The Inner Membrane Protein HrcV from *Xanthomonas* spp. Is Involved in Substrate Docking During Type III Secretion

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Pathogenicity of the gram-negative plant-pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* depends on a membrane-spanning type III secretion (T3S) system, which translocates effector proteins into eukaryotic host cells. In this study, we characterized the T3S system component HrcV, which is a member of the YscV/FliH family of inner membrane proteins. HrcV consists of eight transmembrane helices and a cytoplasmic region (HrcV_C). Mutant and protein–protein interaction studies showed that HrcV_C is essential for protein function and binds to T3S substrates, including the early substrate HrpB2, the pilus protein HrpE, and effector proteins. Furthermore, HrcV_C interacts with itself and with components and control proteins of the T3S apparatus. The interaction of HrcV_C with HrpB2, HrpE, and T3S system components depends on amino acid residues in a conserved motif, designated flagella/hypersensitive response/invasion proteins export pore (FHIPEP), which is located in a cytoplasmic loop between transmembrane helix four and five of HrcV. Mutations in the FHIPEP motif abolish HrcV function but do not affect the interaction of HrcV_C with effector proteins.

The gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* is the causal agent of bacterial spot disease of pepper and tomato plants and one of the model systems to study bacterial virulence strategies. Essential for the pathogenicity of *X. campestris* pv. *vesicatoria* is a type III secretion (T3S) system which translocates approximately 30 effector proteins into the host cell cytosol (Büttner and Bonas 2010; Ryan et al. 2011). T3S systems are conserved in many gram-negative plant- and animal-pathogenic bacteria and are evolutionarily related to the bacterial flagellum, which is also referred to as a T3S system (Chevance and Hughes 2008; Desvaux et al. 2006). Flagellar T3S systems are connected via an extracellular hook to the flagellar filament, which is the key bacterial motility organelle. By contrast, translocation-associated T3S systems are associated with an extracellular pilus (in plant-pathogenic bacteria, up to 2 µm long) or a needle (in animal-pathogenic bacteria, 40 to 80 nm long) that presumably provide protein transport channels to the host–pathogen interface.

T3S pili and needles are directly or indirectly associated with the T3S translocon, which is a predicted oligomeric protein channel and consists of bacterial type III-secreted proteins. The T3S translocon is required for effector protein translocation across the host plasma membrane (Büttner 2012; Mattei et al. 2010; Mueller et al. 2008).

Despite the differences in the extracellular appendages of flagellar and translocation-associated T3S systems, the membrane-spanning basal bodies of both systems share architectural similarities. They consist of ring structures in the inner membrane (IM) and outer membrane (OM) which are probably joined by a periplasmic rod (Büttner 2012; Erhardt et al. 2010). The IM ring is associated with the export apparatus that is built by members of the conserved YscR, YscS, YscT, YscU, and YscV families of transmembrane proteins. The nomenclature of these proteins refers to the Ysc proteins from the animal-pathogenic bacterium *Yersinia* (Bogdanove et al. 1996; He et al. 2004). Because the transport channel of the export apparatus has an inner diameter of 2 to 3 nm, which is too narrow to allow the transport of fully folded proteins, T3S substrates likely travel the secretion apparatus in a partially unfolded conformation (Blocker et al. 2001; Marlovits et al. 2004). Unfolding of T3S substrates is probably mediated by the cytoplasmic ATPase of the T3S system, which associates with the export apparatus and forms a multimeric ring structure (Akeda and Galan 2005). Additional interaction partners of the ATPase include its predicted cytoplasmic regulator (YscL protein family) and components of the putative cytoplasmic (C) ring of the T3S system (YscQ protein family), which is associated with the export apparatus and might act as a T3S substrate docking site (Biemans-Oldehinkel et al. 2011; Johnson et al. 2008; Lorenz et al. 2012; Morita-Ishihara et al. 2006; Spaeth et al. 2009).

Most T3S substrates are targeted for secretion by a signal in the N-terminal region that is not conserved on the amino acid level (Arnold et al. 2010; Lloyd et al. 2001; Petnicki-Ocwieja et al. 2002; Samudrala et al. 2009). Furthermore, in many cases, efficient secretion depends on specific chaperones that bind to T3S substrates and likely promote substrate recognition by components of the T3S system at the IM (Feldman and Cornelis 2003; Parsot et al. 2003). Chaperone-substrate complexes were shown to interact with the C ring and the ATPase of the T3S system. Additional substrate docking sites are probably present in the cytoplasmic regions of members of the YscU and YscV protein families that bind to secreted proteins (Bange et al. 2010; Lorenz et al. 2008; Minamino and MacNab 2000a). YscV family members are proteins of approximately 700 amino acids that are also referred to as the flagella/hypersensitive response/invasion proteins export pore (FHIPEP)

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Fig. 1. Cytoplasmic domain of HrcV is required for protein function. **A**, Topology model of HrcV. Rectangles represent transmembrane regions and numbers refer to amino acid positions. The topology of HrcV was previously studied by the use of a dual alkaline phosphatase-β-galactosidase reporter (Berger et al. 2010). Dots indicate single amino acid positions. C, C terminus; IM, inner membrane; N, N terminus. **B**, Complementation of the $\Delta hrcV$ mutant phenotype. *Xanthomonas campestris* pv. *vesicatoria* strains 85-10, 85-10 $\Delta hrcV$ ($\Delta hrcV$), 85*, and 85* $\Delta hrcV$ ($\Delta hrcV$) carrying the empty vector (–) or expression constructs encoding HrcV-c-Myc (HrcV) and HrcV₃₂₄₋₆₄₅-c-Myc (HrcV₃₂₄₋₆₄₅), respectively, as indicated were inoculated into leaves of susceptible ‘Early Cal Wonder’ (ECW) and resistant ‘ECW-10R’ pepper plants. Disease symptoms were photographed 8 days postinoculation (dpi). For the better visualization of the hypersensitive response (HR), leaves were bleached in ethanol 2 dpi. Dashed lines mark the infiltrated areas. For protein studies, bacteria were grown in minimal medium, and equal amounts of total cell extracts (TE) were analyzed by immunoblotting, using a c-Myc epitope-specific antibody. For the analysis of type III secretion (T3S), bacteria were incubated in secretion medium, and TE and culture supernatants (SN) were analyzed by immunoblotting using antibodies specific for the translocon protein HrpF. HrcV-c-Myc and HrcV₃₂₄₋₆₄₅-c-Myc are indicated by asterisks; additional bands presumably correspond to degradation products. **C**, Deletion of *hrcV*₃₂₄₋₆₄₂ abolishes bacterial pathogenicity and T3S. Strains 85-10 (*hrpG* wt) and 85-10 $\Delta hrcV$ ₃₂₄₋₆₄₂ ($\Delta hrcV$ 324-642) carrying the empty vector (–) or expression constructs encoding HrpG* (*hrpG**), HrcV-c-Myc (HrcV), and HrcV₃₂₄₋₆₄₅-c-Myc (HrcV₃₂₄₋₆₄₅), respectively, as indicated, were inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants. Disease symptoms and the HR were monitored as described in B. For protein studies and the analysis of T3S, TE and SN were analyzed by immunoblotting, using c-Myc epitope- and HrpF-specific antibodies, as described in B. HrcV-c-Myc and HrcV₃₂₄₋₆₄₅-c-Myc are indicated by asterisks; additional bands correspond to degradation products. **D**, HrcV and HrcV_C associate with the bacterial membranes. *X. campestris* pv. *vesicatoria* 85* $\Delta hrcV$ encoding HrcV-c-Myc and HrcV₃₂₄₋₆₄₅-c-Myc, respectively, from corresponding expression constructs was grown in secretion medium. Membrane (M) and soluble fractions (S) were separated by ultracentrifugation and analyzed by immunoblotting using c-Myc epitope- and HrcJ-specific antibodies. One representative blot probed with the HrcJ-specific antibodies is shown.

superfamily (Carpenter and Ordal 1993; Gough et al. 1993; Plano et al. 1991; Wei and Beer 1993). They consist of a conserved N-terminal transmembrane and a less conserved C-terminal cytoplasmic region. Crystal structure analyses of YscV homologs from *Salmonella* spp. (Lilic et al. 2010; Saijo-Hamano et al. 2010; Worrall et al. 2010), *Helicobacter pylori* (Moore and Jia 2010), and *Bacillus subtilis* (Bange et al. 2010) showed that the cytoplasmic region is organized in three to four subdomains that are connected to the transmembrane region of the protein by a flexible linker. Furthermore, the crystal structure of the cytoplasmic region of the YscV homolog MxiA from *Shigella flexneri* revealed the presence of a homo-nonameric ring (Abrusci et al. 2013). Oligomerization was also proposed for the cytoplasmic regions of YscV family members from animal-pathogenic bacteria and, presumably, occurs in the absence of the IM and OM rings of the T3S system (Diepold et al. 2011; Li and Sourjik 2011). Therefore, it was proposed that oligomerization of YscV family members initiates the assembly of the IM-associated portions of the T3S

system that are later connected to the OM ring (Diepold et al. 2011; Li and Sourjik 2011).

The central role of YscV family members in the assembly of the export apparatus probably accounts for the various protein-protein interactions that have been reported for YscV and homologs. YscV family members were shown to bind to OM-associated components of the T3S system and members of the export apparatus, including the flagellar proteins FliO, FliP, and FliQ and the cytoplasmic domain of the YscU homolog FlhB. Furthermore, the YscV homolog FlhA from flagellar T3S systems binds to the IM ring protein FliF, the cytoplasmic ATPase and its regulator, as well as to T3S substrates and chaperones (Bange et al. 2010; Diepold et al. 2011; McMurry et al. 2004; Minamino and MacNab 2000a; Minamino et al. 2011, 2012; Stone et al. 2010; Zhu et al. 2002). The C-terminal domain of FlhA (FlhA_C) also interacts with the chaperone escort protein FliJ, which presumably promotes the binding of chaperone-substrate complexes to FlhA_C (Bange et al. 2010; Fraser et al. 2003a; Minamino et al. 2012). These findings are

Fig. 2. Cytoplasmic region of HrcV interacts with components, control proteins, and substrates of the type III secretion (T3S) system. Glutathione S-transferase (GST) and GST fusions of HrcU₂₅₅₋₃₅₇, HrcN, HrcV₃₂₄₋₆₄₅, HrcC, HpaB, HpaC, HrpB2, HrpE, and the effector protein derivatives XopJ, AvrBs3, AvrBs3 Δ 2, and AvrBs3₁₋₅₀, as indicated, were immobilized on glutathione sepharose and incubated with *Escherichia coli* lysates containing HrcV-c-Myc or HrcV₃₂₄₋₆₄₅-c-Myc. Total cell extracts (TE) and eluted proteins (eluate) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting, using a c-Myc epitope-specific antibody. Blots were routinely reprobed with GST-specific antibodies to ensure that comparable amounts of GST and GST fusion proteins were used.

in agreement with the proposed role of the C-terminal domains of YscV family members in substrate docking.

YscV and homologs have been studied intensively in flagellar and translocation-associated T3S systems from animal-pathogenic bacteria, whereas little is known about the role of YscV homologs from plant pathogens. In the present study, we analyzed the YscV homolog HrcV from *X. campestris* pv. *vesicatoria*. Like other T3S system components, HrcV is encoded by the chromosomal hypersensitive response and pathogenicity (*hrp*) gene cluster, which contains 25 genes and is essential for pathogenicity (Bonas et al. 1991; Rossier et al. 2000; Weber et al. 2005; Wengelnik et al. 1996a). Eleven *hrp* gene products, including HrcV, are conserved in animal- or plant-pathogenic bacteria and, therefore, are referred to as Hrp conserved (Hrc). They presumably constitute the core components of the T3S system (Büttner and Bonas 2002; He et al. 2004). HrcV consists of eight transmembrane helices and a large C-terminal cytoplasmic region (HrcV_C) and is essential for T3S (Berger et al. 2010; Rossier et al. 1999). Interaction studies revealed that HrcV interacts with the T3S chaperone Hrp-associated (Hpa) B and the T3S substrate specificity switch (T3S4) protein HpaC, both of which are involved in the control of T3S (Büttner et al. 2006; Rossier et al. 1999). The T3S chaperone HpaB promotes the efficient secretion and translocation of multiple effector proteins and, presumably, targets them to the ATPase HrcN, which can dissociate HpaB-effector complexes (Büttner et al. 2004, 2006; Lorenz and Büttner 2009). HpaB also interacts with the T3S4 protein HpaC, which promotes the secretion of translocon and effector proteins and suppresses the efficient secretion of the early T3S substrate HrpB2. Thus, HpaC acts similarly to T3S4 proteins and, presumably, switches the substrate specificity of the T3S system from early to late substrates (Lorenz et al. 2008). The HpaC-mediated switch depends on the C-terminal domain of the YscU homolog HrcU, HrcU_C, which interacts with HpaC and presumably undergoes a conformational change that alters the substrate recognition specificity (Lorenz and Büttner 2011; Lorenz et al. 2008). It is not yet known how T3S substrates from *X. campestris* pv. *vesicatoria* are recognized by the secretion apparatus. Substrate binding sites have been identified in HrcU_C, which interacts with the early T3S substrate HrpB2, and in the predicted C ring component HrcQ, which binds to effector proteins. Notably, the T3S signal of effector proteins appears to be dispensable for the interaction with HrcQ, suggesting that HrcQ is not the initial targeting site for type III effectors (Lorenz et al. 2008, 2012).

In the present study, we investigated a potential contribution of HrcV to T3S substrate binding. For this, we performed protein-protein interaction and mutant studies and also analyzed the role of the cytoplasmic region of HrcV and of amino acids of a conserved motif, designated FHIPEP, which is located in a cytoplasmic loop between transmembrane helix four and five. Our experimental findings suggest that HrcV is involved in substrate docking and that amino acid residues of the FHIPEP motif contribute to the binding of specific interaction partners to HrcV_C.

RESULTS

The cytoplasmic domain of HrcV is essential for pathogenicity and T3S.

HrcV (XCV0425) from *X. campestris* pv. *vesicatoria* is encoded by the second gene of the *hrpC* operon and is annotated as a protein of 640 amino acids. Comparative sequence analysis of HrcV and homologs from *Xanthomonas* spp. revealed the presence of an alternative translation start codon (ATG) five codons upstream of the annotated GTG start codon

and one codon upstream of the stop codon of *hrcU*, which is the first gene of the *hrpC* operon (Supplementary Fig. S1). For the analysis of HrcV from *X. campestris* pv. *vesicatoria* 85-10, we generated expression constructs encoding C-terminally c-Myc epitope-tagged derivatives of HrcV_{ATG} (translation start at the ATG codon; hereafter referred to as HrcV), the C-terminal cytoplasmic region spanning amino acids 324 to 645, and the N-terminal region, including amino acids 1 to 336 (Fig. 1A; numbers refer to the position relative to the predicted ATG translation start codon). An expression construct encoding the annotated protein (HrcV_{GTG}) was also generated; however, HrcV_{GTG}-c-Myc was unstable (data not shown). Expression constructs were introduced into the *hrcV* deletion mutant 85-10Δ*hrcV* (deleted in codons 251 to 438 of *hrcV*) and bacteria were inoculated into leaves of susceptible 'Early Cal Wonder' (ECW) and resistant 'ECW-10R' pepper plants. ECW-10R plants carry the *BsI* resistance gene and induce the hypersensitive response (HR) upon recognition of the type III effector AvrBs1, which is delivered by strain 85-10 (Escobar et al. 2001; Ronald and Staskawicz 1988). The HR is a rapid local cell death at the infection site that restricts bacterial ingress (Jones and Dangl 2006). As expected, the wild-type strain led to disease symptoms in ECW and the HR in ECW-10R pepper plants, whereas no reactions were observed for *hrcV* deletion mutants (Fig. 1B). The wild-type phenotype was restored in strain 85-10Δ*hrcV* carrying HrcV-c-Myc but not HrcV₃₂₄₋₆₄₅-c-Myc (Fig. 1B). Immunoblot analysis of bacterial cell extracts revealed that HrcV derivatives except HrcV₁₋₃₃₆-c-Myc (data not shown) were stably synthesized (Fig. 1B and C). When HrcV-c-Myc and HrcV₃₂₄₋₆₄₅-c-Myc were analyzed in the *hrcV* wild-type strain 85-10, they did not interfere with in planta symptom formation, suggesting that the observed loss of function of HrcV₃₂₄₋₆₄₅-c-Myc was not caused by a dominant-negative effect on the plant-pathogen interaction (Supplementary Fig. S2).

To investigate whether the C-terminal cytoplasmic region of HrcV (HrcV_C) is required for protein function, we deleted codons 324 to 642 of the genomic *hrcV* gene in strain 85-10. The resulting *hrcV_C* deletion mutant failed to induce disease symptoms and the HR when inoculated into ECW and ECW-10R plants. The mutant phenotype was complemented by ectopic expression of *hrcV-c-myc* but not of *hrcV₃₂₄₋₆₄₅-c-myc* (Fig. 1C). Taken together, these findings suggest that the C-terminal region of HrcV is essential but not sufficient for protein function and cannot be provided in trans.

In addition to infection experiments, we analyzed the in vitro function of the T3S system in wild-type, *hrcV*, and *hrcV_C* deletion mutant strains carrying the empty vector or HrcV derivatives, as described above. For this, we used derivatives of strain 85-10*hrpG** (85*) that contains a constitutively active version of the key regulator HrpG, which regulates the expression of a genome-wide regulon, including *hrp*, putative virulence, and effector genes (Noël et al. 2001; Rossier et al. 1999; Schulze et al. 2012; Wengelnik et al. 1996b, 1999). Because *hrp* genes are only expressed in planta and in specific minimal media, the presence of HrpG* is crucial for the analysis of T3S in cultures. For T3S assays, strain 85* and derivatives thereof were incubated in secretion medium and total cell extracts and culture supernatants were analyzed by immunoblotting using antibodies against the putative translocon protein HrpF. HrpF was secreted by strain 85* but was not detectable in the culture supernatants of *hrcV* and *hrcV_C* deletion mutant strains as expected (Rossier et al. 1999) (Fig. 1B and C). The secretion deficiency was complemented by *hrcV-c-myc* but not by *hrcV₃₂₄₋₆₄₅-c-myc*, which confirms the results of the infection assays (discussed above). Notably, the presence of *hrpG** in *hrcV* and *hrcV_C*

mutant strains and derivatives thereof did not alter the observed phenotypes in susceptible and resistant pepper plants (Fig. 1B and C).

HrcV presumably associates with the bacterial membranes.

Next, we analyzed the subcellular localization of HrcV-c-Myc and HrcV₃₂₄₋₆₄₅-c-Myc by fractionation studies. For this, strain 85* Δ hrcV expressing hrcV-c-myc or hrcV₃₂₄₋₆₄₅-c-myc

was grown under T3S-inducing conditions and membrane-associated and soluble proteins were separated by ultracentrifugation. Immunoblot analyses of different fractions revealed that HrcV-c-Myc was mainly detected in the membrane fraction, as expected (Fig. 1D). By contrast, HrcV₃₂₄₋₆₄₅-c-Myc was present in the soluble and membrane fractions, suggesting that it was cytoplasmic but also associated with the bacterial membranes (Fig. 1D). As control, blots were reacted with an

Fig. 3. Amino acid residues of the conserved flagella/hypersensitive response/invasion proteins export pore (FHIPEP) motif of HrcV contribute to protein function. **A**, Sequence alignment of the FHIPEP motifs of HrcV and HrcV homologs from plant- and animal-pathogenic bacteria. The amino acid sequences of the FHIPEP motifs of HrcV from *Xanthomonas campestris* pv. *vesicatoria* 85-10 (HrcV_{Xcv}, GenBank accession number YP_362156.1), *Pseudomonas syringae* pv. *tomato* (HrcV_{Psyr}, GenBank accession number AAG33877.1), and *Ralstonia solanacearum* GMI1000 (HrcV_{Rsol}, GenBank accession number NP_522424) as well as of InvA and FlhA from *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* (GenBank accession numbers AAA16867.1 and P40729.1), YscV from *Yersinia pestis* (GenBank accession number AAA27643.1), and EscV from *Escherichia coli* (GenBank accession number AAK26714.1) were aligned using ClustalW2. Numbers refer to amino acid positions. Conserved amino acids are highlighted in black. Nonconserved amino acids of HrcV that were mutated in this study are marked by asterisks. Boxes indicate the mutations of conserved amino acids that were introduced into HrcV from *X. campestris* pv. *vesicatoria* in this study. **B**, HrcV derivatives with mutations in the FHIPEP motif associate with the bacterial membranes. *X. campestris* pv. *vesicatoria* 85* Δ hrcV carrying expression constructs encoding HrcV_{R138A}-c-Myc (HrcV_{R138A}), HrcV_{D149A}-c-Myc (HrcV_{D149A}), or HrcV_{D161A}-c-Myc (HrcV_{D161A}) was grown in secretion medium. Membrane (M) and soluble fractions (S) were separated by ultracentrifugation and analyzed by immunoblotting using c-Myc epitope- and HrcJ-specific antibodies. One representative blot probed with the HrcJ-specific antibodies is shown. TE, total cell extract. **C**, Mutations in the FHIPEP motif abolish HrcV function. *X. campestris* pv. *vesicatoria* strains 85* (wt) and 85* Δ hrcV (Δ hrcV) carrying the empty vector (–) or expression constructs encoding HrcV-c-Myc, HrcV_{R138A}-c-Myc, HrcV_{D149A}-c-Myc, or HrcV_{D161A}-c-Myc were inoculated into leaves of resistant ‘Early Cal Wonder’ (ECW)-10R and susceptible ECW pepper plants. Disease symptoms were photographed 7 days postinoculation (dpi). For the better visualization of the hypersensitive response (HR), leaves were bleached in ethanol 2 dpi. Dashed lines mark the infiltrated areas. For protein and type III secretion (T3S) studies, bacteria were grown in secretion medium, and equal amounts of TE and culture supernatants (SN) were analyzed by immunoblotting using c-Myc epitope- and HrpF-specific antibodies. **D**, HrcV derivatives with mutations in the FHIPEP motif exert a dominant-negative effect on pathogenicity and T3S. Strain 85* (wt) carrying the empty vector (–) or expression constructs encoding HrcV-c-Myc, HrcV_{R138A}-c-Myc, HrcV_{D149A}-c-Myc, or HrcV_{D161A}-c-Myc was inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants. Dashed lines mark the infiltrated areas. The documentation of plant reactions and the analysis of protein levels and T3S were performed as described in C. The lower band detected by the c-Myc-specific antibody corresponds to HrcV-c-Myc and derivatives thereof, and the upper band results from unspecific binding of the antibody.

Fig. 4. Nonconserved amino acids of the flagella/hypersensitive response/invasion proteins export pore (FHIPEP) motif contribute to HrcV function. **A**, Genomic D149A, R138A, and D161A mutations in HrcV abolish bacterial pathogenicity. Strains 85* (wt), 85**hrcV_{D149A}* (*hrcV_{D149A}*), 85**hrcV_{R138A}* (*hrcV_{R138A}*), and 85-10*hrcV_{D161A}*(pFG72-1) (*hrcV_{D161A}*) carrying the empty vector (–) or an expression construct encoding HrcV-c-Myc (HrcV) were inoculated into leaves of susceptible ‘Early Cal Wonder’ (ECW) and resistant ‘ECW-10R’ pepper plants. Disease symptoms were photographed 7 days postinoculation (dpi). For the better visualization of the hypersensitive response (HR), leaves were bleached in ethanol 2 dpi. Dashed lines mark the infiltrated areas. For protein and type III secretion (T3S) studies, bacteria were grown in secretion medium, and equal amounts of cell extracts were analyzed by immunoblotting using a c-Myc epitope-specific antibody. **B**, R138A, D149A, and D161A mutations in HrcV abolish the detectable HrpF secretion. Strains 85* (wt), 85* $\Delta hrcV$ ($\Delta hrcV$), 85* *hrcV_{R138A}* (*hrcV_{R138A}*), 85* *hrcV_{D149A}* (*hrcV_{D149A}*), and 85-10*hrcV_{D161A}*(pFG72-1) (*hrcV_{D161A}*) carrying the empty vector (–) or an expression construct encoding HrcV-c-Myc (HrcV) were incubated in secretion medium. Equal amounts of total cell extracts (TE) and culture supernatants (SN) were analyzed by immunoblotting using HrpF-specific antibodies. **C**, Mutations of nonconserved amino acids of the FHIPEP motif interfere with the ability of HrcV to complement the *hrcV* mutant phenotype and exert a dominant-negative effect on pathogenicity and T3S. *Xanthomonas campestris* pv. *vesicatoria* strains 85* (wt) and 85* $\Delta hrcV$ ($\Delta hrcV$) carrying the empty vector (–) or expression constructs encoding HrcV-c-Myc (HrcV), HrcV_{ASG}-c-Myc (HrcV_{ASG}), HrcV_{RAG}-c-Myc (HrcV_{RAG}), and HrcV_{RSG}-c-Myc (HrcV_{RSG}), respectively, were inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants. Disease symptoms and the HR were documented as described in A. Dashed lines mark the infiltrated areas. For the analysis of protein levels and T3S, bacteria were incubated in secretion medium and TE and SN were analyzed by immunoblotting, using c-Myc epitope- and HrpF-specific antibodies.

antibody against the predicted IM ring protein HrcJ, which was only detectable in the membrane fractions (Fig. 1D).

The cytoplasmic region of HrcV interacts with components of the T3S system and with itself.

Given that HrcV is an essential component of the T3S system, we analyzed whether HrcV can interact with T3S system proteins, including the IM protein HrcU, the cytoplasmic ATPase HrcN, and the OM secretin HrcC. For this, glutathione *S*-transferase (GST) fusions of HrcN, HrcC, and the cytoplasmic region of HrcU (HrcU_C, corresponding to amino acids 255 to 357), respectively, were purified from *Escherichia coli* using glutathione sepharose and incubated with *E. coli* lysates containing HrcV-c-Myc or HrcV₃₂₄₋₆₄₅-c-Myc. Immunoblot analysis showed that comparable amounts of GST and GST fusion proteins were present in the eluates (Supplementary Fig. S3; data not shown). HrcV-c-Myc was detected in the eluates of GST-HrcU₂₅₅₋₃₅₇ and GST-HrcN but not of GST and GST-HrcC (Fig. 2). Similar findings were observed for HrcV₃₂₄₋₆₄₅-c-Myc, suggesting that the cytoplasmic region of HrcV is sufficient for the interaction with HrcN and HrcU_C (Fig. 2). This is in agreement with the finding that YscV homologs interact with members of the YscN and YscU families of T3S system components (Alegria et al. 2004; Diepold et al. 2011; Li and Sourjik 2011; Minamino and MacNab 2000a; Saijo-Hamano et al. 2004; Zhu et al. 2002). We also investigated a self-interaction of the cytoplasmic region of HrcV using immobilized GST-HrcV₃₂₄₋₆₄₅. Because both HrcV-c-Myc and HrcV₃₂₄₋₆₄₅-c-Myc coeluted with GST-HrcV₃₂₄₋₆₄₅ but not with GST, we assume that the cytoplasmic region of HrcV can self-interact (Fig. 2).

The cytoplasmic region of HrcV provides binding sites for HpaB, HpaC, and T3S substrates.

We previously demonstrated an interaction of HrcV with the general T3S chaperone HpaB and the T3S4 protein HpaC (Büttner et al. 2006). To investigate whether the cytoplasmic

region of HrcV is sufficient for these interactions, GST fusions of HpaB and HpaC were immobilized on glutathione sepharose and incubated with bacterial lysates containing HrcV-c-Myc or HrcV₃₂₄₋₆₄₅-c-Myc, as described above. Both HrcV derivatives coeluted with GST-HpaB and GST-HpaC, respectively, but not with GST alone, suggesting that the C-terminal region of HrcV provides binding sites for HpaB and HpaC (Fig. 2).

Because the cytoplasmic regions of YscV family members were proposed to act as substrate docking sites of the T3S system, we also analyzed a potential interaction of HrcV_C with secreted proteins, including extracellular components of the T3S system and effector proteins. When GST fusions of the early T3S substrate HrpB2, the pilus protein HrpE, and the effector proteins XopJ and AvrBs3, respectively, were purified from *E. coli* and incubated with HrcV derivatives, HrcV-c-Myc and HrcV₃₂₄₋₆₄₅-c-Myc were present in the eluates of all GST fusion proteins but not of GST alone (Fig. 2). Therefore, we conclude that the cytoplasmic region of HrcV is a potential binding site for early and late T3S substrates. Given that the secretion of T3S substrates is mediated by a signal in the N-terminal region, we investigated the contribution of this protein region in the effector protein AvrBs3 to the interaction with HrcV_C. The T3S signal of AvrBs3 was previously localized to the N-terminal 50 amino acids (Büttner et al. 2004). Therefore, GST-AvrBs3₁₋₅₀ containing the N-terminal 50 amino acids of AvrBs3 and a GST fusion of AvrBs3 lacking the N-terminal 152 amino acids (GST-AvrBs3Δ2) were used for pull-down assays. HrcV₃₂₄₋₆₄₅-c-Myc coeluted with GST-AvrBs3Δ2 but not with GST and GST-AvrBs3₁₋₅₀ (Fig. 2). Therefore, the N-terminal T3S signal is presumably dispensable for the binding of AvrBs3 to HrcV_C.

Amino acid residues of the FHIPEP motif of HrcV are essential for pathogenicity.

HrcV contains a conserved amino acid motif, designated FHIPEP, which is present in a cytoplasmic loop between trans-

Fig. 5. Amino acid residues of the flagella/hypersensitive response/invasion proteins export pore (FHIPEP) motif contribute to the interaction of HrcV with itself, type III secretion (T3S) system components, the T3S chaperone HpaB, the pilus protein HrpE, and the early substrate HrpB2. Glutathione *S*-transferase (GST) and GST fusions of HrcV₃₂₄₋₆₄₅, HrcN, HrcU₂₅₅₋₃₅₇, HpaB, HrpB2, HpaC, HrpE, and the effector proteins XopJ and AvrBs3 were immobilized on glutathione sepharose and incubated with *Escherichia coli* lysates containing HrcV₁₋₃₃₆-c-Myc, HrcV_{ASG}-c-Myc, HrcV_{RAG}-c-Myc, HrcV_{R138A}-c-Myc, and HrcV_{D161A}-c-Myc, respectively, as indicated. Total cell extracts (TE) and eluted proteins (eluate) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting, using c-Myc epitope-specific antibodies. Blots were routinely reprobed with GST-specific antibodies to ensure that comparable amounts of GST and GST fusion proteins were used.

membrane helices four and five of YscV family members (PROSITE accession number PS00994) (Fig. 1A). To analyze the functional importance of amino acids of the FHIPEP motif in HrcV, we introduced alanine substitutions of the conserved residues R138, D149, or D161 into this motif (Fig. 3A; a FHIPEP deletion mutant derivative of HrcV was unstable; data not shown). Corresponding HrcV derivatives were stably synthesized and associated with the bacterial membranes, as expected (Fig. 3B). Infection and secretion studies revealed that none of the HrcV point mutant derivatives complemented the mutant phenotypes of strains 85-10 Δ hrcV (Supplementary Fig. S4) and 85* Δ hrcV with respect to disease symptoms, HR induction, and HrpF secretion (Fig. 3C). Furthermore, all three HrcV mutant derivatives exerted a dominant-negative effect on bacterial pathogenicity and on HrpF secretion when analyzed in the wild-type strains 85* (Fig. 3D) and 85-10. Thus, it cannot be excluded that the lack of complementation was caused by a dominant-negative effect of these HrcV derivatives on pathogenicity. Therefore, we introduced all three mutations into the genomic *hrcV* gene of strains 85-10 and 85*. Strains 85* and 85-10 carrying genomic *hrcV*_{D149A}, *hrcV*_{R138A}, or *hrcV*_{D161A} mutations did not induce visible plant reactions, suggesting that the mutations abolished HrcV function (Fig. 4A). In agreement with this, the detectable HrpF secretion in strains 85**hrcV*_{D149A}, 85**hrcV*_{R138A}, and 85**hrcV*_{D161A} was abolished (Fig. 4B). HrpF secretion and plant reactions were restored upon ectopic expression of *hrcV-c-myc* (Fig. 4A and B). Therefore, we conclude that the mutant phenotypes were not caused by dominant-negative effects of the mutations on the expression of downstream genes.

In addition to conserved amino acid residues in the FHIPEP motif, we also mutated the amino acid residues at positions 143, 147, and 150 that are not conserved among HrcV and homologs from animal-pathogenic bacteria (Fig. 3A). For this, HrcV mutant derivatives were designed to contain three mutations each; that is, G143A, T147S, and A150G (HrcV_{ASG}); G143R, T147A, and A150G (HrcV_{RAG}); and G143R, T147S, and A150G (HrcV_{RSG}). The sequence of the FHIPEP motif of HrcV_{ASG} is identical to the corresponding amino acid sequence in the YscV homolog InvA from *Salmonella typhimurium* whereas the FHIPEP motifs of HrcV_{RSG} and HrcV_{RAG} differ in one and two amino acids, respectively, when compared with the corresponding motif in InvA (Fig. 3A). All HrcV mutant derivatives were stably synthesized in *X. campestris* pv. *vesicatoria* (Fig. 4C). When analyzed in *hrcV* deletion mutant strains, HrcV_{ASG} partially complemented the in planta phenotype of strain 85-10 Δ hrcV (Supplementary Fig. S5) whereas a partial complementation of disease symptoms and a wild-type HR phenotype was observed with strain 85* Δ hrcV (Fig. 4C). We have previously reported that the presence of *hrpG** can promote plant reactions, presumably because of the overexpression of T3S system genes (Lorenz and Büttner 2009, 2011). When bacteria were incubated in secretion medium, HrcV_{ASG} did not restore detectable HrpF secretion in strain 85* Δ hrcV (Fig. 4C). However, HrpF is presumably secreted in small amounts that are sufficient for in planta symptom formation (discussed above) (Fig. 4C). In contrast to HrcV_{ASG}, HrcV_{RAG} and HrcV_{RSG} did not complement the *hrcV* in planta mutant phenotype and also did not restore detectable HrpF secretion (Fig. 4C). A nonpathogenic phenotype was also observed for a genomic *hrcV*_{RSG} mutant (strain 85-10*hrcV*_{RSG}). When analyzed in strains 85* and 85-10, HrcV_{RAG} exerted a dominant-negative effect on in planta reactions. This effect was less pronounced for HrcV_{RSG} and HrcV_{ASG} (Fig. 4C). However, all three HrcV mutant derivatives abolished the efficient secretion of HrpF in strain 85*, suggesting that enhanced levels of HrcV derivatives with mutations in the FHIPEP motif interfere with T3S (Fig. 4C).

Mutations in the FHIPEP motif affect the binding of HrcV to some of its interaction partners.

Next, we investigated whether mutations in the FHIPEP motif affect the binding of HrcV to its interaction partners. When GST fusions of HrcV interaction partners were purified from *E. coli* and incubated with *E. coli* lysates containing HrcV derivatives with mutations in the FHIPEP motif, HrcV_{ASG}-c-Myc and HrcV_{RAG}-c-Myc coeluted with GST fusions of HrcV₃₂₄₋₆₄₅, HrcN, HpaC, HrpB2, XopJ, and AvrBs3. By contrast, both derivatives were not or only weakly detectable in the eluates of GST, GST-HrcU₂₅₅₋₃₅₇, GST-HpaB, and GST-HrpE (Fig. 5). This suggests that the nonconserved amino acid residues of the FHIPEP motif contribute to the interaction of HrcV with some but not all tested interaction partners.

We also analyzed the contribution of two conserved amino acid residues of the FHIPEP motif to protein-protein interactions. When HrcV_{R138A}-c-Myc and HrcV_{D161A}-c-Myc were incubated with GST or GST fusion proteins as described above, both derivatives coeluted with GST-HpaC, GST-HrpE, and GST fusions of T3S effectors but were not detected in the eluates of GST-HrcV₃₂₄₋₆₄₅, GST-HrcN, GST-HrcU₂₅₅₋₃₅₇, GST-HpaB, and GST-HrpB2 (Fig. 5). The lack of detectable interactions of HrcV_{R138A}-c-Myc and HrcV_{D161A}-c-Myc with HpaB, HrpB2, and T3S system components might explain the loss-of-function phenotypes of both derivatives and suggests that these interactions are required for protein function (discussed above) (Fig. 5).

Notably, the FHIPEP motif is located in the transmembrane region of HrcV whereas the identified HrcV interaction partners presumably bind to the cytoplasmic region of HrcV, as was shown by our interaction studies with HrcV_C (discussed above) (Fig. 2). To investigate whether the transmembrane region of HrcV contains additional binding sites for HrcV interaction partners, GST fusions of T3S system components, HpaB, and HrpB2 were incubated with HrcV₁₋₃₃₆-c-Myc, which lacks the cytoplasmic region of HrcV. HrcV₁₋₃₃₆-c-Myc was stably synthesized in *E. coli* but not in *X. campestris* pv. *vesicatoria* (discussed above) and, therefore, could only be analyzed by in vitro pull-down assays. Because HrcV₁₋₃₃₆-c-Myc was not detected in the eluates of GST and GST fusion proteins, we assume that the N-terminal transmembrane region of HrcV is not sufficient for the binding of HrcV₃₂₄₋₆₄₅, HrcN, HrcU₂₅₅₋₃₅₇, HpaB, and HrpB2 to HrcV (Fig. 5). Taken together, our pull-down assays revealed that amino acid residues of the FHIPEP motif of HrcV are required for the self-interaction of HrcV_C as well as for the interaction with HpaB, HrpB2, HrpE, HrcU_C, and HrcN but are presumably not essential for the binding of effector proteins. To our knowledge, this is the first experimental evidence for a contribution of amino acid residues of the FHIPEP motif to protein-protein interactions that involve the cytoplasmic region of YscV family members.

DISCUSSION

The present study provides the first detailed molecular characterization of an YscV family member of translocation-associated T3S systems. Our results suggest an essential role of the cytoplasmic region of HrcV from *X. campestris* pv. *vesicatoria* during T3S and pathogenicity. The finding that HrcV_C interacts with secreted proteins, including the early substrate HrpB2, the pilus protein HrpE, and effector proteins, suggests that it is involved in T3S substrate binding. Interaction studies with deletion derivatives of the effector protein AvrBs3 showed that the interaction with HrcV_C is independent of the N-terminal region of AvrBs3, which harbors the T3S signal and the HpaB-binding site (Fig. 2). Thus, HrcV_C is probably not the initial targeting site for AvrBs3. Given that the N-terminal signal

of AvrBs3 is also dispensable for the interaction with HrcQ (Lorenz et al. 2012), it is possible that T3S signals are indirectly recognized by components of the T3S system, possibly by the help of T3S chaperones that often bind to N-terminal substrate regions. In this context, it is interesting to note that the T3S chaperone HpaB and also the T3S4 protein HpaC interact with HrcV_C (Fig. 2). An interaction between HrcV_C and HpaB from *X. axonopodis* pv. *citri* was previously also shown in yeast (Alegria et al. 2004). Because HpaB and HpaC presumably contribute to the targeting of T3S substrates to the secretion apparatus, HrcV_C might not only be involved in T3S substrate binding but also in the recognition of HpaB- or HpaC-substrate complexes. A similar function was proposed for YscV family members from flagellar T3S systems which interact with T3S chaperones, chaperone-substrate complexes, and the chaperone escort protein FliJ, as was shown for the cytoplasmic region of FlhA from *Salmonella* spp. (Bange et al. 2010; Minamino et al. 2012). The recognition of T3S substrates and associated control proteins by components of the export apparatus is one of the as-yet-unresolved issues in our understanding of T3S in plant- and animal-pathogenic bacteria.

Apart from HrcV_C, T3S substrate binding sites in *X. campestris* pv. *vesicatoria* have also been localized to the cytoplasmic region of the YscU family member HrcU and the predicted C ring component HrcQ (Lorenz et al. 2008, 2012). The cytoplasmic regions of YscU family members are proteolytically

cleaved and were proposed to be involved in the control of T3S substrate specificity. Thus, a conformational change in the cytoplasmic domains of YscU family members presumably leads to an alteration of substrate recognition and, thus, activates the secretion of translocon and effector proteins after pilus or needle assembly (Björnfort et al. 2009; Ferris et al. 2005; Fraser et al. 2003b; Lorenz and Büttner 2011; Lorenz et al. 2008; Minamino and Macnab 2000b; Riordan and Schneewind 2008; Sorg et al. 2007). The cytoplasmic region of HrcU from *X. campestris* pv. *vesicatoria* interacts with the T3S4 protein HpaC and the early T3S substrate HrpB2 but presumably does not provide binding sites for effector proteins (Lorenz and Büttner 2011; Lorenz et al. 2008). However, effector proteins and HrpB2 interact with HrcQ (Lorenz et al. 2012) and HrcV_C (Fig. 2). The finding that early and late T3S substrates can bind to two or more different components of the T3S system points to the presence of multiple binding sites for a given T3S substrate (Fig. 6). Notably, similar findings were observed for the T3S chaperone HpaB, which binds to HrcU and the ATPase HrcN (Lorenz and Büttner 2009). Furthermore, the T3S4 protein HpaC interacts with HrcV_C, HrcU_C, HrcN, and HrcQ, suggesting that HpaC also binds to multiple T3S system components (Lorenz and Büttner 2009; Lorenz et al. 2008, 2012) (Fig. 6).

HrcV_C does not only interact with secreted proteins but also self-interacts and binds to components of the T3S system, including the ATPase HrcN and the cytoplasmic domain

Fig. 6. Model of type III secretion (T3S) system components at the inner membrane (IM). The IM proteins HrcU, R, S, T, and V are the core components of the export apparatus that is associated with the cytoplasmic ATPase HrcN, which presumably forms a hexameric complex (only three subunits are shown). HrcN interacts with its predicted cytoplasmic regulator HrcL and the predicted C ring protein HrcQ, which is proposed to form a ring structure at the cytoplasmic side of the IM. HrcQ and HrcN both bind to the cytoplasmic regions of HrcU (U_C) and HrcV (V_C). HrcU_C is proteolytically cleaved off at a conserved NTPH amino acid motif and also interacts with HrcV_C. The export apparatus is presumably associated with ring structures at the periplasmic side of the IM (IM ring). Letters refer to individual Hrc proteins. Transparent barrels indicate the predicted multiple copies of HrcV, which might be involved in the formation of the export apparatus. Known protein–protein interactions and corresponding references are listed.

of the YscU homolog HrcU (HrcU_C). The self-interaction of HrcV_C suggests the formation of HrcV protein complexes, as was shown for YscV family members from animal-pathogenic bacteria that presumably oligomerize to initiate the assembly of the T3S export apparatus (Abrusci et al. 2013; Diepold et al. 2011; Li and Sourjik 2011). A similar role of YscV homologs from plant-pathogenic bacteria in the assembly of the T3S system remains to be investigated. The interaction with T3S system components, including HrcN and HrcU_C, probably accounts for the membrane association of HrcV_C. Notably, HrcN and HrcQ also associate with the bacterial membranes under T3S-permissive conditions (Lorenz et al. 2009, 2012). HrcN interacts with HrcQ and the predicted regulator of the ATPase, HrcL, suggesting that a complex of HrcN, HrcL, and HrcQ associates with the export apparatus at the IM upon activation of the T3S system (Lorenz and

Büttner 2009; Lorenz et al. 2012) (Fig. 6). Given that HrcN and HrcQ both interact with the cytoplasmic regions of HrcU and HrcV, HrcU_C and HrcV_C presumably provide a docking platform for the cytoplasmic ATPase complex and the predicted C ring of the T3S system (Lorenz and Büttner 2009; Lorenz et al. 2012) (Fig. 6).

Despite the essential contribution of the cytoplasmic region of HrcV to the identified protein–protein interactions, HrcV_C is presumably not sufficient for protein function, as was revealed by complementation studies. Thus, HrcV function also depends on the transmembrane region of the protein which contains the FHIPEP motif between transmembrane helix four and five. The FHIPEP motif represents the most conserved region in YscV family members but its precise contribution to protein function has not yet been analyzed. Given that point mutations in conserved amino acid residues of the FHIPEP motif of

Table 1. Bacterial strains and plasmids used in this study

Strains, plasmids	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; Kousik and Ritchie 1998
85-10Δ <i>hrcV</i>	85-10 derivative deleted in codons 251-438 of <i>hrcV</i>	Rossier et al. 1999
85-10Δ <i>hrcV</i> ₃₂₄₋₆₄₂	85-10 derivative deleted in codons 324-642 of <i>hrcV</i>	This study
85-10 <i>hrcV</i> _{R138A}	<i>hrcV</i> _{R138A} mutant derivative of strain 85-10	This study
85-10 <i>hrcV</i> _{D149A}	<i>hrcV</i> _{D149A} mutant derivative of strain 85-10	This study
85-10 <i>hrcV</i> _{D161A}	<i>hrcV</i> _{D161A} mutant derivative of strain 85-10	This study
85-10 <i>hrcV</i> _{RSG}	<i>hrcV</i> _{G143R/T147S/A150G} mutant derivative of strain 85-10	This study
85*	85-10 derivative containing the <i>hrpG</i> * mutation	Wengelnik et al. 1999
85*Δ <i>hrcV</i>	85* derivative deleted in codons 251-438 of <i>hrcV</i>	Rossier et al. 1999
85* <i>hrcV</i> _{R138A}	<i>hrcV</i> _{R138A} mutant derivative of strain 85*	This study
85* <i>hrcV</i> _{D149A}	<i>hrcV</i> _{D149A} mutant derivative of strain 85*	This study
<i>Escherichia coli</i>		
BL21(DE3)	F [−] <i>ompT hsdS_B (r_B[−] m_B[−]) gal dcm</i> (DE3)	Stratagene, Heidelberg, Germany
Top10	F- <i>mcrAΔ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araΔ139Δ(ara-leu)7697 galU galK rpsL endA1 nupG</i>	Invitrogen, Karlsruhe, Germany
Plasmids		
pBRM	Golden-Gate-compatible derivative of pBBR1MCS-5, contains the <i>lac</i> promoter and a 3 × c-Myc epitope-encoding sequence, Gm ^r	Szczesny et al. 2010
pBRM <i>hrcV</i> _{ATG}	pBRM derivative encoding HrcV _{ATG} -c-Myc	This study
pBRM <i>hrcV</i> _{GTG}	pBRM derivative encoding HrcV _{GTG} -c-Myc	This study
pBRM <i>hrcV</i> ₁₋₃₃₆	pBRM derivative encoding HrcV ₁₋₃₃₆ -c-Myc	This study
pBRM <i>hrcV</i> ₃₂₄₋₆₄₅	pBRM derivative encoding HrcV ₃₂₄₋₆₄₅ -c-Myc	This study
pBRM <i>hrcV</i> _{Δ138-161}	pBRM derivative encoding HrcV _{Δ138-161} -c-Myc	This study
pBRM <i>hrcV</i> _{R138A}	pBRM derivative encoding HrcV _{R138A} -c-Myc	This study
pBRM <i>hrcV</i> _{D149A}	pBRM derivative encoding HrcV _{D149A} -c-Myc	This study
pBRM <i>hrcV</i> _{D161A}	pBRM derivative encoding HrcV _{D161A} -c-Myc	This study
pBRM <i>hrcV</i> _{ASG}	pBRM derivative encoding HrcV _{G143A/T147S/A150G} -c-Myc	This study
pBRM <i>hrcV</i> _{RAG}	pBRM derivative encoding HrcV _{G143R/T147A/A150G} -c-Myc	This study
pBRM <i>hrcV</i> _{RSG}	pBRM derivative encoding HrcV _{G143R/T147S/A150G} -c-Myc	This study
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	Escolar et al. 2001; Stratagene
pGavrBs3	Derivative of pGEX-2TKM encoding GST-AvrBs3	Szurek et al. 2001
pGavrBs3Δ2	Derivative of pGEX-2TKM encoding GST-AvrBs3Δ2	Büttner et al. 2004
pGavrBs3 ₁₋₅₀	Derivative of pGEX-2TKM encoding GST-AvrBs3 ₁₋₅₀	Büttner et al. 2004
pGhpaB	Derivative of pGEX-2TKM encoding GST-HpaB	Büttner et al. 2006
pGhpaC	Derivative of pGEX-2TKM encoding GST-HpaC	Büttner et al. 2006
pGhrpB2	Derivative of pGEX-2TKM encoding GST-HrpB2	Schulz and Büttner 2011
pGhrpE	Derivative of pGEX-2TKM encoding GST-HrpE	Büttner et al. 2002
pGhrpC	Derivative of pGEX-2TKM encoding GST-HrcC	This study
pGhrN	Derivative of pGEX-2TKM encoding GST-HrcN	Lorenz and Büttner 2009
pGhrU ₂₅₅₋₃₅₇	Derivative of pGEX-2TKM encoding GST-HrcU ₂₅₅₋₃₅₇	Lorenz et al. 2008
pGhrV ₃₂₄₋₆₄₅	Derivative of pGEX-2TKM encoding GST-HrcV ₃₂₄₋₆₄₅	This study
pGxopJ	Derivative of pGEX-2TKM encoding GST-XopJ	Lorenz et al. 2012
pRK2013	ColE1 replicon, TraRK ⁺ Mob ⁺ ; Km ^r	Figurski, 1979
pFG72-1	Derivative of pUFR043 encoding HrpG*, Km ^r	Wengelnik et al. 1999
pOGG2	Golden Gate-compatible derivative of pOK1, Sm ^r	Schulze et al. 2012
pOGG2 <i>hrcV</i> _{Δ324-642}	Derivative of pOGG2 carrying the flanking regions of <i>hrcV</i> ₃₂₄₋₆₄₂	This study
pOGG2 <i>hrcV</i> _{R138A}	Derivative of pOGG2 carrying <i>hrcV</i> _{R138A}	This study
pOGG2 <i>hrcV</i> _{D149A}	Derivative of pOGG2 carrying <i>hrcV</i> _{D149A}	This study
pOGG2 <i>hrcV</i> _{D161A}	Derivative of pOGG2 carrying <i>hrcV</i> _{D161A}	This study
pOGG2 <i>hrcV</i> _{RSG}	Derivative of pOGG2 carrying <i>hrcV</i> _{RSG}	This study

^a GST, glutathione S-transferase; Ap, ampicillin; Km, kanamycin; Rif, rifampicin; Sm, spectinomycin; Gm, gentamycin; r, resistant.

HrcV interfere with protein function, the FHIPEP motif is presumably required for the contribution of HrcV to T3S and pathogenicity (Figs. 3 and 4). This is in line with the finding that deletion of the FHIPEP motif in FlhA from *Salmonella* spp. leads to a loss of protein function (McMurry et al. 2004). Our mutant approaches also revealed a functional importance of the nonconserved amino acids of the FHIPEP motif from HrcV. Mutation of these residues to amino acids that are present at equivalent positions in the FHIPEP motif of the HrcV homolog InvA from *Salmonella* spp. did not significantly affect HrcV function (HrcV_{ASG}), whereas the exchange of one or two of the nonconserved amino acids against alternative residues abolished protein function (Fig. 4). FHIPEP mutant derivatives of HrcV also exerted a dominant-negative effect on pathogenicity that was less pronounced for HrcV_{ASG}. This is in agreement with the finding that the ectopic expression of FlhA derivatives from *Salmonella* spp. with deletions in the FHIPEP motif exerts a dominant-negative effect on bacterial motility (McMurry et al. 2004).

When we analyzed the influence of mutations in the FHIPEP motif on protein–protein interactions, we observed that certain mutations in the FHIPEP motif interfere with the self-interaction of HrcV_C and the binding of HrcV interaction partners, including HrcN, HrcU_C, HpaB, HrpB2, and HrpE (Fig. 5). Because the analyzed interaction partners bind to the cytoplasmic region of HrcV, mutations in the FHIPEP motif might indirectly affect the binding of HrcV interaction partners, presumably by interfering with the conformation of HrcV_C. Notably, however, none of the mutations affected the interaction of HrcV with HpaC and effector proteins, suggesting a differential contribution of the FHIPEP motif to the binding of HrcV_C interaction partners. Therefore, it is tempting to speculate that different conformations of HrcV_C are required for the interaction with specific binding partners. Crystal structure analyses might help to clarify whether mutations in the FHIPEP motif alter the conformation of HrcV_C. However, it will be technically challenging to obtain the crystal structure for the full-length HrcV protein. Thus far, crystal structures have only been published for the cytoplasmic regions of HrcV homologs (Abrusci et al. 2013; Bange et al. 2010; Lilic et al. 2010; Moore and Jia 2010; Saijo-Hamano et al. 2010; Worrall et al. 2010). Notably, experimental evidence suggests that the cytoplasmic region of the YscV homolog FlhA undergoes a conformational change upon binding of FlhA interaction partners (Saijo-Hamano et al. 2010). Therefore, it remains to be investigated in future studies whether conformational changes in the cytoplasmic regions of YscV family members control the specificity of substrate binding during T3S in plant- and animal-pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* cells were grown at 37°C in lysogeny broth or Super medium (Qiagen, Hilden, Germany). *X. campestris* pv. *vesicatoria* strains were cultivated at 30°C in nutrient-yeast-glycerol medium (Daniels et al. 1984) or in minimal medium A (Ausubel et al. 1996) supplemented with sucrose (10 mM) and casamino acids (0.3%). Plasmids were introduced into *E. coli* by chemical transformation 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; and gentamycin, 7.5 µg/ml.

Plant material and plant inoculations.

The near-isogenic ECW and ECW-10R pepper cultivars (Kousik and Ritchie 1998; Minsavage et al. 1990) were grown and inoculated as described previously (Bonas et al. 1991). Briefly, *X. campestris* pv. *vesicatoria* strains were inoculated with a needle-less syringe into the intercellular spaces of leaves at concentrations of 1×10^8 CFU ml⁻¹ in 10 mM MgCl₂ if not stated otherwise. The appearance of disease symptoms and the HR were scored over a period of 1 to 10 days postinoculation. For the better visualization of the HR, leaves were bleached in 70% ethanol. Experiments were repeated at least two times.

Generation of expression constructs.

For the generation of a *hrcV* expression construct encoding c-Myc epitope-tagged HrcV_{ATG}, codons 1 to 270 and 271 to 645 of *hrcV*_{ATG} were amplified from *X. campestris* pv. *vesicatoria* 85-10 by polymerase chain reaction (PCR) using primers that contained a 4-bp overlap and *BsaI* sites. Similarly, codons 6 to 270 and 271 to 645 were amplified to generate a *hrcV*_{GTG}-c-myc expression construct. Primers were designed to introduce silent mutations leading to the removal of internal *BsaI* sites in *hrcV*. Both amplicons were cloned into the Golden-Gate-compatible expression vector pBRM in a single restriction-ligation reaction using *BsaI* and ligase as described, generating pBRMhrcV_{ATG} and pBRMhrcV_{GTG} (Engler et al. 2008). To construct HrcV derivatives, codons 1 to 336 and 324 to 645 of *hrcV* were amplified by PCR and cloned separately into pBRM. For the generation of an HrcV-derivative deleted in codons 138 to 161, which encode the FHIPEP motif, codons 1 to 137 and 162 to 645 were amplified by PCR and both amplicons were cloned into plasmid pBRM, generating pBRMhrcV_{Δ138-161}. To create HrcV point mutant derivatives with amino acid exchanges in the FHIPEP motif, codons 1 to 144 and 145 to 645 of *hrcV* were amplified by PCR and cloned into plasmid pBRM. Mutations leading to amino acid exchanges at positions R138, G143, T147, D149, A150, and D161 were introduced by primer sequences (codon 138, CGC exchanged by CGG; codon 143, GGT exchanged by GCG; codon 147, ACG exchanged by TCT; codon 149, GAC exchanged by GCC; codon 150, GCA exchanged by GGT; and codon 161, GAC exchanged by GCC). Primer sequences are available upon request. To obtain GST expression constructs of *hrcV*₃₂₄₋₆₄₅ and *hrcC*, the corresponding genes or gene fragments were amplified by PCR and cloned into the *EcoRI* and *XhoI* sites of plasmid pGEX-2TKM.

Generation of *X. campestris* pv. *vesicatoria* *hrcV* mutants.

To generate a 957-bp in-frame deletion of *hrcV*_C (deletion of codons 324 to 642), we amplified 700-bp fragments upstream and downstream of the desired deletion by PCR and cloned the PCR products into the Golden-Gate-compatible suicide vector pOGG2. The resulting construct, pOGG2hrcV_{Δ324-642}, was conjugated into *X. campestris* pv. *vesicatoria* 85-10. Double crossovers resulted in strain 85-10ΔhrcV₃₂₄₋₆₄₂ that was selected as described previously (Huguet et al. 1998). Because no transconjugants could be obtained for strain 85*, we introduced plasmid pFG72-1, which encodes HrpG*, into strain 85-10ΔhrcV₃₂₄₋₆₄₂. To introduce R138A, D149A, or D161A mutations individually into the genomic *hrcV* gene, 500- and 1,000-bp fragments flanking codons 138, 149, or 161 of *hrcV* were amplified. Amplicons contained a CGC to GCG exchange of codon 138, creating an *AclI* site (silent mutation of codon 138); a GAC to GCT exchange of codon 149, which creates a *TseI* site (silent mutation of codon 149); or a GAC to GCA exchange of codon 161 to generate a *DraIII* site (silent mutation of codon 161). The PCR products were cloned into the

Golden-Gate-compatible suicide vector pOGG2 and the resulting constructs (pOGG2hrcV_{R138A}, pOGG2hrcV_{D149A}, or pOGG2hrcV_{D161A}) were conjugated into *X. campestris* pv. *vesicatoria* strains 85-10 and 85*, as described above. Because no transconjugants of construct pOGG2hrcV_{D161A} could be obtained for strain 85*, we introduced plasmid pFG72-1 into strain 85-10hrcV_{D161A}.

To generate a genomic *hrcV* mutant encoding HrcV_{RSG}, 500- and 1,000-bp fragments flanking codon 147 of *hrcV* were amplified. The fragments contained a GGT to CGT exchange of codon 143, an ACG to AGC exchange of codon 147, and a GCA to GGA exchange of codon 150, creating a silent *BsmI* site. Both amplicons were cloned into the suicide vector pOGG2, generating pOGG2hrcV_{RSG}, which was conjugated into *X. campestris* pv. *vesicatoria* 85-10, as described above.

In vitro T3S assays and analysis of protein extracts.

For the analysis of in vitro T3S, bacteria were incubated in secretion medium and equal amounts of bacterial total cell extracts and culture supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as was described previously (Rossier et al. 1999). We used antibodies specific for HrpF (Büttner et al. 2002) or the c-Myc epitope (Roche Applied Science, Mannheim, Germany); horseradish peroxidase-labeled anti-rabbit and anti-mouse antibodies (GE Healthcare, Munich) were used as secondary antibodies. Experiments were repeated at least two times. Blots were routinely reacted with polyclonal antibodies specific for the intracellular protein HrcJ to ensure that no bacterial lysis had occurred (Rossier et al. 2000).

GST pull-down assays.

For GST pull-down assays, GST and GST fusion proteins were synthesized in *E. coli* BL21 (DE3). Bacterial cells from 50-ml cultures were resuspended in phosphate-buffered saline (PBS) and broken with a French press. Insoluble cell debris were removed by centrifugation, and soluble GST and GST fusion proteins were immobilized on a glutathione sepharose matrix according to the manufacturer's instructions (GE Healthcare). The matrix was washed twice with PBS and then incubated with 800 µl of *E. coli* lysate containing the putative interaction partner for 2 h at 4°C. Unbound proteins were removed by washing four times with PBS and bound proteins were eluted with 10 mM reduced glutathione at room temperature for 2 h. In all, 10 µl of total protein lysates and 20 µl of eluted proteins were analyzed by SDS-PAGE and immunoblotting using antibodies specific for the c-Myc epitope and GST, respectively (Roche Applied Science and GE Healthcare). Horseradish peroxidase-labeled anti-mouse and anti-goat antibodies (GE Healthcare) were used as secondary antibodies. Experiments were repeated at least twice.

Fractionation studies.

For fractionation studies, bacteria were precultured overnight in 5 ml of minimal medium A (pH 7.0), resuspended in 50 ml of minimal medium A at pH 5.3 (secretion-permissive conditions), and grown overnight to an optical density at 600 nm (OD₆₀₀) of approximately 0.9. Cells were harvested by centrifugation, resuspended in 3 ml of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and broken with a French press. Cell debris was removed by centrifugation and 1 ml of lysate was centrifuged at 244,413 × g for 90 min at 4°C. The pellet, which corresponds to the membrane fraction, was resuspended in 1 ml of 10 mM HEPES. Membrane and soluble fractions were mixed with Laemmli buffer and 20 µl of total bacterial lysates, and membrane and soluble fractions were analyzed by SDS-PAGE and immunoblotting using antibodies

specific for the c-Myc epitope and the intracellular protein HrcJ, respectively. Similar protein levels were adjusted according to OD₆₀₀. Experiments were repeated at least twice.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

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