio-labeled dCTP was purchased from Perkin-Elmer (Wellesley, MA, U.S.A.). Hybond N+ (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) was used for Northern blot hybridization. Rediprime II random prime labeling system and Microspin (Amersham Pharmacia Biotech) were used to prepare probes for Northern blot hybridization. Each kit was used according to the manufacturer's instructions. Sequencing was performed at Kansas State University Sequencing Facilities. Amino acid alignments were constructed using ClustalW (Thompson et al. 1994). Identity and similarity comparisons were calculated using NTI Vector software (Infomax, Bethesda, MD, U.S.A.).

DNA amplification.

The primer sequences used in this study are provided in Table 2. The primers KohrpFxoF and KohrpFxoR were used to amplify a partial fragment of *hrpF* by polymerase chain reaction (PCR) from genomic DNA. The complete *hrpF* gene was amplified from cosmid clone pAS49 using Fshort1 and Rshort1. The *hpaF* gene was amplified from pAS49 by PCR using hpaFF1 and hpaFR1. The *hpa3-hpa4* locus was amplified by PCR using hpa34F and hpa34R. *hrpF* genes from *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri*, and *X. campestris* pv. *vesicatoria* were amplified from the respective genomic DNA by PCR using the following sets of primers: *hrpF_{Xcc}*, *XcchrpFf*, and *XcchrpFr*; *hrpF_{Xac}*, *XachrpFf*, and *XachrpFr*; and *hrpF_{Xcv}*, *XcvhrpFf*, and *XcvhrpFr*, respectively. The *hpa1* gene was amplified from *X. oryzae* pv. *oryzae* genomic DNA using hpa1-BamHI2 and hpa1-KpnI2. Gentamicin 3-acetyltransferase gene was PCR amplified from

pKockoutG (Windgassen et al. 2000) using the primers GmF-Eco and GmR-Eco. The 3' untranslated region of *TFIIA71* was amplified from rice IRBB7 genomic DNA by PCR using Chr1TF2F and Chr1TF2R. The parameters used for PCR were as follows: step 1, 95°C for 2 min; step 2, 95°C for 30 s; step 3, 50°C for 30 s; step 4, 68°C for 1 min per kilobase; 25 cycles from step 2 to step 4; and step5, 68°C for 5 min.

Transposon and deletion mutagenesis.

The *hrpF* mutant ASX41 was constructed by a single homologous recombination using 654 bp of *hrpF*, which was amplified by PCR. The fragment was cloned in pCR2.1-TOPO, which carries genes for resistance to kanamycin and carbenicillin, and the plasmid was designated as pAS18. pAS18 was introduced into *X. oryzae* pv. *oryzae* strain PXO99^A by single homologous recombination. The recombinants were screened by resistance to kanamycin. For the complementation, the complete *hrpF* gene, including approximately 500 bp upstream from the start codon, was amplified from pAS49 and was cloned into pCR2.1-TOPO. The plasmid was termed pAS50 and sequenced. pAS50 was ligated with broad-host-range vector pHM1 at the unique *KpnI* site, resulting in pAS65. To construct *hrpF* mutant ASX85, a *NotI* and *HindIII* fragment was excised from pAS50 and cloned into pBluescript II KS(+) and termed pAS61. Transposons were inserted randomly into pAS61 using an EZ::TN <KAN-2> insertion kit according to the directions of the manufacturer (Epicentre Technologies), and a plasmid with a transposon insertion in the *hrpF* coding sequence at 507 bp from the start codon was selected for further use. The plasmid was designated as pAS64. pAS64 was introduced into PXO99^A, and a double recombinant clone, called ASX85, was selected on the basis of resistance to kanamycin resistance (retention of insertion) and sensitivity to carbenicillin (loss of vector sequences). The recombination event was verified by PCR amplification and sequencing of a fragment containing one end of the transposon sequence and flanking genomic sequence. To generate a

mutation in *hpaF* mutants, a 2.4-kb fragment containing a portion of *hpaF* was obtained by PCR and double digestion with *XbaI* and *SacI*. The fragment was ligated in pBluescript II KS(+), and the clone was subjected to mutagenesis with EZ::TN <KAN-2> transposons. A plasmid with an insertion at 419 bp from the *hpaF* start codon was selected for use. The plasmid was introduced into PXO99^A and recombinants from a double recombination event were selected on the basis of resistance to kanamycin and sensitivity to carbenicillin. The mutation in *hpaF* was verified by PCR and sequence analysis of genomic DNA. To obtain *hpa3* and *hpa4* mutants, the *hpa3* and *hpa4* region was amplified by PCR and cloned into pGEM-T Easy Vector. The resulting plasmid was subjected to mutagenesis with EZ::TN <KAN-2> transposons. Plasmids with insertions at 447 bp from the *hpa3* start codon or 1,140 bp from

Table 2. Primer sequences

Primer name	Sequence
KohrpFxoF	5'-acctgccatcgacgatggatac-3'
KohrpFxoR	5'-cagctcaatcaatccgaactgg-3'
Fshort1	5'-attaatgaggtattcactatctgcgc-3'
Rshort1	5'-gcgcgttatctgcgacgtatcctg-3'
hpaFF1	5'-atcgcttgcgcgacgttcgtatg-3'
hpaFR1	5'-cgcttggcgaagttagctgcc-3'
hpa34F	5'-ccaacggattcgtatttagtaaacac-3'
hpa34R	5'-tcattgctgcgccgcttggccac-3'
XcchrpFf	5'-cctgatcaacacagtgtctccac-3'
XcchrpFr	5'-gtcgtcctcaagcaagatcgttactg-3'
XachrpFf	5'-gagcgaccttgatccgggtaatcac-3'
XachrpFr	5'-ctaacgcgaatgctgctgtggcatg-3'
XcvhrpFf	5'-cagcaaatccagggtcgtcagtcag-3'
XcvhrpFr	5'-cttcgctgtggcatgcgcgttacc-3'
hpa1-BamHI2	5'-atcgcggtatcccgctgcatcaacgcttcctggag-3'
hpa1-KpnI2	5'-gacccgggtaccttgatgcaccgcctggatgac-3'
GmF-Eco	5'-atcacagaattcaggacgcgtcaattctcgaattg-3'
GmR-Eco	5'-tacaatgaattcctcgaattgacgcgtcgcgggaag-3'
Chr1TF2F	5'-gctggagactaagaagagtaatttgc-3'
Chr1TF2R	5'-gagcacactatgtcacacatcac-3'

Table 1. Strains and plasmids used in the study

Strain	Relevant characteristics ^a	Source
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
PXO99 ^A	5-azacytidine resistant, race 6	Hopkins et al. 1992
PXO99AME7	<i>hrpC</i> mutant, Km ^r	Zhu et al. 2000
ASX41	PXO99 ^A <i>hrpF</i> ⁻ , single recombinant of pAS18, Km ^r	This study
ASX85	PXO99 ^A <i>hrpF</i> ⁻ , double recombinant of pAS64, Km ^r	This study
ASX153	PXO99 ^A <i>hrpF</i> ⁻ <i>hpaI</i> ⁻ , double recombinant of pAS127, Km ^r Gn ^r	This study
ASX155	PXO99 ^A <i>hpaI</i> ⁻ , double recombinant of pAS127, Gn ^r	This study
<i>X. campestris</i> pv. <i>vesicatoria</i>	Strain XV157	Gift from J. Jones
<i>X. campestris</i> pv. <i>campestris</i>	Strain ATCC33913	
<i>X. axonopodis</i> pv. <i>citri</i>	Strain 306	
Plasmids		
pBluescript II KS(+)	Phagemid, pUC derivative, Cb ^r	Stratagene
pCR2.1-TOPO	Phagemid, Cb ^r , Km ^r	Invitrogen
pHM1	Broad-host-range vector with pUC19 polylinker, Sp ^r	Hopkins et al. 1992
pAS18	pCR2.1-TOPO with partial <i>hrpF</i> _{oxo2}	This study
pAS49	Cosmid (pHC79) clone of <i>hrpF</i> _{oxo2} region	This study
pAS50	pCR2.1-TOPO with <i>hrpF</i> _{oxo2}	This study
pAS61	pBluescript II KS(+) with <i>hrpF</i> _{oxo2}	This study
pAS64	pAS61 with transposon inserted in <i>hrpF</i> _{oxo2}	This study
pAS65	pAS50 ligated with pHM1	This study
pZWavrXa10	<i>avrXa10</i> fused to lacZ promoter of pBluescript II KS(+)	Yang et al. 2000
pZWavrXa7	<i>avrXa7</i> fused to lacZ promoter of pBluescript II KS(+)	Yang et al. 2000
pAS127	<i>hpaI</i> with gentamicin 3-acetyltransferase(Gn ^r) insertion	This study
pAS76	pHM1 with <i>hrpF</i> _{xcv}	This study
pAS74	pHM1 with <i>hrpF</i> _{xac}	This study
pAS109	pHM1 with <i>hrpF</i> _{xcc}	This study
pHM1::avrBs1-2	pHM1 with <i>avrBs1</i> ORF1 and 2	X. Tang, unpublished
pHM1::avrBs3	pHM1 with <i>avrBs3</i>	Zhu et al. 1998

^a Km = kanamycin, Gn = gentamicin, Cb = carbenicillin, Sp = spectinomycin, and ORF = open reading frame.

hpa4 start codon were isolated and introduced into PXO99^A. Mutants generated by double recombination event were selected as described above for *hrpF* *xoo*.

A 1.8-kb DNA fragment containing *hpa1* was amplified by PCR, digested by *Bam*HI and *Kpn*I, and ligated with pBluescript II KS(+) to construct an *hpa1 hrpF* double mutant. The gene for gentamicin-3-acetyltransferase, which was amplified by PCR using the primers with *Eco*RI sites, was inserted into the *Eco*RI site of *hpa1*. The plasmid was termed pAS127 and was introduced into the *hrpF* mutant ASX85 by electroporation and selection for resistance to gentamicin. *hrpA* single mutant ASX155 was similarly generated by introducing pAS127 into the wild-type strain PXO99^A. Recombinants due to double homologous recombination were identified on the basis of sensitivity to carbenicillin and verified by PCR analysis.

The *hrpF* genes and their respective promoter regions were amplified by PCR from genomic DNA from *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri*, and *X. campestris* pv. *vesicatoria* and each gene was cloned into pCR2.1. Each gene is designated by a three-letter code subscript, which refers to the species of origin, as follows: *X. axonopodis* pv. *citri* (xac), *X. axonopodis* pv. *glycine* (xag), *X. campestris* pv. *campestris* (xcc), *X. campestris* pv. *vesicatoria* (xvc), and *X. oryzae* pv. *oryzae* (xoo). The resulting plasmids then were digested by *Hind*III (*X. campestris* pv. *vesicatoria* and *X. campestris* pv. *campestris*) or *Sac*I (*X. axonopodis* pv. *citri*) and ligated with pHM1, and the plasmids were termed pAS76, 74, and 109, respectively.

Plant assays.

Pathogenicity and hypersensitivity assays were performed as previously described (Hopkins et al. 1992). Tomato cv. VFN-8; pepper cvs. ECW, 10R, and 30R; cabbage cv. Goldenacre; and grapefruit cv. Duncan (*Citrus paradisi*) were used as hosts for hypersensitivity tests of the respective strains. Nine-day-old rice seedlings IRBB10 and IRBB7, containing corresponding resistance genes *Xa10* and *Xa7*, respectively, were used for race-specific resistance assays of *X. oryzae* pv. *oryzae*. Seedlings of IR24 (21 days old) were used for pathogenicity tests in rice. All plants were grown in growth chambers at 28°C with a 12-h photoperiod. Inoculum concentrations were adjusted to an optical density of 0.5 at 600 nm (approximately 0.5×10^8 CFU/ml) using a DU640B spectrophotometer (Beckman Coulter, Fullerton, CA, U.S.A.). For hypersensitivity tests, plants were inoculated by needleless syringe infiltration (Zhu et al. 1998). Symptoms were scored by measuring lesion length after the leaf clip inoculation (Kauffmann et al. 1973). Scores are the mean of three leaves. Bacterial growth in rice leaves was measured by harvesting three leaves (20 cm) for each treatment after leaf clip inoculation. The leaves were ground with a mortar and pestle in 5 ml of sterile water. The solution was diluted serially and spread on TSA plates with either kanamycin (for PXO99^AME7) or spectinomycin, depending on the genotype of the strain. The mean number of colonies in three plates of the proper dilution (10 to 100 colonies) was calculated. Significance between treatments was assessed using log-converted data on the basis of a *P* value <0.05, using the Tukey test for post analysis of variance.

Northern hybridization analysis.

The second leaf of 14-day-old rice plants was inoculated at multiple sites with bacterial suspensions prepared as described above or by sterile water with a needleless syringe. The leaves were harvested 12 and 24 h after inoculation to detect the induction of *POX22.3* and *TFIIAγ1*, respectively. Total RNA was extracted by using Trizol following the instructions of the manufacture (Invitrogen). Approximately 15 μg of total RNA

was loaded for each sample and subjected to electrophoresis in 1% agarose gels. The RNA was transferred to Hybond N+ (Amersham Biosciences). The membrane was hybridized with a specific probe at 65°C in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% sodium dodecyl sulfate, 20 mM Na₂HPO₄/NaH₂PO₄ [pH 6.5], 5 mM EDTA, and 1 mM Tris-HCl). The probe for the peroxidase gene *POX22.3* (NCBI accession AF014467) was amplified from the unique 3' untranslated region by PCR as described previously (Chittoor et al. 1997). The 3' untranslated region of *TFIIAγ1* was amplified by PCR and used as a probe.

ACKNOWLEDGMENTS

This study was supported by funds from the Tillman Scholarship Fund (A. Sugio), USDA-CREES-NRI (F. F. White), and the Kansas Agriculture Experiment Station (publication 05-172-J). We thank J. Jones for providing *X. campestris* pv. *vesicatoria* XV157 and grapefruit plants (*Citrus paradisi* var. Duncan), X. Tang for providing pHM1::avrBs1-2 and pepper plant ECW 10R, A. Bogdanov for providing pepper ECW 30R and helping bacterium inoculation, and M. Leathers for technical assistance.

LITERATURE CITED

- Alfano, J. R., Charkowski, A. O., Deng, W. L., Badel, J. L., Petnicki-Ocwieja, T., van Dijk, K., and Collmer, A. 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 97:4856-4861.
- Bellato, C., Krishnan, H. B., Cubo, T., Temprano, F., and Pueppke, S. G. 1997. The soybean cultivar specificity gene *nolX* is present, expressed in a *nodD*-dependent manner, and of symbiotic significance in cultivar-nonspecific strains of *Rhizobium* (*Sinorhizobium*) *fredii*. *Microbiology* 143(Pt 4):1381-1388.
- Broms, J. E., Sundin, C., Francis M. S., and Forsberg, A. 2003. Comparative analysis of type III effector translocation by *Yersinia pseudotuberculosis* expressing native LcrV or PcrV from *Pseudomonas aeruginosa*. *J. Infect. Dis.* 188:239-249.
- Brown, I. R., Mansfield, J. W., Taira, S., Roine, E., and Romantschuk, M. 2001. Immunocytochemical localization of HrpA and HrpZ supports a role for the Hrp pilus in the transfer of effector proteins from *Pseudomonas syringae* pv. *tomato* across the host plant cell wall. *Mol. Plant-Microbe Interact.* 14:394-404.
- Buttner, D., and Bonas, U. 2002a. Getting across—bacterial type III effector proteins on their way to the plant cell. *EMBO (Eur. Mol. Biol. Organ.) J.* 21:5313-5322.
- Buttner, D., and Bonas, U. 2002b. Port of entry—the type III secretion translocon. *Trends Microbiol.* 10:186-192.
- Buttner, D., Nennstiel, D., Klusener, B., and Bonas, U. 2002. Functional analysis of HrpF, a putative type II translocon protein from *Xanthomonas campestris* pv. *vesicatoria*. *J. Bacteriol.* 184:2389-2398.
- Charkowski, A. O., Alfano, J. R., Preston, G., Yuan, J., He, S. Y., and Collmer, A. 1998. The *Pseudomonas syringae* pv. *tomato* HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. *J. Bacteriol.* 180:5211-5217.
- Chittoor, J. M., Leach, J. E., and White, F. F. 1997. Differential induction of a peroxidase gene family during infection of rice by *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant-Microbe Interact.* 10:861-871.
- da Silva, A. C., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F., Alves, L. M., do Amaral, A. M., Bertolini, M. C., Camargo, L. E., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M., Coutinho, L. L., Cursino-Santos, J. R., El-Dorri, H., Faria, J. B., Ferreira, A. J., Ferreira, R. C., Ferro, M. I., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G., Lemos, M. V., Locali, E. C., Machado, M. A., Madeira, A. M., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A., Silva, C., de Souza, R. F., Spinola, L. A., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I., Trindade dos Santos, M., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C. and Kitajima, J. P. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* 417:459-463.

- Goel, A. K., Rajagopal, L., Nagesh, N., and Sonti, R. V. 2002. Genetic locus encoding functions involved in biosynthesis and outer membrane localization of xanthomonadin in *Xanthomonas oryzae* pv. *oryzae*. J. Bacteriol. 184:3539-3548.
- Hacker, J., and Kaper, J. B. 2000. Pathogenicity islands and the evolution of microbes. Annu. Rev. Microbiol. 54:641-679.
- Hoiczyk, E., and Blobel, G. 2001. Polymerization of a single protein of the pathogen *Yersinia enterocolitica* into needles punctures eukaryotic cells. Proc. Natl. Acad. Sci. U.S.A. 98:4669-4674.
- Holmstrom, A., Olsson, J., Cherepanov, P., Maier, E., Nordfelth, R., Pettersson, J., Benz, R., Wolf-Watz, H., and Forsberg, A. 2001. LcrV is a channel size-determining component of the Yop effector translocon of *Yersinia*. Mol. Microbiol. 39:620-632.
- Hopkins, C. M., White, F. F., Choi, S. H., Guo, A., and Leach, J. E. 1992. Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. 5:451-459.
- Horn, M. A., Heinsteins, P. F., and Low, P. S. 1989. Receptor-mediated endocytosis in plant cells. Plant Cell 1:1003-1009.
- Huguot, E., and Bonas, U. 1997. *hrpF* of *Xanthomonas campestris* pv. *vesicatoria* encodes an 87-kDa protein with homology to NolX of *Rhizobium fredii*. Mol. Plant-Microbe Interact. 10:488-498.
- Kauffman, H. E., Reddy, A. P. K., Hsieh, S. P. V., and Marca, S. D. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis. Rep. 57:537-541.
- Kim, J. F., and Beer, S. V. 1998. HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class. J. Bacteriol. 180:5203-5210.
- Kim, J. G., Park, B. K., Yoo, C. H., Jeon, E., Oh, J., and Hwang, I. 2003. Characterization of the *Xanthomonas axonopodis* pv. *glycines* Hrp pathogenicity island. J. Bacteriol. 185:3155-3166.
- Krishnan, H. B. 2002. NolX of *Sinorhizobium fredii* USDA257, a type III-secreted protein involved in host range determination, is localized in the infection threads of cowpea (*Vigna unguiculata* (L.) Walp.) and soybean (*Glycine max* (L.) Merr.) nodules. J. Bacteriol. 184:831-839.
- Lee, J., Klusener, B., Tsiamis, G., Stevens, C., Neyt, C., Tampakaki, A. P., Panopoulos, N. J., Noller, J., Weiler, E. W., Cornelis, G. R., Mansfield, J. W., and Nurnberger, T. 2001. HrpZ(PspH) from the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* binds to lipid bilayers and forms an ion-conducting pore in vitro. Proc. Natl. Acad. Sci. U.S.A. 98:289-294.
- Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity on bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.
- Meinhardt, L. W., Krishnan, H. B., Balatti, P. A., and Pueppke, S. G. 1993. Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 9:17-29.
- Neyt, C., and Cornelis, G. R. 1999. Insertion of a Yop translocation pore into the macrophage plasma membrane by *Yersinia enterocolitica*: Requirement for translocators YopB and YopD, but not LcrG. Mol. Microbiol. 33:971-981.
- Noel, L., Thieme, F., Nennstiel, D., and Bonas, U. 2002. Two novel type III-secreted proteins of *Xanthomonas campestris* pv. *vesicatoria* are encoded within the hrp pathogenicity island. J. Bacteriol. 184:1340-1348.
- Petnicki-Ocwieja T, van Dijk, K., and Alfano, J. R. 2005. The hrpK operon of *Pseudomonas syringae* pv. *tomato* DC3000 encodes two proteins secreted by the type III (Hrp) protein secretion system: HopB1 and HrpK, a putative type III translocator. J. Bacteriol. 187:649-663.
- Roden, J. A., Belt, B., Ross, J. B., Tachibana, T., Vargas, J., and Mudgett, M. B. 2004. A genetic screen to isolate type III effectors translocated into pepper cells during *Xanthomonas* infection. Proc. Natl. Acad. Sci. U.S.A. 101:16624-16629.
- Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E. L., Kalkkinen, N., Romantschuk, M., and He, S. Y. 1997. Hrp pilus: An hrp-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. Proc. Natl. Acad. Sci. U.S.A. 94:3459-3464.
- Rossier, O., Van den Ackerveken, G., and Bonas, U. 2000. HrpB2 and HrpF from *Xanthomonas* are type III-secreted proteins and essential for pathogenicity and recognition by the host plant. Mol. Microbiol. 38:828-838.
- Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Catolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J., and Boucher, C. A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1987. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.
- Windgassen, M., Urban, A., and Jaeger, K. E. 2000. Rapid gene inactivation in *Pseudomonas aeruginosa*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 193:201-205.
- Yang, B., and White, F. F. 2004. Diverse members of the AvrBs3/PthA family of type III effectors are major virulence determinants in bacterial blight disease of rice. Mol. Plant-Microbe Interact. 17:1192-1200.
- Yang, B., Zhu, W., Johnson, L. B., and White, F. F. 2000. The virulence factor AvrXa7 of *Xanthomonas oryzae* pv. *oryzae* is a type III secretion pathway-dependent nuclear-localized double-stranded DNA-binding protein. Proc. Natl. Acad. Sci. U.S.A. 97:9807-9812.
- Zhu, W., Magbanua, M. M., and White, F. F. 2000. Identification of two novel hrp-associated genes in the hrp gene cluster of *Xanthomonas oryzae* pv. *oryzae*. J. Bacteriol. 182:1844-1853.
- Zhu, W., Yang, B., Chittoor, J. M., Johnson, L. B., and White, F. F. 1998. AvrXa10 contains an acidic transcriptional activation domain in the functionally conserved C terminus. Mol. Plant-Microbe Interact. 11:824-832.