Characterization of HrpB2 from *Xanthomonas campestris* pv. *vesicatoria* identifies protein regions that are essential for type III secretion pilus formation

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The Gram-negative plant-pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* employs a type III secretion (T3S) system to translocate effector proteins into plant cells. T3S depends on HrpB2, which is essential for assembly of the extracellular T3S pilus and is itself weakly secreted. To characterize the role of HrpB2, we used a transposon mutagenesis approach, which led to the insertion of pentapeptide-encoding sequences into *hrpB2*. Complementation studies with HrpB2 mutant derivatives revealed that the N-terminal region of HrpB2 tolerates pentapeptide insertions, whereas insertions in the regions spanning amino acids 60–74 and 93–130, respectively, resulted in a loss of bacterial pathogenicity and T3S, including secretion of HrpB2 itself. The C-terminal region (amino acids 93–130) of HrpB2 contains a conserved VxTLxK amino acid motif that is also present in predicted inner rod proteins from animal-pathogenic bacteria and is required for the contribution of HrpB2 to pilus assembly and T3S. Electron microscopy and fractionation studies revealed that HrpB2 is not a component of the extracellular pilus structure but localizes to the bacterial periplasm and the outer membrane. We therefore propose that the essential contribution of HrpB2 to T3S and pilus assembly is linked to its possible function as a periplasmic component of the T3S system at the base of the pilus.

**INTRODUCTION**

Many Gram-negative plant- and animal-pathogenic bacteria employ a type III secretion (T3S) system to transport bacterial effector proteins directly into the eukaryotic host cell, a process that is hereafter referred to as translocation (Ghosh, 2004). T3S systems are being analysed intensively in several bacterial model organisms, including species of *Yersinia*, *Salmonella*, *Shigella*, *Pseudomonas* and *Xanthomonas* that cause severe disease in humans, animals and/or crop plants. In our laboratory, we are studying T3S in *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease on pepper and tomato plants. The T3S system of *X. campestris* pv. *vesicatoria* translocates approximately 30 effector proteins into the plant cell that manipulate host cellular pathways such as defence responses to the benefit of the pathogen and thus promote bacterial multiplication in the plant apoplast (Büttner & Bonas, 2010; Ryan *et al.*, 2011). However, in resistant plants that possess cognate resistance genes, individual effector proteins [also designated Avr (avirulence) proteins] can be recognized by the plant surveillance system, which activates defence reactions. Avr protein-triggered plant defence is often associated with the hypersensitive response (HR), a rapid local cell death at the infection site which restricts bacterial ingress (Jones & Dangl, 2006). Effector protein translocation depends on the complete assembly of the membrane-spanning T3S apparatus, which is associated with an extracellular pilus that spans the plant cell wall. The pilus is directly or indirectly connected to a bacterial channel-like translocon that presumably inserts into the host plasma membrane and mediates effector protein translocation (Büttner & He, 2009; Ghosh, 2004; Mattei *et al.*, 2011). Components of the T3S system from *X. campestris* pv. *vesicatoria* are encoded by the chromosomal...
*hrp* (hypersensitive response and pathogenicity) gene cluster, which contains 25 genes that are organized in eight operons (Bonas *et al.*, 1991; Büttner *et al.*, 2007; Rossier *et al.*, 2000; Weber *et al.*, 2007; Wengelnik *et al.*, 1996a). Eleven *hrp* gene products (designated Hrc for *hrp* conserved) are conserved among plant- and/or animal-pathogenic bacteria, and presumably constitute the core components of the T3S system (He *et al.*, 2004). The nomenclature of Hrc proteins refers to the homologous Ysc (Yersinia secretion) proteins from the animal-pathogenic bacterium Yersinia. Eight Hrc proteins are also homologous to components of the bacterial flagellum, which is evolutionarily related to translocation-associated T3S systems and is therefore referred to as the flagellar T3S system. Electron microscopy (EM) studies have revealed that the core secretion apparatus of translocation-associated and flagellar T3S systems shares a similar overall architecture and contains ring complexes in the inner membrane (IM) and outer membrane (OM) (Blocker *et al.*, 2001; Kubori *et al.*, 1998; Marlovits *et al.*, 2004; Sani *et al.*, 2007). The OM ring is connected to the extracellular appendages of the secretion system, including the pilus (up to 2 μm long, in plant-pathogenic bacteria) or needle (40–80 nm long, in animal-pathogenic bacteria) of translocation-associated T3S systems and the hook of flagellar T3S systems, which is associated with the filament. The IM ring provides a scaffold for the assembly of transmembrane components of the T3S apparatus, which include members of the YscR, YscS, YscT, YscU, YscV, YscN, YscL and YscQ protein families (Erhardt *et al.*, 2010; Ghosh, 2004). EM studies of isolated needle complexes from Salmonella typhimurium have revealed the presence of an ‘inner rod’ structure in the periplasm, which is composed of PrgJ and is presumably required for the stable anchoring of the extracellular needle (Marlovits *et al.*, 2004, 2006; Wood *et al.*, 2008). Evidence for an inner rod has also been reported for the T3S system from enteropathogenic Escherichia coli (Ogino *et al.*, 2006; Sal-Man *et al.*, 2012), but the presence of similar structures in T3S systems from other animal- and plant-pathogenic bacteria awaits experimental confirmation.

Given the architecture of the T3S system, it is assumed that T3S is a hierarchical process and that components of the pilus/needle are secreted prior to translocon and effector proteins. Pilus and needle components are therefore referred to as ‘early substrates’ of the T3S system. A switch in T3S substrate specificity presumably activates the secretion of translocon and effector proteins (‘late substrates’) after pilus/needle formation. The substrate specificity of T3S systems is controlled by so-called T3S substrate specificity switch (T3S4) proteins that have been identified in translocation-associated and flagellar T3S systems (Agrain *et al.*, 2005; Deane *et al.*, 2010). Several T3S4 proteins have been shown to interact with the C-terminal cytoplasmic domains of members of the FlhB/YscU family of IM proteins that are essential for T3S. FlhB/YscU family members have been proposed to act as substrate docking sites, and are proteolytically cleaved at a conserved NPTH motif (letters refer to amino acids) that is important for protein function (Botteaux *et al.*, 2008; Ferris *et al.*, 2005; Lavander *et al.*, 2002; Lorenz *et al.*, 2008; Minamino & MacNab, 2000; Sorg *et al.*, 2007). It is assumed that the cleavage and/or the interaction with T3S4 proteins induces a conformational change in or around the NPTH loop, which leads to the switch in T3S substrate specificity.

We have previously shown that T3S in X. campestris pv. vesicatoria is controlled by the cytoplasmic T3S4 protein HpaC, which promotes the secretion of translocon and effector proteins and inhibits the efficient secretion of the pathogenicity factor HrpB2 (Lorenz *et al.*, 2008). HrpB2 is essential for T3S and pilus formation, and is presumably one of the first substrates that traverses the T3S system (Rossier *et al.*, 2000; Weber *et al.*, 2005). Previous interaction studies revealed that HrpB2 interacts with HpaC and that both proteins bind to the C-terminal cytoplasmic domain of the FlhB/YscU family member HrcU (HrcUC), which is essential for the substrate specificity switch (Alegria *et al.*, 2004; Lorenz *et al.*, 2008; Schulz & Büttner, 2011). The finding that the HrpB2–HrcUC interaction is probably required for the efficient secretion of HrpB2 is in agreement with the predicted role of HrcUC as a substrate docking site of the T3S system (Lorenz & Büttner, 2011). It is, however, unknown why the secretion of HrpB2 is suppressed by HpaC and how HrpB2 contributes to T3S. As HrpB2 is conserved in species of Xanthomonas, Burkholderia and Acidovorax, and in Ralstonia solanacearum, it is presumably involved in multiple plant–pathogen interactions. Previous sequence analyses of HrpB2 homologues have revealed the presence of two conserved motifs in the central (FQALM, letters refer to amino acids 35–39) and the C-terminal region (TLMKNQ, amino acids 125–130) (Cappelletti *et al.*, 2011). In contrast to the FQALM motif, the C-terminal TLMKNQ motif appears to be essential for the contribution of HrpB2 from Xanthomonas axonopodis pv. citri to pathogenicity towards citrus plants (Cappelletti *et al.*, 2011).

In this study, we aimed to characterize HrpB2 from *X. campestris* pv. vesicatoria. To identify functionally important protein regions, we used a transposon mutagenesis approach that led to the insertion of pentapeptides into HrpB2. Complementation studies revealed that the C-terminal region of HrpB2 is essential for protein function and does not tolerate insertions. Interestingly, this region of HrpB2 contains a conserved VxTLxK amino acid motif, which is also present in predicted inner rod proteins from animal-pathogenic bacteria and overlaps with the previously identified TLMKNQ motif. Analysis of point mutant derivatives of HrpB2 and the results of EM and infection studies suggest that the VxTLxK motif is essential for pilus assembly, T3S and pathogenicity, and that the contribution of single amino acids of this motif to protein function is not conserved among HrpB2 from *X. campestris*.
pv. vesicatoria and X. axonopodis pv. citri. As HrpB2 is itself not associated with extracellular pilus structures but localizes to the bacterial periplasm and the OM, we suggest that it promotes pilus assembly as an internal component of the membrane-spanning T3S system.

**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 (LB) or Super medium (Qiagen). X. campestris pv. vesicatoria strains were cultivated at 30 °C in nutrient-yeast-glycerol (NYG) 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 (Figsz & Helsinki, 1979). Antibiotics were added to the media at the following final concentrations: ampicillin, 100 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; rifampicin, 100 µg ml⁻¹; spectinomycin, 100 µg ml⁻¹; gentamicin, 7.5 µg ml⁻¹.

**Plant material and plant inoculations.** The near-isogenic pepper cultivars Early Cal Wonder (ECW) and ECW-10R (Kousik & Ritchie, 1998; Mimsavage et al., 1990) were grown as described previously (Bonas et al., 1991). X. campestris pv. vesicatoria strains were inoculated with a needle-less syringe into the intercellular spaces of leaves at concentrations of 2 × 10⁶ cf.u. ml⁻¹ in 1 mM MgCl₂ if not stated otherwise. The appearance of disease symptoms and the HR was scored over a period of 1–9 days post-inoculation (days p.i.). For better visualization of the HR, leaves were bleached in 70 % ethanol. Experiments were repeated at least twice.

**Generation of expression plasmids and deletion mutants.** For the generation of hrpB2 expression constructs, hrpB2 was amplified by PCR from X. campestris pv. vesicatoria strain 85-10 and inserted into the Smal site of vector pUC57ABsal in a restriction-ligation reaction (Bolchi et al., 2005). pUC57ABsal is a derivative of pUC57 in which the internal Bsal site is mutated (Morbitzer et al., 2011). Then, hrpB2 point mutant and deletion derivatives were generated by PCR using pUC57hrpB2 as a template and phosphorylated primers that annealed back to back to the template and were designed to introduce the desired deletions. The amplicons were religated and transformed into E. coli, and corresponding inserts were subsequently cloned into the Golden Gate-compatible vector pBRM downstream of a single lac promotor using the type II restriction enzyme Bsal (Engler et al., 2008; Szczesny et al., 2010). Amplicons contained an internal stop codon to prevent translation of the 3 × c-Myc epitope-encoding sequence of pBRM.

For generation of glutathione S-transferase (GST) expression constructs, hrpB2 as well as point mutant and deletion derivatives thereof were amplified by PCR and cloned into the EcoRI and Xhol sites of plasmid pGEX-2TKM. Primer sequences are available upon request.

For the generation of strain 85°AΔhpaCAhrpB2, the suicide vector construct pOKAhrpB2 that harbours the flanking regions of hpaC was conjugated into X. campestris pv. vesicatoria strain 85°AhrpB2. Homologous recombinations led to deletion of the genomic hpaC gene, and double deletion mutants were selected as described previously (Huguet et al., 1998).

**Generation of HrpB2 derivatives with pentapeptide insertion.** We used the MGS Mutation Generation System kit (Finnzymes) to generate a library of transposon-mutagenized hrpB2 derivatives. The in vitro transposition reaction was performed with the Golden Gate-compatible expression plasmid pBRMhrpB2STOP, which is a derivative of pBRMhrpB2STOP and contains an internal mutated NotI restriction site. To generate pBRMN, pBRM was amplified by PCR using phosphorylated primers that annealed back to back to the vector backbone and contained the mutation (see Table 1). The amplicon was religated to generate pBRMN, and hrpB2 (with stop codon) was inserted as described above, giving pBRMhrpB2STOP. According to the manufacturer’s instructions, the target plasmid pBRMhrpB2STOP was mixed with the MuA Transposase and the Entranceposon M1-Kan6, which inserts into random sites of the target DNA and leads to 5 bp duplications of the target sequence (Finnzymes). The in vitro transposition reaction mixture was transformed into E. coli and transformants were selected for gentamicin and kanamycin resistance. Positive clones with a transposon insertion in hrpB2 were identified by colony PCR using vector-specific primers that annealed to hrpB2-flanking regions. Corresponding plasmids were digested with NotI to remove most of the Entranceposon. Subsequent religation of plasmids led to the generation of 15 bp insertions consisting of a 10 bp Entranceposon sequence and 5 bp duplicated target site (see manufacturer’s instructions). Insertion sites were determined by sequencing. The amino acid sequences encoded by the 15 bp insertions varied depending on the reading frame in which the insertion had occurred (see manufacturer’s instructions). Insertion sites and inserted pentapeptides are listed in Fig. 1.

**GST pull-down assays.** For GST pull-down assays, GST and GST fusion proteins were synthesized in E. coli BL21(DE3). Bacterial cells from 30 ml cultures were resuspended in PBS and broken with a French press. Insoluble cell debris was 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). Unbound proteins were removed by washing twice with PBS, and the glutathione Sepharose matrix was incubated with 600 µl E. coli cell lysates containing the putative interaction partners for 1–2 h at 4 °C. Unbound proteins were removed by washing twice with PBS and bound proteins were eluted with 10 mM reduced glutathione at room temperature for 2 h. Then, 10 µl total protein lysates and 20 µl eluted proteins were analysed by SDS-PAGE and immunoblotting using antibodies specific for the c-Myc epitope and GST, respectively (Roche Applied Science and GE Healthcare) (Rossier et al., 2000). Horseradish peroxidase-labelled anti-mouse and anti-goat antibodies (GE Healthcare) were used as secondary antibodies. Antibody reactions were visualized by enhanced chemiluminescence (GE Healthcare).

**T3S assays.** T3S assays were performed as described previously (Rossier et al., 1999). Briefly, bacteria were incuolated in secretion medium, and equal amounts of bacterial total cell extracts and culture supernatants were analysed by SDS-PAGE and immunoblotting using polyclonal antibodies specific for HrpB2 and HrpF (Böttner et al., 2002; Rossier et al., 1999, 2000). Horseradish peroxidase-labelled anti-rabbit antibodies (GE Healthcare) were used as secondary antibodies. Experiments were repeated at least twice. Blots were routinely reacted with antibodies specific for the intracellular proteins HrcN or HrcJ to ensure that no bacterial lysis had occurred (Rossier et al., 2000) (data not shown).

**Subfractionation studies.** To separate bacterial cytoplasmic, periplasmic, IM and OM fractions, we used a protocol previously described by Thein et al. (2010). For this, bacteria were preincubated overnight in 5 ml minimal medium A (pH 7.0) supplemented with sucrose and Casamino acids, and subsequently grown in 1 l minimal medium A at pH 5.3 to induce T3S up to an OD₆⁰₀ of approximately 0.8. Bacterial cells were harvested by centrifugation, resuspended in
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics†</th>
<th>Reference(s) or source</th>
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<tr>
<td><strong>X. campestris pv. vesicatoria strains†</strong></td>
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<tr>
<td>85-10</td>
<td>hrpB2 deletion mutant of strain 85-10</td>
<td>Rossier et al. (2000)</td>
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<td>85*</td>
<td>85-10 derivative containing the hrpG* mutation</td>
<td>Wengelnik et al. (1999)</td>
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<td>85*ahpaC</td>
<td>hpaC deletion mutant of strain 85*&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Rossier et al. (2000)</td>
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<td>hpaC hrpB2 double deletion mutant of strain 85*&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>E. coli strains</strong></td>
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<td>BL21(DE3)</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; amp&lt;sup&gt;T&lt;/sup&gt; hsdS&lt;sub&gt;80&lt;/sub&gt; (r&lt;sub&gt;F&lt;/sub&gt; m&lt;sub&gt;T&lt;/sub&gt;) gal dcm BL21(DE3)</td>
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<td>DH5x</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; recA hsdR17&lt;sub&gt;16&lt;/sub&gt;L&lt;sub&gt;15&lt;/sub&gt; m&lt;sub&gt;C&lt;/sub&gt; &lt;del&gt;80&lt;/del&gt;lacZ &lt;del&gt;ΔM15&lt;/del&gt;</td>
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<td>DH5x &lt;del&gt;λpir&lt;/del&gt;</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; recA hsdR17&lt;sub&gt;16&lt;/sub&gt;L&lt;sub&gt;15&lt;/sub&gt; m&lt;sub&gt;C&lt;/sub&gt; &lt;del&gt;80&lt;/del&gt;lacZ &lt;del&gt;ΔM15&lt;/del&gt; [λpir]</td>
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<td><strong>Plasmids</strong></td>
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<td>pBBR1MCS-5</td>
<td>Broad-host-range vector; lac promoter; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pBlueskript(II) KS</td>
<td>Phagemid, pUC derivative; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Derivative of pBRM encoding HrpB2</td>
<td>This study</td>
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<td>pBRMhrpB2&lt;sub&gt;STOP&lt;/sub&gt;§</td>
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<td>pDSK604</td>
<td>Derivative of the broad-host-range vector pDSK602 with modified polylinker; contains triple lacUV5 promoter; Sm&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>ColE1 replicon, TraRK&lt;sup&gt;+&lt;/sup&gt; Mob&lt;sup&gt;+&lt;/sup&gt;; Km&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Derivative of pUC57 with mutated BsaI site</td>
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†Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Rif, rifampicin; Sm, spectinomycin; †, resistant.
§X. campestris pv. vesicatoria strain 85-10 was reclassified as Xanthomonas euvesicatoria (Jones et al., 2004). However, as this nomenclature has not yet been widely used, we refer to the classical name X. campestris pv. vesicatoria in this study.

†Transposon mutant derivatives of pBRMhrpB2 are listed in Fig. 1.
centrifuged at 85,000 g for 30 min at 4 °C. The supernatant contained the IM-enriched fraction. The pellet corresponds to the OM-enriched fraction and was washed in 1 ml 50 mM Tris/HCl, pH 8.0, 2 % (w/v) Triton X-100 and 10 mM MgCl2. The sample was centrifuged as above and the pellet was washed three times in 500 ml deionized water before it was resuspended in 1 ml Laemmli buffer. Proteins in the periplasm-, cytoplasm- and IM-enriched fractions were precipitated using TCA and sodium deoxycholate (DOC). For this, one volume of sample was mixed with 1/100 volume of 2 % (w/v) DOC and incubated for 30 min at 4 °C; 1/10 volume of 100 % TCA was added and the samples were incubated for 30 min on ice. After centrifugation at 10,000 g for 15 min at 4 °C, the pellet was dried, washed with ice-cold acetone and resuspended in 1 ml Laemmli buffer. Subsequently, 15 μl of each fraction was analysed by SDS-PAGE and immunoblotting using antibodies specific for HrpB2 and HrcC (Wengelnik et al., 1996a).

EM studies. For EM studies, bacteria were grown on gold grids (300 Square, #G2300A; Plano) covered with a Formvar film for 6 h at 30 °C under secretion-permissive conditions. Excess liquid was removed with filter paper and the grids were air-dried for 10 s, washed three times with H2O and stained with 1 % aqueous uranyl acetate solution. The samples were inspected with an EM 900 transmission electron microscope (Zeiss SMT). Micrographs were taken with an SSCCD SM-1k-120 camera (TRS).

For immunogold labelling experiments, bacteria were cultivated as described above and fixed with 2 % paraformaldehyde in PBS at room temperature for 20 min. The grids were washed three times with PBS for 1 min and treated with a blocking reagent (1 % acetylated BSA and 0.1 % Tween 20 in PBS) for 30 min. After incubation with the primary anti-HrpB2 and anti-HrpE antibodies (Weber et al., 2005) for 12 h at 4 °C, the grids were washed four times for 5 min each with blocking reagent and incubated with the secondary antibody (goat anti-rabbit 10 nm gold conjugate, Sigma-Aldrich) for 90 min at room temperature. After washing four times with H2O for 5 min each, the samples were stained with 1 % aqueous uranyl acetate solution and inspected by EM as described above.

Fig. 1. Overview of the positions of pentapeptide insertions in HrpB2 and their effects on bacterial pathogenicity. HrpB2 is represented by a rectangle, and boxes correspond to single amino acids. The positions and amino acid sequences of pentapeptide insertions that were identified in this study are indicated below [e.g. P6 (VRPHP/VAAAP) refers to two different pentapeptide insertions after the proline residue at amino acid position 6 with the amino acid sequences VRPHP and VAAAP, respectively]. HrpB2 mutant derivatives were analysed in X. campestris pv. vesicatoria strains 85-10ΔhrpB2 (hrpG wt) and 85ΔhrpB2 (hrpG*) for their abilities to complement the mutant phenotype with respect to disease symptoms and the HR in susceptible and resistant pepper plants, respectively. Green rectangles refer to mutant derivatives that restored the wild-type phenotype, whereas HrpB2 derivatives that partially complemented the hrpB2 mutant phenotype are indicated in yellow. Red rectangles refer to HrpB2 derivatives that did not restore symptom formation in hrpB2 mutant strains. The results of the complementation studies differed for three HrpB2 mutant derivatives depending on the amino acid sequences of the pentapeptide insertions. In these cases, single rectangles were subdivided into triangles that refer to each pentapeptide insertion (the upper triangle always corresponds to the first pentapeptide insertion sequence that is given in parentheses).
RESULTS

Generation of HrpB2 mutant derivatives by a transposon mutagenesis approach

This study aimed to identify functionally important protein regions in HrpB2 from X. campestris pv. vesicatoria strain 85-10. For this, we performed a random transposon mutagenesis of hrpB2 (see Methods) using plasmid pBRMNhrpB2 as template. pBRMNhrpB2 contains hrpB2 downstream of a lac promoter (see Table 1). In total, 1389 transposon-mutagenized derivatives of pBRMNhrpB2 were obtained and screened by PCR. Transposon sequences within hrbB2 were excised by NotI digestion (see Methods), and after religation an in-frame insertion of 15 bp random sequence was left, including a 5 bp duplicated target sequence. This led to five-amino-acid insertions with variable sequences in the corresponding gene products. In total, 106 mutagenized hrpB2 derivatives were generated that contained insertions at 64 different positions. Several insertions occurred at similar positions but encoded pentapeptides with different amino acid sequences. The positions of the insertions in HrpB2 and the pentapeptide sequences are summarized in Fig. 1. Hereafter, we refer to the corresponding HrpB2 derivatives by indicating the position of the insertion (e.g. HrpB2P6 contains a pentapeptide insertion after the proline residue at position 6 of HrpB2).

Infection studies with HrpB2 mutant derivatives identify functionally important protein regions

To characterize HrpB2 mutant derivatives functionally, corresponding expression constructs were introduced into X. campestris pv. vesicatoria strain 85-10ΔhrpB2, which is a non-pathogenic derivative of the wild-type strain 85-10 and lacks the chromosomal hrpB2 gene (Rossier et al., 2000). For infection studies, strains 85-10 and 85-10ΔhrpB2 carrying pBRMNhrpB2STOP (wild-type hrpB2) or mutant derivatives thereof were inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants. ECW-10R pepper plants carry the Bsl1 resistance gene and induce the HR upon recognition of the type III effector AvrBs1 that is delivered by strain 85-10 (Escolar et al., 2001; Ronald & Staskawicz, 1988). As expected, strain 85-10 induced disease symptoms, so-called water-soaked lesions, in ECW, and the HR in ECW-10R pepper plants, whereas no symptoms were observed for strain 85-10ΔhrpB2 (Rossier et al., 2000) (Fig. 2a). The hrbB2 mutant phenotype was fully or partially complemented by HrbB2 and HrbB2 derivatives with insertions in the N-terminal region (amino acids 6–55, except for insertion F35, which led to a loss of protein function) or the region spanning amino acids 80–91 (summarized in Fig. 1; the phenotypes of selected mutants are shown in Fig. 2a). By contrast, most pentapeptide insertions in the region spanning amino acids 60–74 and the C-terminal region (amino acids 93–130) of HrbB2 abolished HrbB2 function (Figs 1 and 2a). The observed phenotypic differences were not caused by differences in HrbB2 protein levels because all HrbB2 derivatives were stably synthesized (Fig. 2a; data not shown).

We also analysed HrbB2 mutant derivatives in hrpG* strains that contain a constitutively active version of the key regulatory protein HrpG. HrpG* leads to the constitutive expression of T3S genes and is therefore key for in vitro secretion assays (Wengelnik et al., 1996b, 1999). In most cases, similar phenotypes were observed for derivatives of hrpG wild-type and hrpG* strains (Fig. 1). Exceptions were HrbB2L31, HrbB2P55 and HrbB2P84, which fully restored pathogenicity of strain 85*ΔhrpB2 but only partially complemented the mutant phenotype of strain 85-10ΔhrpB2 (Fig. 1). These differences were presumably caused by the enhanced and accelerated symptom formation that is observed with hrpG* strains (Wengelnik et al., 1999). The mutant derivatives HrbB2L38, HrbB2M86 and HrbB2Q89, however, restored the wild-type phenotype of strain 85-10ΔhrpB2 but only partially complemented the mutant phenotype of strain 85*ΔhrpB2, suggesting that the presence of hrpG* can also interfere with the ability of some derivatives to restore pathogenicity of the hrpB2 deletion mutant (Fig. 1). The infection experiments with hrpG* strains were included in the study of HrbB2 mutant derivatives because these strains were further analysed by in vitro T3S assays that cannot be performed with hrpG wild-type strains.

HrbB2 mutant derivatives that lead to a loss of pathogenicity are not detectable in the culture supernatant and do not promote secretion of the translocon protein HrpF

For in vitro T3S assays, hrpG* derivatives were incubated in secretion medium, and total cell extracts and culture supernatants were analysed by immunoblotting, using antibodies specific for the translocon protein HrpF. As expected, HrpF was secreted by strain 85* but was not detectable in the culture supernatant of strain 85*ΔhrpB2 (Fig. 2b). The HrpF secretion deficiency was complemented by HrbB2 and the mutant derivatives HrbB2P6 and HrbB2P55. The derivative HrbB2S80, which fully restored pathogenicity of hrpB2 deletion mutants, only partially complemented the HrpF secretion deficiency, suggesting that even small amounts of secreted HrpF are sufficient for the interaction with the plant (Figs 1 and 2b). All other tested HrbB2 derivatives (HrbB2F35, HrbB2G60, HrbB2R73, HrbB2L102, HrbB2V113, HrbB2V123, HrbB2T125, HrbB2L126 and HrbB2K128) did not restore detectable levels of HrpF secretion, in agreement with the finding that they did not complement the hrpB2 mutant phenotypes in planta (Figs 1 and 2b).

We also investigated the secretion of HrbB2 mutant derivatives using an HrbB2-specific polyclonal antiserum. As HrbB2 is only weakly secreted by strain 85* (at the detection limit of the HrbB2-specific antiserum), secretion assays were performed with strain 85*ΔhpaCΔhrpB2.
carrying HrpB2 or selected mutant derivatives thereof as above. Deletion of the T3S4 gene hpaC leads to the oversecretion of HrpB2 and thus facilitates the detection of HrpB2 and derivatives in the culture supernatant (Lorenz et al., 2008). As expected, the amounts of the native HrpB2 protein in the culture supernatant of strain 85* were at the detection limit of the HrpB2-specific antiserum, whereas significantly increased amounts of HrpB2 were detected in the culture supernatant of strain 85*ΔhpaC. Similar results were observed for HrpB2, which was ectopically expressed in strain 85*ΔhpaCΔhrpB2 (Fig. 2b; note that the ectopic expression also leads to increased amounts of HrpB2 in the total cell extracts). Pentapeptide insertions at positions 6 and 55 did not significantly interfere with the secretion of corresponding HrpB2 derivatives, whereas the insertion at position 80 led to reduced HrpB2 secretion. Secretion of all other tested HrpB2 mutant derivatives with insertions at positions 35, 60, 73, 102, 111, 123, 125, 126 and 128 was

Fig. 2. Infection and secretion studies with selected HrpB2 mutant derivatives. (a) Infection studies with HrpB2 mutant derivatives. X. campestris pv. vesicatoria strains 85-10 (wt) and 85-10ΔhrpB2 (ΔhrpB2) carrying plasmid pBRM (−), HrpB2 or mutant derivatives thereof as indicated were inoculated at a density of 10⁸ c.f.u. ml⁻¹ into leaves of susceptible ECW and resistant ECW-10R pepper plants. Disease symptoms were photographed 7 days p.i. For better visualization of the HR, leaves were bleached in ethanol 2 days p.i. Dashed lines mark the inoculated areas. For analysis of HrpB2 and mutant derivatives thereof, bacterial protein extracts were analysed by immunoblotting using an HrpB2-specific antiserum. (b) Pentapeptide insertions in the regions spanning amino acids 60–74 and 93–130, respectively, of HrpB2 abolish the detectable secretion of the translocon protein HrpF and of HrpB2 mutant derivatives. X. campestris pv. vesicatoria strains 85* (wt), 85*ΔhrpB2 (ΔhrpB2), 85*ΔhpaC (ΔhpaC) and 85*ΔhpaCΔhrpB2 (ΔhpaCΔhrpB2) carrying plasmid pBRM (−), HrpB2 or mutant derivatives thereof with pentapeptide insertions as indicated [compare with (a)] were incubated in secretion medium. Total cell extracts (TE) and culture supernatants (SN) were analysed by SDS-PAGE and immunoblotting, using HrpF- and HrpB2-specific antibodies.
severely reduced (Fig. 2b). As these mutations also led to a loss of pathogenicity and detectable HrpF secretion (see above), we conclude that the essential role of HrpB2 for pathogenicity is linked to its contribution to T3S, including its own type III-dependent export.

**HrpB2 contains a functionally important VxTLxK motif that is also present in predicted inner rod proteins from animal-pathogenic bacteria**

Comparative sequence analyses revealed that HrpB2 contains a C-terminal amino acid motif (VxTLxK, amino acids 123–128) that is also present in HrpB2 homologues from *Xanthomonas* spp. and HrpF from *R. solanacearum*. Notably, we found a similar motif in the predicted inner rod proteins YscL from *Yersinia* spp., MxiL from *Shigella flexneri* and Prgl from *Salmonella* spp. (Fig. 3a, b). The VxTLxK motif overlaps with the TLKNG motif (amino acids 125–130), which was identified in HrpB2 homologues from *Xanthomonas* spp. and *R. solanacearum* and was shown to contribute to the pathogenicity of *X. axonopodis pv. citri* (Cappelletti et al., 2011). To characterize the functional importance of the VxTLxK motif in HrpB2 from *X. campestris pv. vesicatoria*, we generated HrpB2 derivatives lacking amino acids 123–128 and 123–130, respectively. HrpB2L126A and HrpB2K128A failed to complement the in planta hrpB2 mutant phenotypes and did not restore the secretion of the translocon protein HrpF (Fig. 3c), suggesting that amino acids 123–128 of HrpB2 are required for T3S and pathogenicity. As ectopic expression of hrpB2L126A or hrpB2K128A in strain 85-10 did not alter the wild-type phenotype, the observed phenotypes were presumably not caused by negative effects of the two HrpB2 deletion derivatives on pathogenicity (Fig. 3d).

We have previously shown that HrpB2 interacts with the T3S4 protein HpaC and the C-terminal domain of the IM protein HrcU, HrcUC (Lorenz et al., 2008). Here, we investigated whether the C-terminal region of HrpB2 is required for the interaction with HpaC and HrcUC. For this, we performed GST pull-down assays with GST, GST-HrpB2 and GST-HrpB2A123–130 that were immobilized on glutathione Sepharose and incubated with bacterial lysates containing HpaC-c-Myc and HrcUC1265–357-c-Myc, respectively. Fig. 3(e) shows that HpaC-c-Myc and HrcUC1265–357-c-Myc coeluted with both GST-HrpB2 and GST-HrpB2A123–130 but not with GST alone. We have previously observed that the NPTH motif of HrcU is required for HrpB2 binding and that a GST-HrcUC1265–357 fusion protein does not interact with HrpB2 (Lorenz & Büttner, 2011). However, it is possible that the conformation of the PTH loop (amino acids 265–267) of HrcU is altered in the context of the GST-HrcUC1265–357 fusion protein and thus affects the efficient interaction with HrpB2. Our finding that HrcUC1265–357 interacts with HrpB2 when analysed as a C-terminally c-Myc epitope-tagged derivative (Fig. 3e) suggests that the conserved asparagine residue of the NPTH motif is dispensable for the binding of HrcUC to HrpB2.

Notably, while comparable amounts of HrcUC1265–357-c-Myc coeluted with GST-HrpB2 and GST-HrpB2A123–130, the amounts of HpaC-c-Myc that were detected in the eluate of GST-HrpB2A123–130 were slightly reduced when compared with the eluate of GST-HrpB2. This suggests that the C-terminal eight amino acids of HrpB2 that harbour the VxTLxK motif might contribute to the interaction with HpaC but are dispensable for the binding of HrpB2 to HrcUC1265–357. In addition to GST-HrpB2A123–130 we also analysed the interaction of GST-HrpB2D1–89, which harbours only the C-terminal 40 amino acids of HrpB2, with HpaC and HrcUC1265–357. GST-HrpB2D1–89 appeared to interact with HpaC-c-Myc, although reduced amounts of HpaC-c-Myc were detected in the eluate of GST-HrpB2D1–89 when compared with GST-HrpB2 (Fig. 3e). It is therefore possible that the C-terminal region of HrpB2 contains a binding site for HpaC. However, the presence of an additional HpaC-binding site in the N-terminal region of HrpB2 cannot be excluded. In contrast to HpaC, HrcUC1265–357-c-Myc was not detected in the eluate of GST-HrpB2D1–89 suggesting that the C-terminal 40 amino acids of HrpB2 are not sufficient for the binding of HrcUC (Fig. 3e).

**Analysis of HrpB2 point mutant derivatives confirms the functional importance of the VxTLxK motif**

To further characterize the C-terminal region of HrpB2, we introduced point mutations, leading to individual exchanges of L102, V111, V123, T125, L126 and K128 by alanine. Complementation studies revealed that L102A and V111A exchanges in HrpB2 did not interfere with bacterial pathogenicity and *in vitro* secretion of HrpF (Fig. 4), although pentapeptide insertions at both positions abolished protein function (see above, Fig. 1). HrpB2 mutant derivatives with V123A and T125A exchanges restored symptom formation on susceptible and resistant pepper plants when analysed in strains 85-10hrpb2 and 85Δhrpb2 but only partially complemented the HrpF secretion deficiency of strain 85Δhrpb2 (Fig. 4). By contrast, HrpB2L126A and HrpB2K128A led to reduced *in planta* reactions and did not restore wild-type HrpF secretion levels (Fig. 4). We conclude from these data that the L126A and K128A exchanges affect the ability of HrpB2 torestore pathogenicity and T3S. Similarly to the C-terminal HrpB2 deletion derivatives, the observed phenotypes for HrpB2 point mutant derivatives were presumably not caused by possible negative effects of the proteins because ectopic expression of the corresponding genes in the wild-type strain 85-10 did not interfere with bacterial pathogenicity (data not shown).

**The C-terminal region of HrpB2 contributes to pilus formation**

Next, we investigated the contribution of HrpB2 and mutant derivatives thereof to the formation of T3S pili. For this, bacteria were incubated in secretion medium and pili...
Fig. 3. The C-terminal region of HrpB2 contains a functionally important amino acid motif that is also present in HrpJ and putative inner rod proteins. (a) Sequence alignment of HrpB2, HrpJ and putative inner rod proteins. The C-terminal amino acid sequences of HrpB2 from \textit{X. campestris pv. vesicatoria} (GenBank accession no. YP\_362159), HrpJ from \textit{R. solanacearum} GMI1000 (NP\_522427), MxiI from \textit{Shigella flexneri} (AAP79001), YscI from \textit{Yersinia enterocolitica} (AAD16842) and PrgJ from \textit{S. enterica} subsp. \textit{enterica} sv. Typhimurium (AAB60190) were aligned using CLUSTALW 2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Conserved amino acids are highlighted in black, and numbers refer to amino acid positions. Amino acids of HrpB2 that were replaced by alanine in this study are marked by asterisks. (b) Results of a motif search with HrpB2, HrpJ and predicted inner rod proteins. Amino acid frequencies are displayed in a logo (http://meme.sdsc.edu/meme/intro.html). The sequences correspond to amino acids 116–129 of HrpB2, 114–127 of HrpJ, 84–97 of MxiI, 101–114 of YscI and 88–101 of PrgJ [GenBank accession numbers as in (a)]. (c) The C-terminal eight amino acids of HrpB2 are essential for T3S and pathogenicity. \textit{X. campestris pv. vesicatoria} strains 85* (wt) and 85*\_hrpB2 (\textit{D}hrpB2) carrying plasmid pBRM (–), HrpB2, HrpB2\textsubscript{D123–130} or HrpB2\textsubscript{D123–128} as indicated were inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants. Disease symptoms were photographed 8 days p.i. For better visualization of the HR, leaves were bleached in ethanol 1 day p.i. Dashed lines indicate the infiltrated areas. Similar phenotypes were observed for \textit{hrpG} wild-type strains (data not shown). For in vitro T3S assays, the same strains were incubated in secretion medium. Total cell extracts (TE) and culture supernatants (SN) were analysed by SDS-PAGE and immunoblotting using an HrpF-specific antiserum. (d) C-terminal HrpB2 deletion derivatives do not exert a dominant-negative effect on the host–pathogen interaction. Strain 85-10 carrying plasmid pBRM (–) or expression constructs encoding HrpB2, HrpB2\textsubscript{D123–130} or HrpB2\textsubscript{D123–128} as indicated were inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants. Disease symptoms and the HR were documented as described in (c). (e) Interaction studies with HrpB2\textsubscript{D123–130}. GST, GST-HrpB2 and GST-HrpB2\textsubscript{D123–130} were immobilized on glutathione Sepharose and incubated with bacterial lysates containing HpaC-c-Myc and HrcU\textsubscript{265–357}-c-Myc, respectively. Total cell extracts (TE) and eluted proteins (eluates) were analysed by SDS-PAGE and immunoblotting, using c-Myc epitope- and GST-specific antibodies. GST and GST fusion proteins are marked by asterisks, and lower bands correspond to degradation products. One representative blot reacted with the GST-specific antiserum is shown.
were analysed by EM as described by Weber et al. (2005). T3S pili were visible for strain 85* but not for the hrpB2 deletion mutant 85*ΔhrpB2, which confirms the previous observation that HrpB2 is required for pilus formation (Fig. 5a) (Weber et al., 2005). To analyse the influence of HrpB2 derivatives with point mutations and deletions within the C-terminal VxTLxK motif on pilus formation, we counted the number of pili of approximately 300 cells for each strain. The majority of cells contained one or no pilus (Fig. 5). However, the total number of pili in every sample is presumably higher because the pilus structures facing the EM grid could not be counted. Furthermore, as T3S pili are very fragile and easily break during the experimental procedure, we counted only pili structures that were still attached to bacterial cells. Ectopic expression of hrpB2 in strain 85*ΔhrpB2 increased the number of detectable pili when compared with samples of the wild-type strain 85* (Fig. 5b). No significant difference in the number of pili were observed for strain 85*ΔhrpB2 carrying HrpB2, HrpB2_{V123A} and HrpB2_{T125A}, whereas fewer pili were detected in the presence of HrpB2_{L126A} and HrpB2_{K128A} (Fig. 5b). HrpB2_{A123–130} and HrpB2_{A123–128} abolished detectable pilus formation. Our findings therefore suggest that the C-terminal region of HrpB2 that harbours the VxTLxK motif is required for the formation of stable pilus structures. It remains to be investigated whether this motif is directly involved in pilus biogenesis or exerts an indirect influence, possibly by affecting the folding and thus the binding of HrpB2 to its interaction partners. A contribution of this motif in HrpB2 homologues and predicted inner rod proteins from animal-pathogenic bacteria to the formation of pili or needles has not yet been studied.

**HrpB2 is not a major component of the T3S pilus**

Given the previous finding that HrpB2 is secreted by the T3S system and is essential for pilus assembly (Rossier et al., 2000), we wondered whether it is an extracellular pilus component. We therefore performed EM and immunogold labelling studies using polyclonal antisera raised against HrpB2 and the major pilus subunit HrpE. Binding of primary antibodies was detected by secondary antibodies that were coupled to gold particles. As observed by Weber et al. (2005), T3S pili of strain 85* were labelled by the HrpE-specific antibody (Fig. 6). When the samples were
incubated with the HrpB2-specific antiserum, only a few gold particles were detected in the surrounding milieu, but not associated with the pili (Fig. 6). The labelling was HrpB2-specific because no gold particles were found in preparations of strain 85*ΔhrpB2, which lacks HrpB2 (data not shown). We also performed immunogold labelling studies with strain 85*ΔhpaC, which oversecretes HrpB2 (Lorenz et al., 2008). Similarly to strain 85*, strain 85*ΔhpaC produced T3S pili that were labelled by the HrpE-specific antiserum (Fig. 6). After incubation of the samples with the HrpB2-specific antiserum, we detected significantly increased amounts of gold particles in the surrounding milieu of the bacteria but not attached to the pilus structures (Fig. 6). The increased labelling efficiency

Fig. 5. The C-terminal VxTLxK motif of HrpB2 is required for pilus formation. (a) EM studies of T3S pili in hrpB2 wild-type and mutant strains. Strains 85* (wt) and 85*ΔhrpB2 (ΔhrpB2) carrying plasmid pBRM (−), HrpB2 or mutant derivatives thereof as indicated were grown on EM grids under secretion-permissive conditions. Transmission electron micrographs of negatively stained bacteria are shown. Visible surface appendages are T3S pili. Bars, 1 μm. (b) L126A and K128A mutations in HrpB2 lead to reduced pilus formation. Bacteria were incubated on grids as described in (a). T3S pili were visualized by EM and the number of pili that were attached to bacterial cells was counted for approximately 300 cells per strain in three different samples. The diagram displays the percentage of bacteria with zero, one, two and three pili. Asterisks indicate significant differences between the numbers of pili observed for strain 85*ΔhrpB2 carrying the HrpB2 wild-type protein or mutant derivatives thereof (P<0.05, based on the results of an unpaired Student’s t test). The experiment was repeated twice with similar results. Numbers in columns are means ±sd.
with the HrpB2-specific antibody presumably reflects the oversecretion of HrpB2 by strain 85ΔhpaC. We conclude from these observations that HrpB2 is not a major component of the extracellular T3S pilus.

**HrpB2 localizes to the bacterial periplasm and the OM**

To investigate the localization of HrpB2 in bacterial cells, we separated fractions enriched in the cytosol, periplasm, IM and OM by ultracentrifugation as described by Thein et al. (2010) (see Methods). Strain 85* was incubated under secretion-permissive conditions, and proteins of different fractions were analysed by SDS-PAGE and immunoblotting. HrpB2 was mainly present in fractions corresponding to the periplasm and the OM. By contrast, HrpB2 was not detectable in the cytosol- and IM-enriched fractions (Fig. 7a). Similar results were obtained for the C-terminal HrpB2 deletion derivative HrpB2Δ123–128 (Fig. 7b). This suggests that the C-terminal VxTLxK motif is not required for the localization of HrpB2. As a control, the blots were reprobed with an antibody specific for the OM secretin HrcC, which was predominantly detectable in the OM fraction. However, small amounts of HrcC were also present in cytosol-, periplasm- and IM-enriched fractions, which confirms the results of previous localization studies (Wengelnik et al., 1996a) (Fig. 7a). As HrcC is presumably transported across the IM by the Sec system, the HrcC-specific signals in the cytosol-, periplasm- and IM-enriched fractions might correspond to transport intermediates. Taken together, these findings suggest that HrpB2 localizes to the periplasm and the OM under secretion-permissive conditions but is also weakly secreted in the culture supernatant, as shown previously. We speculate that HrpB2 promotes pilus formation from the periplasm and that the weak secretion of HrpB2 into the culture supernatant might correspond to ‘leakage’ of HrpB2 across the OM once pilus assembly is initiated. As HrpB2 was not detected in association with the pilus structure (see above), secreted HrpB2 is unlikely to be an extracellular pilus component.

We have previously shown that secretion of HrpB2 is controlled by HpaC and depends on an active T3S system (Lorenz et al., 2008). We therefore wondered whether the periplasmic localization of HrpB2 is also type III-dependent and performed additional subcellular fractionation experiments with strain 85-10ΔhrpB2 carrying hrpB2 on a plasmid under control of the lac promoter. Bacteria were cultivated in complex NYG medium, which does not induce T3S gene expression. Unexpectedly, the localization of HrpB2 in the periplasm- and OM-enriched fractions was unaltered when compared with strain 85* (Fig. 7a), suggesting that the transport of HrpB2 into the periplasm does not depend on the presence of a functional T3S system.

**DISCUSSION**

This study aimed at the functional characterization of HrpB2 from *X. campestris pv. vesicatoria*, which has been shown to be crucial for T3S and is itself weakly secreted by the T3S system (Lorenz et al., 2008; Rossier et al., 2000). To identify functional protein regions in HrpB2, we analysed 64 HrpB2 mutant derivatives with pentapeptide insertions...
that were generated by a transposon mutagenesis approach. Complementation studies revealed that the C-terminal region of HrpB2 is presumably essential, while most insertions in the N-terminal region (amino acids 1–55) and the region spanning amino acids 80–91 did not interfere with protein function. One exception was the insertion at position F35 that abolished HrpB2 function. F35 is part of the conserved FQALM motif previously identified in HrpB2 from X. axonopodis pv. citri. Mutation of this motif in HrpB2\textsubscript{Xac} however, does not affect bacterial pathogenicity (Cappelletti et al., 2011). Given that HrpB2 mutant derivatives from X. campestris pv. vesicatoria contain five additional amino acids that might interfere with protein folding, it is conceivable that a loss of protein function resulted from structural alterations that are not tolerated. This hypothesis is supported by the finding that the substitutions of amino acid residues L102 and V111 by alanine did not significantly affect T3S and pathogenicity, whereas pentapeptide insertions at both positions led to a loss of protein function (Figs 1 and 4).

Notably, computational analysis of the predicted secondary structure of HrpB2 by PHYRE (http://www.sbg.bio.ic.ac.uk/~phyre/) revealed that the regions spanning amino acids 55–82 and 88–130, respectively, contain α-helices and β-sheets. By contrast, predicted secondary structural motifs are mainly absent from the N-terminal region and the region between amino acids 83 and 87 of HrpB2 that tolerate pentapeptide insertions.

Comparative sequence analysis of HrpB2 and homologues revealed the presence of a conserved C-terminal VxTLxK amino acid motif (Fig. 3). Interestingly, this motif is also present in predicted inner rod proteins from animal-pathogenic bacteria that are generally not highly conserved and do not share significant sequence similarity with HrpB2. Furthermore, an overlapping motif has been identified in HrpB2 from X. axonopodis pv. citri as part of an essential protein region. Thus, deletion of the C-terminal seven amino acids from HrpB2\textsubscript{Xac} encompassing this motif leads to a loss of pathogenicity (Cappelletti et al., 2011). We observed a similar finding for a C-terminal HrpB2 deletion derivative from X. campestris pv. vesicatoria (Fig. 3). Analysis of HrpB2 derivatives with single amino acid exchanges in the VxTLxK motif revealed that V123A and T125A mutations did not significantly alter HrpB2 function, whereas L126A and K128A exchanges abolished efficient T3S and pathogenicity. Our observations are in contrast to the finding that a T125A mutation in HrpB2 from X. axonopodis pv. citri leads to a loss of protein function, while L126A and K128A exchanges have no or only a slight effect (Cappelletti et al., 2011). Thus, despite conservation of the C-terminal amino acid sequence, the contribution of single amino acid residues of the VxTLxK motif to protein function appears to differ in HrpB2 homologues from Xanthomonas spp. The precise role of the VxTLxK motif in HrpB2 remains to be investigated. In the present study, our protein–protein interaction and fractionation studies did not uncover a contribution of this motif to the interaction of HrpB2 with HpaC or to the localization of the protein. By contrast, the interaction of HrpB2 with HpaC was slightly reduced in the absence of the C-terminal eight amino acids, suggesting that the VxTLxK motif could be involved in the interaction of HrpB2 with the T3S4 protein HpaC (Fig. 3). However, it is not clear why the reduced interaction of HrpB2\textsubscript{Δ123–130} with HpaC would lead to a complete loss of protein function. In future studies, we will therefore also investigate whether the VxTLxK motif is required for the binding of HrpB2 to other as yet unknown interaction partners. Furthermore, it remains to be clarified whether the mutation of the VxTLxK motif interferes with the folding of HrpB2 and thus affects the incorporation of HrpB2 into or its association with the T3S system.

The presence of a conserved amino acid motif in HrpB2 and predicted inner rod proteins from animal-pathogenic bacteria suggests that these proteins could share a common function. Inner rod proteins from animal-pathogenic bacteria have been proposed to promote the assembly and stable anchoring of the needle to the secretion apparatus and can themselves be secreted by the T3S
system (Marlovits et al., 2004, 2006; Ogino et al., 2006; Wood et al., 2008). This is reminiscent of the finding that HrpB2 is required for pilus assembly and is weakly secreted by the T3S system. The essential contribution of HrpB2 to pilus formation also explains why HrpB2 promotes its own type III-dependent export (Fig. 2). Given that HrpB2 is secreted and required for pilus formation, it has been proposed to be an extracellular component of the T3S system. However, the results of our EM and fractionation studies revealed that HrpB2 localizes to the periplasm and the OM and is not present in the close vicinity of T3S pili (Figs 6 and 7). Thus, HrpB2 is unlikely to be a component of the extracellular pilus but rather promotes pilus assembly from the base of the pilus. It is therefore tempting to speculate that HrpB2 forms a periplasmic substructure of the T3S system similarly to the inner rod from animal-pathogenic bacteria. It is not yet known whether T3S systems from plant-pathogenic bacteria contain an inner rod that could provide an assembly platform for the pilus. If they do, the pilus probably protrudes into the periplasm, as was proposed for T3S needles from Salmonella spp. that sit atop the inner rod structure (Marlovits et al., 2004, 2006; Sani et al., 2007; Wood et al., 2008).

Notably, the localization of HrpB2 to the periplasm and the OM was even observed in the absence of the T3S system (Fig. 7). This was an unexpected finding and suggests the contribution of an alternative transport system to the export of HrpB2 across the IM. As the N-terminal region of HrpB2 contains a predicted Sec signal (predicted by the software tool PrediSi: http://www.prediSi.de/), it remains to be investigated whether the transport of HrpB2 into the periplasm can also be mediated by the Sec system. However, the secretion of HrpB2 across the OM into the extracellular milieu is clearly type III-dependent and was previously shown to require IM components of the T3S system such as HrcU and HrcV as well as the pilus protein HrpE (Lorenz et al., 2008; Lorenz & Büttner, 2011; Rossier et al., 2000). The type III-dependency of HrpB2 secretion into the extracellular milieu is furthermore supported by the finding that the efficient secretion of HrpB2 is suppressed by the cytoplasmic T3S4 protein HpaC (Lorenz et al., 2008). This is reminiscent of the finding that secretion of the predicted inner rod protein YscI from Yersinia spp. is significantly reduced in the presence of the T3S4 protein YscP (Wood et al., 2008). In this context, it is interesting to note that T3S4 proteins might be involved in the control of inner rod formation, as was shown for the T3S4 protein InvJ from Salmonella spp. that is required for the assembly of PrgJ into inner rod structures (Marlovits et al., 2006).

In X. campestris pv. vesicatoria, we are just beginning to understand how secretion of early and late T3S substrates is controlled and which mechanisms are employed by HpaC to suppress the efficient secretion of HrpB2. Experimental evidence suggests that HpaC and HrpB2 both compete for the same binding site in HrcUC and that the interaction of HrpB2 with HrcUC might therefore be required to control its export across the OM (Lorenz & Büttner, 2011). It remains to be investigated whether the targeting of HrpB2 to HrcUC, the periplasm and across the OM depends on the N-terminal protein region, which not only harbours a predicted Sec signal but also contains the signal for the type III-dependent secretion of HrpB2 (Lorenz et al., 2008; Schülz and D. Büttner, unpublished data). Furthermore, in future studies we will analyse whether HrpB2 forms protein complexes and also interacts with additional periplasmic and OM-associated components of the T3S system. Candidates are components of the IM and OM rings of the T3S system, including members of the YscC, YscD and YscJ protein families. Analysis and functional characterization of HrpB2-containing protein complexes might help to clarify whether HrpB2 is part of a predicted periplasmic substructure of the T3S system.

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