Characterization of the Nonconserved *hpaB-hrpF* Region in the *hrp* Pathogenicity Island from *Xanthomonas campestris* pv. *vesicatoria*

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The interaction of the gram-negative phytopathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* with its host plants pepper and tomato is mediated by a type III secretion (T3S) system that translocates bacterial effector proteins into the plant cell. The T3S system is encoded by the chromosomal *hrp* (hypersensitive response and pathogenicity) gene cluster. Here, we report on the analysis of the *hpaB-hrpF* region, which encodes the novel virulence factor HpaE, the effector protein XopF1, and two proteins with unknown functions, HpaD and HpaI. Promoter and transcript analyses revealed that the corresponding genes are coexpressed with the *hrp* genes and that *hpaD*, *hpaI*, and *xopF1* form a novel operon. In vitro and in vivo assays showed that the efficient T3S and translocation of XopF1 depends on the global T3S chaperone HpaB and the putative lytic transglycosylase HpaH, which specifically contributes to the secretion of a certain set of effectors. Taken together, our data suggest that the efficient secretion of effector proteins in *X. campestris* pv. *vesicatoria* requires the contribution of several different Hpa proteins.

Additional keywords: bacterial spot disease.

Gram-negative plant-pathogenic bacteria cause diseases in a variety of economically important crop plants and lead to considerable yield losses worldwide. In most cases, bacterial pathogenicity depends on a specialized type III protein secretion (T3S) system that is activated upon interaction of the bacteria with the plant and is highly conserved among plant and animal pathogenic bacteria. The T3S system spans both bacterial membranes and is associated with an extracellular pilus-like appendage (He et al. 2004; Koebnik 2001). The secretion apparatus mediates the Sec-independent secretion of proteins into the extracellular milieu and the translocation of bacterial effector proteins into the host cell cytosol. Effector protein translocation depends on the presence of the T3S translocon, which is probably inserted as a channel-like protein complex into the host plasma membrane (Büttner and Bonas 2002b).

Targeting of secretion substrates to the T3S system is often mediated by a signal within the first 20 N-terminal residues that is not conserved at the amino acid level (Guttman et al. 2002; Lloyd et al. 2001). Furthermore, for some proteins, a

signal within the 5' region of the corresponding mRNA was proposed, suggesting cotranslational secretion (Anderson and Schneewind 1997; Ramamurthi and Schneewind 2005). In addition to the secretion signal, effector proteins contain a translocation signal within the N-terminal 50 to 100 amino acids (Ghosh 2004). In many cases, the efficient translocation of effector proteins also requires the assistance of specialized chaperones that promote the stability or secretion, or both, of their corresponding interaction partners. Generally, T3S chaperones are small, acidic, and leucine-rich proteins (Feldman and Cornelis 2003; Parsot et al. 2003). They presumably keep their substrates in a partially unfolded and, thus, secretion-competent conformation and guide them to the secretion apparatus (Akeda and Galan 2005; Gauthier and Finlay 2003; Ghosh 2004). Based on their substrate specificities, T3S chaperones were divided into several classes. Class IA chaperones bind to one or several homologous effector proteins and are encoded next to the genes for their interaction partners. By contrast, class IB chaperones have a wide substrate specificity and bind to different type III effectors. Class II chaperones are specific for translocon proteins (Parsot et al. 2003).

One of the model systems to study T3S in plant-pathogenic bacteria is *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease in pepper and tomato. During natural infections, *X. campestris* pv. *vesicatoria* enters the plant via stomata or wounds and colonizes the intercellular spaces of the plant tissue (Stall 1995). Essential for the host-pathogen interaction is the T3S system, which injects more than 20 effector proteins (Xop, *Xanthomonas* outer proteins) into the plant cell (Büttner and Bonas 2002a; F. Thieme and U. Bonas, *unpublished data*). In susceptible plants, effector proteins presumably interfere with host cellular pathways such as basal defense responses to the benefit of the pathogen (Alfano and Collmer 2004; Chang et al. 2004, 2005; Guttman et al. 2002). In a number of cases, however, individual effectors, also termed avirulence (Avr) proteins, elicit specific defense responses in resistant plants that express a corresponding disease-resistance protein. Avr protein-triggered plant defense often culminates in the hypersensitive response (HR), a local programmed plant-cell death at the infection site that is accompanied by an arrest of bacterial multiplication (Dangl and Jones 2001; Klement 1982).

The T3S system of *X. campestris* pv. *vesicatoria* is encoded by a 23-kb chromosomal *hrp* (hypersensitive response and pathogenicity) gene cluster, embedded in a 35.3-kb pathogenicity island (Noël et al. 2002; Thieme et al. 2005). The *hrp* gene cluster contains more than 20 genes that are organized in at least seven transcriptional units (Bonas et al. 1991; Büttner and Bonas 2002a; Weber et al. 2007). *hrp* genes are expressed

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during infection or when the bacteria are incubated in specific minimal media. Two regulatory proteins, HrpG and HrpX, are encoded outside the *hrp* pathogenicity island. HrpG belongs to the OmpR family of two-component system response regulators and controls, in most cases via the AraC-type transcriptional activator HrpX, the expression of a genome-wide regulon including *hrp* and effector genes (Koebnik et al. 2006; Noël et al. 2001; Wengelnik and Bonas 1996; Wengelnik et al. 1996). Deletion of individual *hrp* genes leads to a complete loss of bacterial pathogenicity, suggesting that the corresponding gene products are essential for a functional T3S apparatus (Huguet and Bonas 1997; Huguet et al. 1998; Rossier et al. 2000). Eleven *hrp* genes (termed *hrc* for *hrp* conserved) are conserved among plant and animal pathogenic bacteria and presumably encode core components of the T3S apparatus (He 1998; Huguet et al. 1998; Rossier et al. 2000). Notably, several Hrp proteins including the T3S translocon protein HrpF and the pilus protein HrpE are themselves secreted by the T3S system and build

up extracellular components of the T3S system. The Hrp pilus is a filamentous surface appendage and presumably serves as a transport channel for secreted proteins to the plant-pathogen interface (Büttner et al. 2002; Weber et al. 2005).

The *hrp* region from *X. campestris* pv. *vesicatoria* also contains *hpa* (*hrp*-associated) genes that contribute to but are not essential for the interaction with the plant (Büttner et al. 2004, 2006; Huguet et al. 1998). One example is HpaB, which acts as a global class IB T3S chaperone for multiple sequence-unrelated effector proteins (Büttner et al. 2004). The results of protein-protein interaction studies suggest that HpaB is part of an export control protein complex that guides T3S substrates to the secretion apparatus at the inner membrane (Büttner et al. 2004, 2006). It was shown that effector proteins can be grouped into two classes according to their HpaB-dependency; while translocation of class A effector is abolished in the absence of HpaB, class B effectors are still translocated by the *hpaB* mutant, albeit in reduced amounts (Büttner et al. 2006). It is

Fig. 1. Schematic overview of the *hpaB*-*hrpF* region in different *Xanthomonas* species. **A**, Overview of the *hpaB*-*hrpF* region from *X. campestris* pv. *vesicatoria* 85-10 (*Xcv*, GenBank accession number AM039952). Genes are represented by arrows. Bars refer to the deletions that were generated in this study. **B**, Schematic representation of *hpaB*-*hrpF* regions from different sequenced *Xanthomonas* species. Gray areas represent homologous sequences (more than 75% amino acid sequence identity). Striped boxes indicate transposable elements. Sequences of the following xanthomonads were analyzed: *X. oryzae* pv. *oryzicola* (*Xoc*) RS105 (GenBank accession number AY875714), *X. oryzae* pv. *oryzae* (*Xoo*) strains PX099A (GenBank accession number AY536514), MAFF 311018 (GenBank accession number AP008229), and KACC10331 (GenBank accession number AE013598), and *X. campestris* pv. *campestris* 8004 (*Xcc*, GenBank accession number CP000050). Due to space limitations, only part of *hrpF* from *X. oryzae* pv. *oryzae* MAFF 311018 and KACC10331 is shown.

noteworthy that specific T3S chaperones of individual effectors have not yet been described in *X. campestris* pv. *vesicatoria*. Genomic sequence analysis revealed that most known or predicted type III effector genes lack putative T3S chaperone genes in their vicinity (Thieme et al. 2005). Exceptions are the effector proteins XopF1 and XopF2, which share 60% amino acid sequence identity (Roden et al. 2004; D. Büttner and U. Bonas, unpublished data). XopF1 is encoded in the *hrp* region, between *hpaB* and *hrpF*, whereas XopF2 is encoded elsewhere on the chromosome.

In this study, we report on the analysis of the *hpaB-hrpF* region, which is highly sequence-variable among different xanthomonads. We identified the novel virulence factor HpaE and investigated the role of two *hpa* genes, *hpaD* and *hpaI*, flanking *xopF1*. The analysis of XopF1 secretion and translocation suggests that T3S in *X. campestris* pv. *vesicatoria* is not only controlled by the global T3S chaperone HpaB but also involves the assistance of additional Hpa proteins.

RESULTS

Sequence analysis of the *hpaB-hrpF* region.

Sequence and codon preference analysis of the *hrp* region between *hpaB* and *hrpF* in *X. campestris* pv. *vesicatoria* 85-10 led to the identification of four open reading frames (ORF), termed *hpaE*, *hpaD*, *hpaI*, and *xopF1*, which probably belong to two different operons (Fig. 1A). Database analyses using BLASTP and 3D-PSSM programs revealed that the corresponding predicted gene products do not share sequence or structural similarity with proteins of known functions. *hpaD* and *hpaI* are located adjacent to the type III effector gene *xopF1* and encode predicted proteins of 53 and 69 amino acids, respectively, with a low isoelectric point and a high content of leucine residues. *hpaD* and *hpaI* gene products are homologous to the N and C terminus, respectively, of a putative protein (XCV2943) encoded next to *xopF2*, which is located outside of the *hrp* region (Table 1). *xopF2* encodes an effector protein that shares 60% amino acid sequence identity with XopF1 (Roden et al. 2004).

The availability of DNA sequences from different *Xanthomonas* strains allowed comparative sequence analyses of the region between *hpaB* and *hrpF* and revealed that HpaE, HpaD,

HpaI, and XopF1 share homology with predicted proteins encoded in the *hpaB-hrpF* regions of different *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains. Furthermore, the C terminus of XopF1 is homologous to a putative protein encoded in the *hpaB-hrpF* region of *X. campestris* pv. *campestris* (Fig. 1B; Table 1). Notably, homologs of *hpaE*, *hpaD*, *hpaI*, and *xopF1* are not present in the *hpaB-hrpF* region of *X. axonopodis* pv. *glycines* and *X. axonopodis* pv. *citri*, indicating that this region is not highly conserved among different *Xanthomonas* strains. This is in contrast to the overall high conservation in the sequence and order of genes within the *hrp* operons *hrpA* to *hrpE*.

Expression of *hpaE* is regulated by HrpG and HrpX.

To analyze *hpaE* expression, we performed reverse transcription-polymerase chain reaction (RT-PCR)-based gene expression studies with strains 85-10, 85*, and 85* Δ *hrpX*, which differ in *hrp* gene expression. Strain 85* is a derivative of strain 85-10 and contains *hrpG**, a mutated version of the key regulatory gene *hrpG* that leads to constitutive expression of the *hrp* gene cluster (Wengelnik et al. 1999). Strain 85* Δ *hrpX* is deleted in the regulatory gene *hrpX*. Using *hpaE*-specific primers, a transcript of the expected size was amplified from strain 85* but not from strains 85-10 and 85* Δ *hrpX*, indicating that expression of *hpaE* is regulated by *hrpG* and *hrpX* (Fig. 2A). Since *hpaE* is located directly downstream of the global T3S chaperone gene *hpaB*, we investigated by RT-PCR whether both genes are cotranscribed. Using gene-specific primers, we could amplify a transcript that spans *hpaB* and *hpaE* in strain 85* (Fig. 2B). This indicates that *hpaB* and *hpaE* belong to the same operon. By contrast, the pilus gene *hrpE* that is located upstream of *hpaB* is part of a separate operon (Weber et al. 2007).

To investigate whether HpaE is secreted by the T3S system, we expressed *hpaE* as a C-terminally c-Myc epitope-tagged derivative in strain 85*. As mentioned above, strain 85* contains the *hrpG** mutation that is crucial for *hrp* gene expression under secretion-inducing conditions (Rossier et al. 1999). When bacteria were incubated in secretion medium, a protein of the expected size (9.1 kDa + 5-kDa c-Myc epitope) was detected by a c-Myc epitope-specific antibody in total bacterial cell extract but not in the culture supernatant (Fig. 2C). The blot was reincubated with an antibody against the secreted protein HrpF. As

Table 1. Characteristics of proteins encoded in the *Xanthomonas campestris* pv. *vesicatoria* *hpaB-hrpF* region

Protein ^a	Characteristics ^b	Closest homologs ^c	% ^d
HpaE (CAJ22046)	86 aa; pI = 9.3; 12.8% leucine	HrpE3 (<i>X. oryzae</i> pv. <i>oryzicola</i> RS105; ABH07404) ^e	82/84
HpaD(CAJ22043)	53 aa; pI = 5.5; 15.1% leucine	Hpa3 (<i>X. oryzae</i> pv. <i>oryzae</i> PX099A; AAX14048) ^e	92/92
		Hypothetical protein XOO0104 (<i>X. oryzae</i> pv. <i>oryzae</i> MAFF 311018; BAE66859) ^e	92/92
		Hpa3 (<i>X. oryzae</i> pv. <i>oryzicola</i> RS105; ABH07392) ^e	92/92
		Hypothetical protein XCV2943 (<i>X. campestris</i> pv. <i>vesicatoria</i> 85-10; AAV74204)	57/65
HpaI (CAJ22044)	69 aa; pI = 4.3; 23.2% leucine	Hpa3 (<i>X. oryzae</i> pv. <i>oryzae</i> PX099A; AAX14048) ^e	97/98
		Hypothetical protein XOO0104 (<i>X. oryzae</i> pv. <i>oryzae</i> MAFF 311018; BAE66859) ^e	97/98
		Hpa3 (<i>X. oryzae</i> pv. <i>oryzicola</i> RS105; ABH07392) ^e	94/98
		Hypothetical protein XCV2943 (<i>X. campestris</i> pv. <i>vesicatoria</i> 85-10; AAV74204)	76/92
XopF1 (CAJ22045)	670 aa; pI = 8.8; 9.5% leucine	Hypothetical protein XOO0074 (<i>X. oryzae</i> pv. <i>oryzae</i> KACC10331; AAW73328) ^e	82/86
		Hypothetical protein XOO0103 (<i>X. oryzae</i> pv. <i>oryzae</i> MAFF 311018; BAE66858) ^e	82/86
		Hpa4 (<i>X. oryzae</i> pv. <i>oryzae</i> PX099A; AAX14049) ^e	81/86
		Hpa4 (<i>X. oryzae</i> pv. <i>oryzicola</i> RS105; ABH07393) ^e	78/83
		XopF2 (<i>X. campestris</i> pv. <i>vesicatoria</i> 85-10; AAV74205)	63/73
		Conserved hypothetical protein XC_3024 (<i>X. campestris</i> pv. <i>campestris</i> 8004; AAY50072) ^{e,f}	69/81

^a GenBank accession no. in parentheses.

^b aa, amino acids; refers to the predicted size of the encoded protein; pI, isoelectric point.

^c Homologous proteins were found using BLASTP in nonredundant databases based on the matrix BLOSUM 62. Organism and GenBank accession no. in parentheses.

^d Percent Identity/similarity. Amino acid identities and similarities were determined using BLAST 2 based on the matrix BLOSUM 62.

^e These proteins are encoded in the *hrp* gene cluster.

^f Homology is restricted to the C terminus of XopF1.

HrpF was detected in the culture supernatant (Fig. 2C), we conclude that T3S had occurred but that HpaE-c-Myc is not secreted or not detectable in the culture supernatant.

To analyze a potential contribution of HpaE to T3S and virulence, we deleted *hpaE* from the genome of *X. campestris* pv. *vesicatoria* strains 85-10 and 85*, using the suicide plasmid

Fig. 2. *hpaE* belongs to the *hrpG* regulon but is dispensable for type III secretion (T3S). **A**, Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *hpaE* from *Xanthomonas campestris* pv. *vesicatoria* strains 85-10, 85*, and 85*Δ*hrpX*. RNA was isolated from bacteria grown in NYG medium (Daniels et al. 1984). 16S ribosomal DNA (rDNA) was amplified as a constitutive control. DNA fragments were separated on a 1% agarose gel and were stained with ethidium bromide. **B**, *hpaB* and *hpaE* are cotranscribed. RT-PCR analysis of *X. campestris* pv. *vesicatoria* 85* was performed as described in A, using primers that anneal to the 5' end of *hpaB* and the 3' end of *hpaE*, respectively. Water (-) and genomic DNA from strain 85* (DNA) served as negative and positive controls, respectively. **C**, In vitro secretion analysis of HpaE. Total cell extracts (TE) and culture supernatants (SN) of *X. campestris* pv. *vesicatoria* 85* carrying the empty vector (-) or a *hpaE*-c-myc expression construct (+) were analyzed by immunoblotting, using a c-Myc epitope-specific antibody. To demonstrate that T3S had occurred, the blot was reprobed with an antibody against HrpF. The upper band detected by the antibody corresponds to HrpF; lower bands are degradation products. **D**, In vitro T3S is not affected in *hpaE* deletion mutants. In vitro secretion of HrpF and AvrBs3 was analyzed in *hpaE* wild-type (wt) and *hpaE* deletion mutant (Δ*hpaE*) strains. The following strains were used: 85* and 85*Δ*hpaE* for HrpF secretion and 82* and 82*Δ*hpaE* for AvrBs3 secretion. Bacteria were incubated in secretion medium and TE and SN were analyzed by immunoblotting, using specific antibodies against HrpF and AvrBs3, respectively. The upper band detected by the AvrBs3-specific antibody corresponds to AvrBs3; lower bands are degradation products. Blots were routinely reacted with an antibody against the cytoplasmic protein HrcN to ensure that no bacterial lysis had occurred (data not shown).

pOK1 (Fig. 1; discussed below). Total protein extracts and culture supernatants of bacteria grown under secretion-permissive conditions were analyzed by immunoblotting, using a polyclonal antibody directed against the translocon protein HrpF. Figure 2D shows that comparable amounts of HrpF were detected in the culture supernatants of strains 85* and 85*Δ*hpaE*. We also analyzed the secretion of the effector protein AvrBs3 in *X. campestris* pv. *vesicatoria* strains 82* and 82*Δ*hpaE*, which are derivatives of strain 82-8 and express *avrBs3* from an endogenous plasmid (Table 2). Since secretion of AvrBs3 was not affected in strain 82*Δ*hpaE* (Fig. 2D), we conclude that HpaE is not a general regulator of T3S.

HpaE is a novel virulence factor.

Next, we investigated the contribution of *hpaE* to the host-pathogen interaction in vivo. Strains 85-10 and 85-10Δ*hpaE* were infiltrated into leaves of the susceptible pepper line Early Cal Wonder (ECW) and the resistant pepper line ECW-10R. ECW-10R plants carry the *BsI* resistance gene and induce the HR upon recognition of the effector protein AvrBs1, which is delivered by strain 85-10 (Escobar et al. 2001; Minsavage et al. 1990). Strain 85-10Δ*hpaE* displayed reduced bacterial growth and reduced disease symptom formation in susceptible plants and a partial and delayed HR in resistant plants when compared with the wild-type strain 85-10 (Fig. 3A and B). The mutant phenotype was complemented by plasmid pDM*hpaE*, which encodes HpaE-c-Myc (Fig. 3A and B). We therefore conclude that *hpaE* significantly contributes to pathogenicity and that the C-terminal c-Myc epitope did not interfere with protein function.

To confirm these observations, we analyzed the in vivo translocation of two effector proteins, XopC and XopJ, in the *hpaE* deletion mutant 85-10Δ*hpaE*. For this, we used the reporter protein AvrBs3Δ2, which is a derivative of the effector protein AvrBs3. AvrBs3Δ2 lacks amino acids 2 to 152 and, thus, the T3S and translocation signal. However, it still induces the HR in AvrBs3-responsive ECW-30R pepper plants when fused to a functional T3S and translocation signal (Noël et al. 2003; Szurek et al. 2002). When XopC₁₋₂₀₀-AvrBs3Δ2 and XopJ₁₋₁₅₅-AvrBs3Δ2, respectively, were delivered by the wild-type strain 85-10, they induced the AvrBs3-specific HR in ECW-30R pepper plants as expected (Fig. 3C) (Noël et al. 2003). HR induction was severely reduced when both proteins were delivered by strain 85-10Δ*hpaE*, suggesting that *hpaE* is required for the efficient translocation of both effector proteins (Fig. 3C).

XopF1 and XopF2 do not significantly contribute to bacterial virulence.

In addition to *hpaE*, we studied a possible virulence function of the putative *xopF1* operon encoding HpaD, HpaI, and XopF1. We deleted all three genes from the genome of *X. campestris* pv. *vesicatoria* 85-10 (Fig. 1; discussed below). The resulting deletion mutant strain 85-10Δ*EF* displayed a wild-type phenotype when infiltrated into susceptible ECW and resistant ECW-10R plants, respectively (data not shown). To investigate a possible functional redundancy due to homologous genes, we also deleted *xopF2* and the flanking ORF *XCV2943* in strain 85-10Δ*EF*. Since the resulting multiple mutant strain 85-10Δ*EF*Δ*xopF2* also behaved like the wild type in infection tests (data not shown), we conclude that the *xopF1* and *xopF2* regions do not play an obvious role in the bacterial interaction with the host plant under the conditions tested. Our data are in agreement with the observation that deletion of single or multiple effectors often does not significantly affect bacterial pathogenicity (Grant et al. 2006; Noël et al. 2003; Roden et al. 2004).

hpaD, *hpaI*, and *xopF1* belong to a separate operon.

To study the expression of *hpaD*, *hpaI*, and *xopF1*, we performed RT-PCR analyses as described above, using gene-specific primers. Figure 4A shows that *hpaD*, *hpaI*, and *xopF1* transcripts were amplified from strain 85* but not, or only in significantly reduced amounts, from strains 85-10 and 85* Δ *hrpX*, indicating that expression of all three genes is regulated by *hrpG* and *hrpX*. We could also amplify transcripts that span *hpaD* and *hpaI* as well as *hpaI* and the 5' end of *xopF1*, respectively, from strain 85* (Fig. 4B). These results suggest that *hpaD*, *hpaI*, and *xopF1* belong to the same operon.

For promoter studies, we fused a fragment starting 325 bp upstream of the predicted translation initiation start codon of *hpaD* and including *hpaI* to a promoterless β -glucuronidase (GUS) gene (*uidA*) in the broad-host-range vector pLAFR6. The resulting construct pLhpaDGUS was introduced into *X. campestris* pv. *vesicatoria* strains 85-10, 85* and 85* Δ *hrpX*. When bacteria were grown in NYG medium (Daniels et al. 1984) in which *hrp* gene expression of the wild-type strain 85-10 is not activated, GUS activity in strain 85* was 26-fold higher than in strains 85-10 and 85* Δ *hrpX* (Fig. 4C). No GUS activity was detectable for construct pLhpaIGUS encompass-

ing *hpaI* and the 5' 449 bp of *xopF1* fused to *uidA* (Fig. 4C). These observations confirm that *hpaD*, *hpaI*, and *xopF1* are cotranscribed as an operon and show that the promoter located upstream of *hpaI* is regulated by HrpX. This is corroborated by the presence of a PIP (plant-inducible promoter; consensus TTCGC-N15-TTCGC) box-like element (TTCGC-N5-TTCGC) 85 bp upstream of the putative translation start codon of *hpaD*. The PIP box is a conserved DNA motif that is present in the promoter regions of several but not all operons regulated by HrpG and HrpX and provides the binding site for HrpX (Fenselau and Bonas 1995; Koebnik et al. 2006; Tsuge et al. 2005; Wengelnik and Bonas 1996).

HpaD and HpaI are cytoplasmic proteins.

Next, we investigated whether HpaD and HpaI are secreted by the T3S system. Since both proteins are very small (5.5 and 7.5 kDa, respectively), they were difficult to detect as His₆ and c-Myc epitope-tagged derivatives (data not shown). We therefore expressed *hpaD* and *hpaI* as fusion partners of HrpF Δ N, which is a deletion derivative of the translocon protein HrpF and is detectable by a specific anti-HrpF polyclonal antiserum. HrpF Δ N is deleted in amino acids 2 to 152 and thus lacks the

Table 2. Bacterial strains and plasmids used in this study

Relevant characteristics ^a		Reference or source
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>		
85-10	Pepper-race 2; wild type; Rif ^r	Canteros 1990
82-8	Pepper-race 1; wild type; Rif ^r	Minsavage et al. 1990
82*	82-8 derivative containing the <i>hrpG</i> * mutation	Wengelnik et al. 1999
85*	85-10 derivative containing the <i>hrpG</i> * mutation	Wengelnik et al. 1999
85* Δ <i>hpaB</i>	<i>hpaB</i> deletion mutant of strain 85*	Büttner et al. 2004
85* Δ <i>hrpF</i>	<i>hrpF</i> deletion mutant of strain 85*	Büttner et al. 2002
85-10 Δ <i>hrpG</i>	<i>hrpG</i> deletion mutant of strain 85-10	Wengelnik et al. 1999
85* Δ <i>hrpX</i>	<i>hrpX</i> deletion mutant of strain 85*	Noël et al. 2001
85* Δ <i>hrcV</i>	<i>hrcV</i> deletion mutant of strain 85*	Rossier et al. 1999
85* Δ L	Derivative of strain 85* deleted in the left <i>hrp</i> gene flanking region	Noël et al. 2002
85* <i>hpaH</i> -oof	<i>hpaH</i> frameshift mutant of strain 85*	Noël et al. 2002
85-10 <i>hpaH</i> -oof	<i>hpaH</i> frameshift mutant of strain 85-10	Noël et al. 2002
85* Δ <i>xopA</i>	<i>xopA</i> deletion mutant of strain 85*	Noël et al. 2002
<i>Escherichia coli</i>		
DH5 α	F ⁻ <i>recA hsdR17</i> (r _k ⁻ , m _k ⁺) Φ 80 <i>dlacZ</i> Δ M15	Bethesda Research Laboratories, Bethesda, MD, U.S.A.
DH5 α λ pir	F ⁻ <i>recA hsdR17</i> (r _k ⁻ , m _k ⁺) Φ 80 <i>dlacZ</i> Δ M15 [<i>λpir</i>]	Ménard et al. 1993
BL21(DE3)	F ⁻ <i>ompT hsdS20</i> (r _b ⁻ , m _b ⁻) <i>gal</i>	Stratagene, Heidelberg, Germany
Plasmids		
pBluescript(II) KS	Phagemid, pUC derivative; Ap ^r	Stratagene
pUC119	ColE1 replicon; Ap ^r	Vieira and Messing 1987
pC3003	pUC19 containing a triple c-myc tag; Ap ^r	J. Kämper
pDSK602	Broad-host-range vector; contains triple <i>lacUV5</i> promoter; Sm ^r	Murillo et al. 1994
pDSK604	Derivative of pDSK602 with modified polylinker	Escobar et al. 2001
pBGUS	pBluescript(II) KS derivative containing the <i>E. coli uidA</i> gene	Escobar et al. 2001
pGEX-2TKM	GST expression vector; p _{lac} GST <i>lacI</i> ^q pBR322 <i>ori</i> ; Ap ^r , derivative of pGEX-2TK with polylinker of pDSK604	Stratagene
pOK1	Suicide vector; <i>sacB sacQ mobRK2 oriR6K</i> ; Sm ^r	Escobar et al. 2001
pRK2013	ColE1 replicon, TraRK ⁺ Mob ⁺ ; Km ^r	Huguet et al. 1998
pLAFR3	RK2 replicon, Mob ⁺ Tra ⁻ ; contains p _{lac} ; Tc ^r	Figurski and Helinski 1979
pLAFR6	RK2 replicon, Mob ⁺ Tra ⁻ ; multicloning site flanked by transcription terminators; Tc ^r	Staskawicz et al. 1987
pXV2	pLAFR3 derivative, contains the <i>hrpB-hrpF</i> region from <i>X. campestris</i> pv. <i>vesicatoria</i> 75-3	Bonas et al. 1989
pUS356F	pUC119 containing <i>avrBs3Δ2</i> fused to a FLAG epitope-coding sequence	Bonas et al. 1991
pDS356F	pDSK602 derivative encoding AvrBs3Δ2; FLAG-tagged	Szurek et al. 2002
pDS300F	pDSK602 derivative encoding AvrBs3; FLAG-tagged	Szurek et al. 2002
pUhrpFΔN	pUC119 derivative containing <i>hrpF</i> deleted in the first 456 nucleotides	Van den Ackerveken et al. 1996
pDhrpFΔN	pDSK602 derivative expressing HrpF deleted in the first 152 amino acids	Büttner et al. 2002
pDxopF1356	pDSK602 derivative encoding XopF1 ₁₋₂₀₀ -AvrBs3Δ2	Büttner et al. 2002
pLhpaH	pLAFR3 derivative encoding HpaH	Büttner et al. 2006
pL6avrBs3356	pLAFR6 derivative encoding AvrBs3 ₁₋₂₀₀ -AvrBs3Δ2 under control of the native promoter	L.N. and U.B., unpublished
pL6xopC356	pLAFR6 derivative encoding XopC ₁₋₂₀₀ -AvrBs3Δ2 under control of the native promoter	Noël et al. 2003
pL6xopJ356	pLAFR6 derivative encoding XopJ ₁₋₁₅₅ -AvrBs3Δ2 under control of the native promoter	Noël et al. 2003

^a Ap = ampicillin; Km = kanamycin; Rif = rifampicin; Sm = spectinomycin; Tc = tetracycline; and the superscript r = resistant.

Fig. 3. *hpaE* contributes to the interaction of *Xanthomonas campestris* pv. *vesicatoria* with pepper plants. **A**, Disease symptoms and the hypersensitive response (HR) induced by *X. campestris* pv. *vesicatoria* wild-type and *hpaE* mutant strains. Strains 85-10, 85-10 $\Delta hpaE$, and 85-10 $\Delta hpaE$ (pDMhpaE) were infiltrated into leaves of susceptible Early Cal Wonder (ECW) and resistant ECW-10R pepper plants. Plasmid pDMhpaE encodes HpaE-c-Myc. Disease symptoms were photographed five days after infiltration. For better visualization of the HR, leaves of ECW-10R plants were bleached in ethanol two days after infiltration. Dashed lines indicate the infiltrated areas. **B**, In planta growth of the *hpaE* deletion mutant strain 85-10 $\Delta hpaE$ is reduced. Strains 85-10, 85-10 $\Delta hpaE$, 85-10 $\Delta hpaE$ (pDMhpaE), and 85-10 $\Delta hrpG$ (negative control) were infiltrated at a density of 10^4 CFU/ml into susceptible ECW pepper plants, and bacterial growth was determined over a period of 7 days. Values are the mean of three samples from three plants. Error bars represent standard deviations. The result of one representative experiment is shown. **C**, In vivo translocation of XopC₁₋₂₀₀-AvrBs3 Δ 2 and XopJ₁₋₁₅₅-AvrBs3 Δ 2 is reduced in the absence of *hpaE*. Strains 85-10 (wt) and 85-10 $\Delta hpaE$ ($\Delta hpaE$) delivering XopC₁₋₂₀₀-AvrBs3 Δ 2 and XopJ₁₋₁₅₅-AvrBs3 Δ 2, respectively, were inoculated into AvrBs3-responsive ECW-30R pepper plants. Leaves were bleached in ethanol three days after inoculation to better visualize the HR. Dashed lines indicate the infiltrated areas.

T3S signal (Büttner et al. 2002). To avoid detection of the native HrpF protein, HpaD- and HpaI-HrpF Δ N fusion proteins were expressed in the *hrpF* deletion mutant 85* $\Delta hrpF$, which is not pathogenic but secretes T3S substrates like the wild type (Rossier et al. 2000). Both fusion proteins were detected in total cell extracts by the HrpF-specific antibody when expressed under control of the *lac* or the native *hpaD/hpaI* promoter (Fig. 5A). When bacteria were incubated in secretion medium,

Fig. 4. *hpaD*, *hpaI*, and *xopF1* form an operon. **A**, Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *hpaD*, *hpaI*, and *xopF1* from *Xanthomonas campestris* pv. *vesicatoria* strains 85-10, 85*, and 85* $\Delta hrpX$ grown in NYG medium (Daniels et al. 1984). 16S ribosomal DNA (rDNA) was amplified as a constitutive control. DNA fragments were separated on a 1% agarose gel and were stained with ethidium bromide. **B**, *hpaD*, *hpaI*, and *xopF1* are transcribed as an operon. RT-PCR was performed with *X. campestris* pv. *vesicatoria* 85* grown in NYG medium. Transcripts spanning *hpaD* and *hpaI* as well as *hpaI* and the 5' end of *xopF1* were amplified using gene-specific primers. Water (-) and genomic DNA from strain 85* (DNA) served as negative and positive controls, respectively. **C**, Analysis of *hpaD* and *hpaI* promoter activity using the *uidA* reporter gene. *X. campestris* pv. *vesicatoria* strains 85-10, 85*, and 85* $\Delta hrpX$ carrying *uidA* fusion constructs as indicated were grown in NYG medium. β -glucuronidase (GUS) activities are the average of two independent cultures with duplicates; error bars represent the standard deviations. The result of one representative experiment is shown. One unit of GUS activity is defined as 1 nM of 4-methylumbelliferone released per minute per bacterium. CFU = colony forming units.

HpaD- and HpaI-HrpFAN fusion proteins were not detected in the culture supernatant (Fig. 5B), suggesting that HpaD and HpaI do not contain a functional T3S signal that targets HrpFAN for T3S. Lack of secretion was not due to the HrpFAN reporter protein, because a fusion protein between the N-terminal 200 amino acids of the effector XopF1 and HrpFAN was secreted (Fig. 5B).

In vitro secretion of XopF1 is independent of HpaD and HpaI.

Since HpaD and HpaI are small, acidic, and leucine-rich proteins that are not secreted by the T3S system, they share typical features of T3S chaperones. Often, specific T3S chaperones of effector proteins are encoded adjacent to their respective interaction partners (Feldman and Cornelis 2003; Ghosh 2004). To investigate a possible contribution of HpaD and HpaI to the stability and secretion of the effector protein XopF1, we raised a specific polyclonal antibody against XopF1 (discussed below). The antibody detected XopF1 in protein extracts from *Escherichia coli* strains carrying the *xopF1* expression construct but not in total cell extracts of *X. campestris* pv. *vesicatoria* 85* grown in vitro (Fig. 5C) or reisolated 24 h postinfection from susceptible pepper plants (data not shown). The lack of protein detection in strain 85* was presumably not due to a low sensitivity of the antibody given the ease of protein detection in strain 85* expressing *xopF1* under control of the *lac* promoter from plasmid pDxopF1 (Fig. 5C). We therefore reasoned that XopF1 is unstable or only weakly expressed in strain 85*.

For the analysis of XopF1 secretion, we introduced the *xopF1* expression construct pDxopF1 into *X. campestris* pv. *vesicatoria* strains 85*, 85* Δ EF, the T3S mutant 85* Δ hrpF, and 85* Δ hpaB, which is deleted in the global T3S chaperone gene *hpaB*. When bacteria were incubated in secretion medium, XopF1 was detected in the culture supernatant of strain 85* but not of strains 85* Δ hrpF and 85* Δ hpaB, indicating that secretion of XopF1 depends on both a functional T3S system and the global T3S chaperone HpaB (Fig. 5C). Notably, secretion of XopF1 was not affected in strain 85* Δ EF, which lacks

hpaD and *hpaI* (Fig. 5C). To rule out that the *hpaD/hpaI* homolog *XCV2943* present upstream of *xopF2* compensates for the loss of *hpaI* and *hpaD* in strain 85* Δ EF, the *xopF1* expression construct was introduced into strain 85* Δ EF Δ *xopF2* (discussed above). Figure 5D shows that XopF1 secretion was not affected in strain 85* Δ EF Δ *xopF2*, suggesting that HpaD, HpaI, and the predicted *XCV2943* gene product do not function as classical T3S chaperones of XopF1.

Efficient secretion of XopF1 depends on the putative lytic transglycosylase HpaH.

Next, we analyzed the influence of additional Hpa proteins on XopF1 secretion and found that secretion of XopF1 was significantly reduced in strain 85* Δ L, which is deleted in the left *hrp* gene-flanking region (Fig. 6A). This region encodes HpaH, which is homologous to lytic transglycosylases, and the secreted proteins XopD and XopA (Noël et al. 2002). It was previously

Fig. 5. Type III secretion assays with HpaD, HpaI, and XopF1. **A**, Expression analysis of HrpFAN fusion proteins in *Xanthomonas campestris* pv. *vesicatoria*. Protein extracts were prepared from strain 85* Δ hrpF carrying the empty vector (–) or plasmids expressing HrpF, HrpFAN, HpaD-HrpFAN, and HpaI-HrpFAN. All proteins were expressed under the control of the *lac* promoter (plac). Additionally, HpaD-HrpFAN and HpaI-HrpFAN were expressed under control of the native promoter (pnat). Bacteria were grown in NYG medium (Daniels et al. 1984), and similar protein amounts were analyzed by immunoblotting, using a HrpF-specific antibody. Bands corresponding to the respective fusion proteins are marked by asterisks; lower bands represent degradation products. **B**, In vitro secretion assays of HrpF and HrpFAN fusion proteins. *X. campestris* pv. *vesicatoria* 85* Δ hrpF carrying the empty vector (–) or plasmids expressing HrpF, HrpFAN, HpaD-HrpFAN, HpaI-HrpFAN, and XopF1₁₋₂₀₀-HrpFAN under control of the *lac* promoter was incubated in secretion medium. Total cell extracts (TE) and culture supernatants (SN) were analyzed by immunoblotting, using a HrpF-specific antibody. The protein bands corresponding to HrpF, HrpFAN, and the respective fusion proteins are marked by asterisks; lower bands are degradation products. **C**, In vitro secretion of XopF1 is independent of HpaD and HpaI. Strains 85* (wt), 85* Δ hrpF (Δ hrpF), 85* Δ hpaB (Δ hpaB), 85* Δ EF (Δ EF), and 85* Δ xopF2 (Δ xopF2) carrying the empty vector (–) or the XopF1 expression plasmid pDxopF1, as indicated, were incubated in secretion medium. TE and SN were analyzed by immunoblotting, using polyclonal antibodies directed against HrpF and XopF1, respectively. **D**, In vitro secretion analysis of XopF1 in strains 85* (wt) and 85* Δ EF Δ *xopF2* (Δ EF Δ *xopF2*), both carrying the XopF1 expression construct pDxopF1. TE and SN were analyzed as described in C.

shown that XopA and HpaH contribute to pathogenicity of *X. campestris* pv. *vesicatoria* (Noël et al. 2002). XopA is presumably involved in the formation of the T3S translocon, whereas the precise role of HpaH has not yet been elucidated.

To investigate whether the reduced secretion of XopF1 in strain 85* ΔL was due to the deletion of *hpaH* or *xopA*, we in-

roduced the *xopF1* expression plasmid into the *xopA* deletion mutant 85* $\Delta xopA$ and the *hpaH* frameshift mutant 85**hpaH*-oof, respectively. When bacteria were incubated in secretion medium, significantly reduced amounts of XopF1 were detected in the culture supernatant of strain 85**hpaH*-oof. By contrast, secretion of XopF1 in strain 85* $\Delta xopA$ was like wild type

Fig. 6. The efficient secretion and translocation of XopF1 depends on HpaH. **A**, HpaH contributes to the in vitro secretion of XopF1. In vitro secretion assays with *Xanthomonas campestris* pv. *vesicatoria* strains 85* (wt), 85* ΔL (ΔL), 85* $\Delta xopA$ ($\Delta xopA$), and 85**hpaH*-oof (*hpaH*-oof) carrying construct pDxopF1 or pDMxopC, respectively. Bacteria were incubated in secretion medium, and total protein extracts (TE) and culture supernatants (SN) were analyzed by immunoblotting, using antibodies directed against XopF1, HrpF, and the c-Myc epitope, respectively. **B**, Translocation of XopF1₁₋₂₀₀-AvrBs3 $\Delta 2$ is type III secretion-dependent. *X. campestris* pv. *vesicatoria* strains 85* (wt) and 85* $\Delta hrcV$ ($\Delta hrcV$) expressing AvrBs3, AvrBs3 $\Delta 2$, and XopF1₁₋₂₀₀-AvrBs3 $\Delta 2$, as indicated, were infiltrated into AvrBs3-responsive Early Cal Wonder (ECW)-30R plants. Leaves were bleached in ethanol two days after infiltration. Dashed lines indicate the infiltrated areas. **C**, The efficient translocation of XopF1₁₋₂₀₀-AvrBs3 $\Delta 2$ depends on HpaH. XopF1₁₋₂₀₀-AvrBs3 $\Delta 2$, AvrBs3₁₋₂₀₀-AvrBs3 $\Delta 2$, XopC₁₋₂₀₀-AvrBs3 $\Delta 2$, and XopJ₁₋₁₅₅-AvrBs3 $\Delta 2$ were delivered by strains 85-10 (wt) and 85-10*hpaH*-oof (*hpaH*-oof), as indicated. Translocation of XopF1₁₋₂₀₀-AvrBs3 $\Delta 2$ in strain 85-10*hpaH*-oof was complemented by construct pLhpaH. Bacteria were infiltrated into AvrBs3-responsive ECW-30R pepper plants at concentrations of 5×10^7 CFU/ml. Leaves were bleached in ethanol two days after infiltration. Dashed lines indicate the infiltrated areas.

(Fig. 6A). We also analyzed the secretion of the translocon protein HrpF and the effector protein XopC, which was synthesized as a C-terminally c-Myc epitope-tagged derivative. Interestingly, secretion of HrpF and XopC-c-Myc was not affected in strains 85* Δ L, 85* Δ xopA and 85**hpaH*-oof, respectively (Fig. 6A). Taken together, these results suggest that HpaH contributes to the secretion of XopF1 but is not required for the efficient secretion of HrpF and XopC.

HpaH is required for the efficient translocation of XopF1.

In addition to secretion assays, we performed in vivo translocation assays, using AvrBs3 Δ 2 as a reporter. It was previously shown that a fusion protein between the N-terminal 200 amino acids of XopF1 and AvrBs3 Δ 2 induces the AvrBs3-specific HR when delivered by *X. campestris* pv. *vesicatoria* 85*, suggesting that the N-terminal 200 amino acids of XopF1 contain a functional translocation signal (Fig. 6B) (Büttner et al. 2006). No HR induction was observed with a T3S mutant, which lacks the conserved *hrcV* gene, indicating that translocation of XopF1 is T3S-dependent (Fig. 6B). To investigate the contribution of HpaH to the translocation of XopF1, we introduced the XopF1₁₋₂₀₀-AvrBs3 Δ 2 expression construct into strains 85-10 and 85-10*hpaH*-oof, respectively. Fig. 6C shows that HR induction in ECW-30R pepper plants by strain 85-10*hpaH*-oof delivering XopF1₁₋₂₀₀-AvrBs3 Δ 2 was significantly reduced when compared to the wild-type strain 85-10. The mutant phenotype was complemented by plasmid pLhpaH, which expresses *hpaH* under control of the *lac* promoter (Fig. 6C).

We also analyzed the translocation of AvrBs3 Δ 2 fusion proteins containing the N termini of the effector proteins XopC, AvrBs3, and XopJ in strains 85-10 and 85-10*hpaH*-oof. The induction of the AvrBs3-specific HR by strain 85-10*hpaH*-oof delivering AvrBs3₁₋₂₀₀-AvrBs3 Δ 2 and XopC₁₋₂₀₀-AvrBs3 Δ 2 was like wild type at the given bacterial inoculation density (Fig. 6C), suggesting that HpaH does not significantly contribute to the translocation of AvrBs3 and XopC. By contrast, HR induction by strain 85-10*hpaH*-oof delivering XopJ₁₋₁₅₅-AvrBs3 Δ 2 was significantly reduced (Fig. 6C). The observed phenotypes were presumably not due to intrinsic properties of the fusion proteins that interfered with the recognition of the reporter, since it was previously shown that XopC₁₋₂₀₀-AvrBs3 Δ 2 and XopF1₁₋₂₀₀-AvrBs3 Δ 2 fusion proteins are both efficiently recognized, even at low bacterial inoculation densities (Büttner et al. 2006). We therefore conclude that HpaH specifically contributes to the translocation of XopF1 and XopJ. Notably, both effectors were recently grouped as class A effector proteins that depend on the global T3S chaperone HpaB for translocation. By contrast, AvrBs3 and XopC belong to class B, which is translocated even in the absence of HpaB (Büttner et al. 2006).

DISCUSSION

In this study, we characterized the *hpaB*-*hrpF* region from *X. campestris* pv. *vesicatoria*. This region contains *hpaE*, *hpaD*, *hpaI*, and *xopF1*, which are coexpressed with the *hrp* genes, suggesting that they are involved in the host-pathogen interaction. Indeed, mutant studies revealed that *hpaE* is a novel virulence factor in *X. campestris* pv. *vesicatoria*. HpaE contributes to bacterial growth and disease symptom formation in susceptible plants and to the HR induction in resistant plants. In vitro secretion assays gave no hints that HpaE is secreted by the T3S system, suggesting that HpaE acts in the bacterial cytoplasm. Since deletion of *hpaE* has no obvious effect on T3S in vitro (Fig. 2) but affects bacterial pathogenicity, we propose that HpaE contributes to effector protein translocation. So far, it is not clear how the translocation of effector proteins across the plant plasma membrane can be controlled by a protein in

the bacterial cytosol. It is possible that HpaE interacts with components of the secretion apparatus and, thus, indirectly contributes to the formation of extracellular structures of the T3S system, such as the insertion of the translocon into the plant plasma membrane. In *X. campestris* pv. *vesicatoria*, there is already one known example of a cytoplasmic protein that controls protein translocation: the global T3S chaperone HpaB inhibits translocation of the putative translocon proteins HrpF and XopA. Notably, however, in vitro secretion of HrpF and XopA is not affected in the absence of HpaB (Büttner et al. 2004). This is reminiscent of our finding that deletion of *hpaE* affects the translocation but not the in vitro T3S of effector proteins.

In addition to *hpaE*, we analyzed the role of *hpaD*, *hpaI*, and *xopF1*. Since HpaD and HpaI are small, acidic, and leucine-rich and are not secreted by the T3S system, they display typical features of T3S chaperones (Feldman and Cornelis 2003). Genomic sequence analysis of strain 85-10 revealed that HpaD, HpaI, and the predicted homolog encoded next to *xopF2* are the only known candidates for specific class IA T3S chaperones of *X. campestris* pv. *vesicatoria* that are encoded next to type III effectors (Thieme et al. 2005). However, our data do not support a role of these proteins as classical T3S chaperones of XopF1. In vitro secretion assays revealed that the secretion of XopF1 is independent of HpaD and HpaI but requires the presence of the global T3S chaperone HpaB (Fig. 5C). Similar findings were previously reported for the in vivo translocation of a XopF1₁₋₂₀₀-AvrBs3 Δ 2 fusion protein (Büttner et al. 2006). Since XopF1 interacts with HpaB, it is conceivable that T3S and translocation of XopF1 is governed by HpaB rather than by specific T3S chaperones (Büttner et al. 2006).

The precise role of *hpaD* and *hpaI* remains to be elucidated. Since both genes are homologous to a single continuous ORF (*XCV2943*) in the genome of *X. campestris* pv. *vesicatoria* as well as in the *hrp* gene clusters of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains, it was suggested that they result from the insertion of a stop codon into an ancestral ORF and are, thus, pseudogenes (Fig. 1) (Sugio et al. 2005). However, we show here that *hpaD* and *hpaI* are transcribed and contain functional translation initiation start sites that allowed the synthesis of HpaD-HrpFAN and HpaI-HrpFAN fusion proteins when expressed under control of the native promoter (Fig. 5A). Therefore, we conclude that *hpaD* and *hpaI* are indeed expressed.

Interestingly, in addition to HpaB, the efficient secretion of XopF1 depends on HpaH, which is encoded in the left *hrp* gene-flanking region and is conserved among xanthomonads. HpaH shares sequence identity with lytic transglycosylases and contains a predicted signal peptide and a signal cleavage site between amino acids 34 and 35, suggesting that it is localized in the periplasm (prediction with SignalP). Lytic transglycosylases are periplasmic proteins that are associated with macromolecular transport systems in gram-negative bacteria. They are thought to mediate rearrangements in the peptidoglycan layer and presumably facilitate the assembly of membrane-spanning secretion systems (Koraimann 2003). In most known cases, however, predicted lytic transglycosylases do not significantly contribute to bacterial pathogenicity (Allaoui et al. 1993; Zhu et al. 2000). Exceptions are the HpaH proteins from *X. campestris* pv. *vesicatoria* and *X. axonopodis* pv. *glycines*, which contribute to both pathogenicity and HR induction (Kim et al. 2003; Noël et al. 2002). However, a lytic transglycosylase activity has so far not been demonstrated for HpaH and homologous proteins.

We previously reported that HpaH from *X. campestris* pv. *vesicatoria* is not required for the efficient secretion of the translocon protein HrpF and the effector protein AvrBs3 (Noël et al.

2002). This is also the case for the secretion of the effector protein XopC (Fig. 6A) and was confirmed by an HR-based in vivo translocation assay. By contrast, translocation of XopF1 and XopJ was reduced in the absence of HpaH as compared with the wild-type strain (Fig. 6C), suggesting that HpaH specifically contributes to the secretion and translocation of a certain set of effectors, including XopF1 and XopJ. Our findings are reminiscent of the HpaB-dependent translocation of XopF1 and XopJ, which both belong to class A of effector proteins and strictly depend on HpaB for translocation. By contrast, AvrBs3 and XopC were grouped as class B effectors that are translocated even in the absence of HpaB (Büttner et al. 2006). In future studies, we will investigate whether HpaH specifically contributes to the translocation of other class A effectors and whether there is a physical interaction between HpaH and class A effectors or components of the secretion apparatus.

In summary, our data suggest that the efficient T3S and translocation of effector proteins in *X. campestris* pv. *vesicatoria* depends on the global T3S chaperone HpaB but also involves additional Hpa proteins. A similar control mechanism has not been reported for any other plant-pathogenic bacterium.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids.

Published bacterial strains and plasmids that were used in this study are described in Table 2. *X. campestris* pv. *vesicatoria* strains were cultivated at 30°C in complex medium NYG (Daniels et al. 1984) or in minimal medium A (Ausubel et al. 1996) supplemented with sucrose (10 mM) and casamino acids (0.3%). *E. coli* cells were cultivated at 37°C in Luria-Bertani medium. Plasmids were introduced into *E. coli* by electroporation and into *X. campestris* pv. *vesicatoria* by conjugation, using pRK2013 as a helper plasmid in triparental matings (Figurski and Helinski 1979). Antibiotics were added to the media at the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 25 µg/ml; rifampicin, 100 µg/ml; spectinomycin, 100 µg/ml; tetracycline, 10 µg/ml.

Plant material and plant inoculations.

The near-isogenic pepper cultivars ECW, ECW-10R (contains the *Bs1* resistance gene), and ECW-30R (contains the *Bs3* resistance gene; Minsavage et al. 1990) were grown and inoculated with *X. campestris* pv. *vesicatoria* as described (Bonas et al. 1991). Bacteria were hand-infiltrated into the intercellular spaces of fully expanded leaves at concentrations of 2×10^8 CFU/ml in 1 mM MgCl₂, if not stated otherwise. The appearance of disease symptoms and the HR was scored over a period of 5 days after inoculation. For better visualization of the HR, leaves were bleached in ethanol. For in planta growth curves, bacteria were inoculated at a density of 10^4 CFU/ml into leaves of susceptible pepper ECW plants. Bacterial growth was determined as described (Bonas et al. 1991). Experiments were repeated at least three times.

Generation of deletion mutants.

To delete *hpaE*, 1.1-kb upstream and 1-kb downstream sequences were amplified by PCR from *X. campestris* pv. *vesicatoria* 85-10. PCR products were digested with *EcoRV*/*EcoRI* and *EcoRI*/*BamHI*, respectively, and ligated into the *SmaI*/*BamHI* sites of the suicide plasmid pOK1. The resulting construct pOKΔ*hpaE* was conjugated into strains 85-10 and 85* as described (Huguet et al. 1998). Double crossing-over events resulted in strains 85-10Δ*hpaE* and 85*Δ*hpaE*.

For the generation of a triple deletion mutant in *hpaD*, *hpaI*, and *xopF1*, a 7.5-kb *BamHI*/*EcoRI* fragment, which was derived from cosmid pXV2 and contained the *hpaB*-*hrpF* region,

was cloned into pUC119, was digested with *ClaI*, and was religated. In the resulting construct pUΔEF, the 635-bp upstream region, *hpaD*, *hpaI*, and the first 1,853 bp of *xopF1* are deleted (Fig. 1A). The 4.6-kb insert of pUΔEF was cloned into pOK1 and was introduced into strains 85-10 and 85*, resulting in 85-10ΔEF and 85*ΔEF, respectively.

For the deletion of *xopF2* and the upstream ORF *XCV2943*, sequences of 1 kb upstream of *XCV2943* and downstream of *xopF2*, respectively, were amplified from *X. campestris* pv. *vesicatoria* 85-10. PCR products were digested with *Sall*/*SpeI* and *SpeI*/*XbaI* and were cloned into the *Sall* and *XbaI* sites of pOK1. The resulting construct pOKΔ*xopF2* was introduced into strains 85-10ΔEF and 85*ΔEF, producing 85-10ΔEFΔ*xopF2* and 85*ΔEFΔ*xopF2*, respectively.

Sequences of primers used in this study are available upon request.

RT-PCR analysis.

RNA extraction, cDNA synthesis, and RT-PCR analysis were performed as described (Noël et al. 2001).

Construction of promoter-GUS fusions.

For the construction of promoter-GUS fusions, the *uidA* gene encoding GUS was excised from pBGUS (Table 2) and was introduced into the *BamHI*/*HindIII* sites of pUC119, giving pUGUS. A 780-bp insert spanning *hpaD*, *hpaI*, and a 325-bp upstream region was ligated into the *SacI*/*SmaI* sites of pUGUS. The insert of the resulting construct was cloned into the *EcoRI*/*HindIII* sites of pLAFR6, giving pLhpaDGUS. For the generation of construct 2, a 650-bp *PstI* fragment spanning *hpaI* and the first 449 bp of *xopF1* was cloned into pBGUS and the resulting *uidA* fusion was introduced into the *EcoRI*/*HindIII* sites of pLAFR6, giving pLhpaIGUS. GUS assays were performed with exponentially growing *X. campestris* pv. *vesicatoria* strains as described (Rossier et al. 1999). One GUS unit is defined as 1 nmol of 4-methylumbelliferone released per minute per bacterium. Experiments were repeated at least three times.

Generation of protein expression constructs.

To generate a HpaE-c-Myc expression construct, *hpaE* was amplified from *X. campestris* pv. *vesicatoria* 85-10 by PCR. The PCR product was cloned into the *EcoRI*/*SacI* sites of pC3003, in-frame with a triple-c-myc epitope-encoding sequence. The resulting insert was introduced into the *EcoRI*/*XhoI* sites of pDSK604, giving pDMhpaE.

For the generation of a GST-XopF1 fusion protein, *xopF1* was amplified from strain 85-10 by PCR and was cloned into the *EcoRI*/*SacI* sites of pGEX-2TKM, giving pGxopF1. For expression of *xopF1* in *X. campestris* pv. *vesicatoria*, the *EcoRI*/*SacI* insert of pGxopF1 was subcloned into pUC119 and was introduced as an *EcoRI*/*HindIII* fragment into pDSK602, giving pDxopF1.

To synthesize a C-terminally c-Myc epitope-tagged derivative of XopC, *xopC* was amplified from *X. campestris* pv. *vesicatoria* 85-10 and was cloned into the *EcoRI*/*SmaI* sites of pC3003. The resulting insert was introduced into the *EcoRI*/*HindIII* sites of pDSK604, giving pDMxopC.

To generate HpaD-HrpFΔN, *hpaD* was amplified from strain 85-10 by PCR and was cloned into the *EcoRI*/*XhoI* sites of plasmid pDhrpFΔN, giving pDhpaDhrpFΔN. To express *hpaD*-*hrpF*ΔN under control of the *hpaD* promoter, *hpaD* and 325 bp of upstream sequence were amplified by PCR and were cloned into the *EcoRI*/*XhoI* sites of pUhrpFΔN (Table 2). The resulting insert was ligated into pLAFR6, giving pL6hpaDhrpFΔN. To construct HpaI-HrpFΔN, a 323-bp fragment containing *hpaI* was amplified from strain 85-10 and was cloned into the

EcoRI/XhoI sites of pUhrpFAN. The resulting insert was ligated into the *EcoRI/HindIII* sites of pLAFR3, giving pLhpalhrpFAN. Similarly, a fragment spanning *hpaI*, *hpaD*, and 325 bp of upstream sequence was amplified by PCR, was subcloned into the *EcoRI/XhoI* sites of pUhrpFAN, and was ligated into pLAFR6, giving pL6hpalhrpFAN. For the generation of a XopF₁₋₂₀₀-HrpFAN construct, the first 200 codons of *xopF1* were amplified and cloned into the *EcoRI/XhoI* sites of plasmid pDhrpFAN, giving pDxopF1hrpFAN.

Generation of a XopF1-specific antiserum.

For the production of an anti-XopF1 polyclonal antiserum, GST-XopF1 was expressed in *E. coli* BL21(DE3). Bacteria were grown in Super medium (Qiagen, Hilden, Germany) at 37°C, and expression was induced at an optical density at 600 nm = 0.7 with isopropyl-β-D-thiogalactoside (2 mM final concentration) for 2 h at 37°C. Cells were harvested and broken with a French press, and inclusion bodies were pelleted by centrifugation. After extensive washing with phosphate-buffered saline, inclusion bodies were resuspended in Laemmli buffer, and the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were stained with Coomassie. The major protein band corresponding to GST-XopF1 was excised from the gel and used for immunization of two rabbits (Eurogentec, Herstal, Belgium).

Secretion experiments and protein analysis.

For the analysis of T3S, bacteria were incubated in secretion medium as described previously (Büttner et al. 2002). Total cell extracts and culture supernatants were concentrated 10 and 100 times, respectively. Equal amounts of bacterial total cell extracts and culture supernatants were analyzed by SDS-PAGE and immunoblotting. Polyclonal antibodies specific for HrpF (Büttner et al. 2002), AvrBs3 (Knoop et al. 1991), and XopF1, respectively, and a monoclonal anti-c-Myc antibody (Qiagen, Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) were used. The anti-XopF1 antibody was diluted 1:3,000. To ensure that no bacterial lysis had occurred, blots were routinely reacted with an antibody specific for the intracellular protein HrcN (Rossier et al. 2000; data not shown). Horseradish peroxidase-labeled anti-rabbit and anti-mouse antibodies (Amersham Pharmacia Biotech) were used as secondary antibodies and reactions were visualized by enhanced chemiluminescence as recommended (Amersham Pharmacia Biotech).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BLASTP program: www.ncbi.nlm.nih.gov/80/BLAST
 3D-PSSM program: www.sbg.bio.ic.ac.uk/~3dpssm
 SignalP: www.cbs.dtu.dk/services/SignalP

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In the article “Characterization of the nonconserved *hpaB-hrpF* region in the *hrp* pathogenicity island from *Xanthomonas campestris* pv. *vesicatoria*” by D. Büttner, L. Noël, J. Stuttmann and U. Bonas, there was an error in Figure 6C, in which an incorrect image was inadvertently used to show the plant reaction to the translocation of XopC₁₋₂₀₀-AvrBs3Δ2 by the *X. campestris* pv. *vesicatoria* *hpaH*oof mutant in comparison to the wild-type strain. Despite this error, the data that is presented is correct and fully supports the conclusions drawn. A corrected version of Figure 6C is given here. None of the conclusions of the paper are altered. We apologize to the readership of *MPMI* for this error and for any inconvenience caused.

Revised Fig. 6

Previously Published Version