The YscU/FlhB homologue HrcU from *Xanthomonas* controls type III secretion and translocation of early and late substrates

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The majority of Gram-negative plant- and animal-pathogenic bacteria employ a type III secretion (T3S) system to deliver effector proteins to eukaryotic cells. Members of the YscU protein family are essential components of the T3S system and consist of a transmembrane and a cytoplasmic region that is autocatalytically cleaved at a conserved NPTH motif. YscU homologues interact with T3S substrate specificity switch (T3S4) proteins that alter the substrate specificity of the T3S system after assembly of the secretion apparatus. We previously showed that the YscU homologue HrcU from the plant pathogen *Xanthomonas campestris* pv. *vesicatoria* interacts with the T3S4 protein HpaC and is required for the secretion of translocon and effector proteins. In the present study, analysis of HrcU deletion, insertion and point mutant derivatives led to the identification of amino acid residues in the cytoplasmic region of HrcU (HrcUC) that control T3S and translocation of the predicted inner rod protein HrpB2, the translocon protein HrpF and the effector protein AvrBs3. Mutations in the vicinity of the NPTH motif interfered with HrcU cleavage and/or the interaction of HrcUC with HrpB2 and the T3S4 protein HpaC. However, HrcU function was not completely abolished, suggesting that HrcU cleavage is not crucial for pathogenicity and T3S. Given that mutations in HrcU differentially affected T3S and translocation of HrpB2 and effector proteins, we propose that HrcU controls the secretion of different T3S substrate classes by independent mechanisms.

INTRODUCTION

Many Gram-negative plant- and animal-pathogenic bacteria utilize a type III secretion (T3S) system to translocate effector proteins into eukaryotic cells and thus manipulate host cellular pathways for their own benefit (Büttner & Bonas, 2010; Ghosh, 2004). T3S systems consist of a membrane-spanning basal body which is composed of ring structures in the inner membrane (IM) and outer membrane that are presumably linked by a periplasmic inner rod (Blocker et al., 2001; DePamphilis & Adler, 1971; Francis et al., 1994; Kubori et al., 1998; Marlovits et al., 2004; Sani et al., 2007). Additional components include at least five IM proteins that belong to the YscR, S, T, U and V families (He et al., 2004). YscU and YscV family members contain large cytoplasmic domains that are associated with the predicted cytoplasmic (C) ring and the cytoplasmic ATPase complex of the T3S system, and were proposed to be involved in substrate docking (Büttner, 2012). The membrane-spanning basal body of translocation-associated T3S systems is associated with an extracellular needle (in animal pathogens) or pilus structure (in plant pathogens), which provides a transport channel for secreted proteins to the host–pathogen interface (Büttner, 2012). Translocation of effector proteins is mediated by a channel-like bacterial translocon, which inserts into the host plasma membrane (Büttner et al., 2002; Mattei et al., 2011; Mueller et al., 2008).

Given the architecture of the T3S system, the assembly of extracellular needles or pili presumably precedes the translocation of effector proteins. It was therefore suggested that the T3S substrate specificity switches from early substrates including extracellular components of the T3S system to late substrates, i.e. effector proteins. The switch in substrate specificity is controlled by T3S substrate specificity switch (T3S4) proteins, which interact with the cytoplasmic regions of members of the YscU family of IM components of the T3S system (Agrain et al., 2005; Büttner, 2012; Cornelis et al., 2006; Deane et al., 2010). The cytoplasmic region of YscU family members is autoproteolytically cleaved between the asparagine and proline residues of a conserved NPTH

**Abbreviations:** ECW, Early Cal Wonder; GST, glutathione S-transferase; HR, hypersensitive response; IM, inner membrane; p.i., post-inoculation; SN, culture supernatants; TE, total cell extracts; T3S, type III secretion; Xcv, *Xanthomonas campestris* pv. *vesicatoria*.

One supplementary table and four supplementary figures are available with the online version of this paper.
In the present study, we aimed to characterize the HrcU homologue HrcUC by inducing a conformational change in HrcUC that alters the T3S substrate specificity. The model of an HpaC-induced conformational change in HrcUC was supported by the finding that the hpaC mutant phenotype can be suppressed by exchange of the conserved tyrosine residue at position 318 of the C-terminal LARxLY amino acid motif of HrcU against aspartic acid (Lorenz & Büttner, 2011). Notably, however, the Y318D mutation in HrcU does not suppress the oversecretion of HrpB2 in the hpaC mutant (Lorenz & Büttner, 2011). Extragenic suppressor mutations in the C-terminal regions of YscU family members were also identified in T3S4 mutants from animal-pathogenic bacteria and presumably affect the protein conformation in or around the NPTH motif (Deane et al., 2008; Edqvist et al., 2003; Kutsukake et al., 1994; Williams et al., 1996; Zarivach et al., 2008).

In the present study, we aimed to characterize the HrcU-dependent T3S substrate specificity switch in Xcv at the molecular level. The results of random and targeted mutagenesis approaches suggest that the LARxLY motif of HrcU and the linker region between the transmembrane domain and the NPTH motif are required for T3S and pathogenicity. Furthermore, infection and secretion studies revealed that HrcU cleavage per se is dispensable for protein function and that HrcU controls the secretion and translocation of early and late substrates by independent mechanisms that can be uncoupled.

### METHODS

**Bacterial strains and growth conditions.** Xcv strains were grown at 30 °C in nutrient-yeast-glycerol (NYG) medium (Daniels et al., 1984) or in minimal medium A (pH 7.0) (Ausbel et al., 1996), which was supplemented with sucrose (10 mM) and Casamino acids (0.3 %). *Escherichia coli* was grown at 37 °C in lysogenic broth (LB) medium (Bertani, 1951). Plasmids were introduced into Xcv by conjugation, using pRK2013 as a helper plasmid in triparental matings (Figurski & Helinski, 1979) and into *E. coli* by chemical transformation. Antibiotics were used at the following final concentrations: ampicillin, 100 µg ml⁻¹; gentamicin, 15 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; rifampicin, 100 µg ml⁻¹; spectinomycin, 100 µg ml⁻¹.

**Infection experiments.** Xcv strains were inoculated with a needleless syringe into the intercellular spaces of leaves of the near-isogenic pepper cultivars Early Cal Wonder (ECW) and ECW-10R at concentrations of 1 x 10⁸ c.f.u. ml⁻¹ in 1 mM MgCl₂ if not stated otherwise (Kousik & Ritchie, 1998; Minsavage et al., 1990). Disease symptoms and the hypersensitive response (HR) were monitored over a period of 1–14 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 HrcU derivatives with pentapeptide insertions.** To generate HrcU derivatives with pentapeptide insertions, hrcU₁₋₂₀₆ and hrcU₂₅₅₋₃₅₇ were amplified by PCR from Xcv strain 85-10 and inserted into the Smal site of vector pUC57Basa in a restriction-ligation reaction (Bolchi et al., 2005; Morbitzer et al., 2011). Transposon-mutagenized derivatives of pUC57hrCU₂₅₅₋₃₅₇ were generated with the MGS Mutation Generation System kit (Finnzymes) according to the manufacturer’s instructions and transposon insertions were identified by PCR after transformation of the reaction into *E. coli*. The Entranceposon was removed by digestion of the plasmid DNA by NotI. Re-ligation led to the generation of 15 bp insertions consisting of a 10 bp Entranceposon sequence and 5 bp duplicated target site. The amino acid sequences of the corresponding pentapeptides varied according to the reading frame in which the Entranceposon had inserted and are listed below in Fig. 3(b). Derivatives of hrcU₂₅₅₋₃₅₇ with 15 bp insertions were ligated with hrcU₁₋₂₀₆ into the Golden Gate-compatible vector pBRM downstream of a single lac promoter and in-frame with a triple c-Myc epitope-encoding sequence (Engler et al., 2008; Szczesny et al., 2010). Plasmids that were generated in this study are listed in Table S1 (available in the online Supplementary Material).

**Generation of expression plasmids and deletion mutants.** hrcU-c-myc point mutant and deletion derivatives were generated by PCR using pBRMhrcU as template and phosphorylated primers that introduced the individual mutations and annealed back to back to the hrcU sequence. Similarly, point and deletion mutant derivatives ofgst–hrcU andgst–hrcU₂₅₅₋₃₅₇ were generated by PCR using phosphorylated primers and constructs pGhrcU and pGhrcU₂₅₅₋₃₅₇ as templates (see Table S1; Lorenz & Büttner, 2011). Amplicons were ligated and transformed into *E. coli*. To generate pBRMhrcU₂₅₅₋₃₅₇, the first 76 codons of hrb2 were amplified by PCR and ligated into the EcoRI/Clal sites of pDS356F, in-frame withavrBs3A2, resulting in construct pDhrpB2₁₋₇₆–avrBs3A2. Primer sequences are available upon request.

**T3S assays and immunoblot analysis.** *In vitro* T3S assays were performed as described previously (Rossier et al., 1999). Equal amounts of bacterial total cell extracts and culture supernatants...
were analysed by SDS-PAGE and immunoblotting, using polyclonal antibodies specific for HrpF (Böttner et al., 2002), HrpB2 (Rossier et al., 2000), AvrBs3 (Knoop et al., 1991) and monoclonal anti-c-Myc (Roche Applied Science) and anti-GST antibodies (Amersham Pharmacia Biotech). Horseradish peroxidase-labelled anti-rabbit, anti-mouse and anti-goat antibodies (GE Healthcare) were used as secondary antibodies. Blots were routinely incubated with an antibody specific for HrcJ to ensure that no bacterial lysis had occurred (Rossier et al., 2000) (data not shown). Experiments were repeated at least twice.

**Glutathione S-transferase (GST) pull-down assays.** GST pull-down assays were performed as described previously (Hartmann et al., 2012). Fifteen microlitres of total protein lysates and 20 μl eluted proteins were analysed by SDS-PAGE and immunoblotting using antibodies specific for the c-Myc epitope, HrcQ (Lorenz et al., 2012) and GST, respectively. Experiments were repeated at least twice.

**RESULTS**

The N-terminal 76 aa of the early T3S substrate HrpB2 contain a translocation signal

We previously showed that HpaC and HrcU suppress the efficient secretion of the predicted inner rod protein HrpB2 (Lorenz et al., 2008). To investigate whether they also interfere with a possible translocation of HrpB2 into the plant cell, we generated a fusion protein between the N-terminal 76 aa of HrpB2 and the reporter protein AvrBs3Δ2, which is a derivative of the effector protein AvrBs3. AvrBs3Δ2 contains the effector domain but is deleted in the secretion and translocation signal and is therefore only translocated by the T3S system as the C-terminal fusion partner of a functional translocation signal (Noël et al., 2003; Szurek et al., 2002). Translocation of AvrBs3 or AvrBs3Δ2 fusions into cells of AvrBs3-responsive ECW-30R pepper plants leads to induction of an HR, i.e. rapid local cell death at the infection site (Dangl & Jones, 2001; Römer et al., 2009). HrpB2_{1–76}–AvrBs3Δ2 induced the HR in ECW-30R pepper plants when delivered by Xcv strain 85ΔHpaC but not by strain 85-10, suggesting that it was translocated in the absence of HpaC (data not shown). Similar results were obtained with strains 85–10 hrpG* (85*) and 85ΔhpaC (Fig. 1a). Derivatives of strain 85* contain HrpG*, which is a constitutively active version of the key regulator HrpG and activates the expression of T3S genes also under non-inducing conditions (Noël et al., 2001; Rossier et al., 1999; Wengelnik et al., 1996, 1999). As control, bacteria were inoculated into leaves of resistant ECW-10R pepper plants, which induce the HR upon recognition of the type III effector AvrBs1 (Escolar et al., 2001; Ronald & Staskawicz, 1988). As expected, strain 85* elicited the HR in ECW-10R plants whereas a partial HR induction was observed for strain 85ΔhpaC (Fig. 1a) (Böttner et al., 2006).

In vitro T3S assays showed that HrpB2_{1–76}–AvrBs3Δ2 was detected in the culture supernatant of strain 85ΔhpaC but not of strain 85* (Fig. 1b), suggesting that it was efficiently secreted in the absence of HpaC. This is in contrast to the translocation protein HrpF, which is secreted in reduced amounts by strain 85ΔhpaC, as was reported previously (Böttner et al., 2006). We conclude from these findings.

![Fig. 1. HpaC and HrcU suppress the translocation of HrpB2_{1–76}–AvrBs3Δ2.](image)
that the N-terminal 76 aa of HrpB2 contain a functional T3S and translocation signal that is suppressed by HpaC.

HrpB2\textsubscript{1–76}–AvrBs3\Delta2 is translocated in the presence of N264A and P265A mutations in HrcU

To investigate a possible contribution of HrcU to the control of HrpB2\textsubscript{1–76}–AvrBs3\Delta2 translocation, we performed translocation assays with strain 85*\textsubscript{hrcU} containing HrcU or HrcU derivatives with point mutations in the NPTH motif. We previously showed that mutations of the conserved asparagine or proline residues of the NPTH motif of HrcU against alanine (N264A and P265A mutations) abolish HrcU cleavage and the interaction with HpaC, whereas the exchange of P265 against glycine (P265G mutation) additionally interferes with the interaction of HrcU with HrpB2 (Lorenz & Büttner, 2011). When bacteria were inoculated into leaves of ECW-30R pepper plants, HrpB2\textsubscript{1–76}–AvrBs3\Delta2 induced the HR when delivered in the presence of HrcU derivatives with N264A and P265A mutations but not with P265G, T266A or H267A mutations (Fig. 1c). As reported previously, N264A and P265A mutations abolished the HR induction in ECW-10R plants (Lorenz & Büttner, 2011) (Fig. 1c). However, given that they promote translocation of HrpB2\textsubscript{1–76}–AvrBs3\Delta2, they did not completely abolish the formation of a functional translocon. We conclude from these findings that the cleavage of HrcU and/or the interaction with HpaC suppress the predicted translocation of HrpB2. Notably, the secretion of HrpB2\textsubscript{1–76}–AvrBs3\Delta2 in hrcU mutant strains was not significantly increased, suggesting that even small amounts of protein are sufficient to trigger the HR (Fig. 1d). This is in contrast to the wild-type HrpB2 protein, which is oversecreted upon ectopic expression of hrcU or derivatives thereof (Lorenz & Büttner, 2011). The different secretion behaviour of both proteins might be due to the lack of the C-terminal region in HrpB2\textsubscript{1–76}–AvrBs3\Delta2 or the presence of the AvrBs3\Delta2 fusion partner.

We also investigated the influence of a double point mutation (P265G and Y318D) in HrcU on the translocation of HrpB2\textsubscript{1–76}–AvrBs3\Delta2. Previous pull-down assays revealed that individual Y318D and P265G mutations interfere with the binding of HrcU to HrpB2 and HpaC (Lorenz & Büttner, 2011). We also observed for the double mutant derivative HrcU\textsubscript{P265G/Y318D} (Fig. 2a). The Y318D mutation in HrcU activates effector protein secretion in the absence of the T3S4 protein HpaC whereas the P265G mutation leads to a loss of HrcU function (Lorenz & Büttner, 2011) (Fig. 2b, c). Notably, HrcU\textsubscript{P265G/Y318D}–c-Myc partially restored plant reactions in ECW and ECW-10R plants as well as HrpB2 secretion in strain 85*\textsubscript{AhrcU} (Fig. 2b, c). Furthermore, HrpB2\textsubscript{1–76}–AvrBs3\Delta2 induced the AvrBs3-specific HR when delivered by strain 85*\textsubscript{AhrcU} in the presence of HrcU\textsubscript{P265G/Y318D}–c-Myc, but not of HrcU\textsubscript{P265G–c-Myc or HrcU\textsubscript{Y318D}–c-Myc (Fig. 2d). This suggests that the Y318D mutation, which presumably leads to a conformational change in HrcU (see above; Lorenz & Büttner, 2011), allows the translocation of HrpB2 in the absence of HrcU cleavage and a detectable HrcU–HrpB2 interaction.

Generation of HrcU transposon mutant derivatives

To gain further insights into functionally important regions of HrcU\textsubscript{C}, we performed a random transposon mutagenesis of hrcU\textsubscript{207–357} (hrcU\textsubscript{C}), which encodes the C-terminal cytoplasmic region of HrcU (Fig. 3a; see Methods) (Berger et al., 2010). Transposon sequences in hrcU\textsubscript{C} were removed by NolI digestion and re-ligation, resulting in the in-frame insertions of a 15 bp random sequence including a 5 bp target duplication. In total, 33 mutagenized hrcU\textsubscript{C} derivatives were generated and ligated in-frame to hrcU\textsubscript{1–206} in the expression vector pBRM in fusion with a C-terminal c-Myc epitope-encoding sequence. The resulting HrcU derivatives are hereafter referred to by the position of the pentapeptide insertion (e.g. HrcU\textsubscript{A243} contains an insertion after the alanine residue at position 243). The positions and amino acid sequences of the insertions are summarized in Fig. 3(b). Six insertions were located in the N-terminal linker region of HrcU\textsubscript{C} (HrcU\textsubscript{CN}, amino acids 207–264), whereas 27 insertions were identified in the C-terminal region (HrcU\textsubscript{CO}, amino acids 265–357), which is separated from HrcU\textsubscript{CN} by the NPTH cleavage motif (amino acids 264–267) (Fig. 3a) (Berger et al., 2010; Lorenz & Büttner, 2011). Expression constructs encoding HrcU mutant derivatives were introduced into Xcv strains 85-10\textsubscript{AhrcU} and 85*\textsubscript{AhrcU}. Immunoblot analysis revealed that 17 insertions in the central region of HrcU\textsubscript{C} interfered with HrcU cleavage, suggesting that they affected the protein conformation around the NPTH motif (Fig. 3b; data not shown).

Identification of amino acid regions in HrcU that contribute to the secretion of early and/or late substrates

When strains 85-10\textsubscript{AhrcU} and 85*\textsubscript{AhrcU} containing HrcU or mutant derivatives thereof were incubated in secretion medium, the putative translocon protein HrpF was secreted by strain 85* but was not detectable in the culture supernatant of strain 85*\textsubscript{AhrcU} (Lorenz & Büttner, 2011) (Fig. 4a). Wild-type levels of HrpF secretion were restored by HrcU–c-Myc whereas HrcU insertion mutant derivatives led to a reduced or undetectable HrpF secretion (Fig. 3b; see also Fig. 4a). We also analysed the influence of selected insertions after residues Y268, A269, V270, A298, D324, P327, E328, R339 and W340 on T3S of the early substrate HrpB2 and the effector protein AvrBs3. As observed previously, HrpB2 was oversecreted upon ectopic expression of hrcU–c-myc in strain 85*\textsubscript{AhrcU} (Fig. 4a) (Lorenz & Büttner, 2011). Reduced levels of HrpB2 were secreted in the presence of HrcU derivatives with insertions after positions 268, 269 or 270. By contrast, secretion of HrpB2 was not detectable in the presence of HrcU derivatives with insertions after positions 298, 327 and 328 whereas...
HrcU

Δ

– HrcUP265GP265G/ Y318D–

Fig. 4a) but allow the translocation of HrpB2–1–76–AvrBs3
V270 of HrcU interfere with HrpB2 secretion (see above; because we observed that insertions at positions Y268 and
nisms to control the secretion and translocation of HrpB2,
isms. We also assume that HrcU employs different mecha-
different T3S substrates by independent molecular mechan-
HrcU probably controls the secretion and translocation of
AvrBs3 but did not lead to a reduction in HrpB2 secretion, and 340 of HrcU interfered with the secretion of HrpF and
Fig. 4a, see also above). Given that insertions at positions 339 and 340 of HrcU interfered with the secretion of HrpF and
AvrBs3 but did not lead to a reduction in HrpB2 secretion, HrcU probably controls the secretion and translocation of
different T3S substrates by independent molecular mechan-
isms. We also assume that HrcU employs different mecha-
nisms to control the secretion and translocation of HrpB2,
because we observed that insertions at positions Y268 and
V270 of HrcU interfere with HrpB2 secretion (see above; Fig. 4a) but allow the translocation of HrpB2–1–76–AvrBs3Δ2 (Fig. S1). Notably, HrpB2–1–76–AvrBs3Δ2 did not induce the

AvrBs3-specific HR when delivered in the presence of the other HrcU insertion derivatives (Fig. S1; data not shown).

Uncleaved HrcU derivatives partially restore pathogenicity in hrcU deletion mutant strains

Next, bacteria were inoculated into leaves of susceptible and resistant pepper plants. As expected, strains 85-10 and
85* induced disease symptoms in the form of water-soaked lesions that later became necrotic in ECW and the HR in
ECW-10R pepper plants. No plant reactions were observed for the hrcU deletion mutant strains 85-10ΔhrcU and
85*ΔhrcU (Fig. 4b) (Lorenz & Büttner, 2011). The wild-type phenotype was restored by HrcU–c-Myc but not by
derivatives thereof, with insertions at positions A243, K244,
The linker region of HrcU is essential for protein function and the interaction with HrpB2

We also generated HrcU derivatives with deletions of amino acids 217–228, 243–257 and 247–261, respectively, in the linker region (see Fig. 3a). Immunoblot analysis of bacterial cell extracts revealed that HrcUA217–228–c-Myc and HrcUA243–257–c-Myc were proteolytically cleaved whereas no cleavage product was detected for HrcUA247–261–c-Myc (Fig. 5a). None of the HrcU deletion derivatives complemented the mutant phenotype of strain 85*AhrCU with respect to T3S and pathogenicity, suggesting that they were not functional (Fig. 5a).

To investigate whether the deletions in HrcU affect protein–protein interactions, we performed GST pull-down assays with selected HrcU interaction partners including HrpB2, the general T3S chaperone HpaB and the predicted C ring protein HrcQ (Lorenz & Büttner, 2009; Lorenz et al., 2008, 2012). A contribution of the linker region of HrcU to the interaction with HpaC could not be tested because HpaC interacts only with the cytoplasmic region of HrcU including amino acids 255–357 (Lorenz et al., 2008). Immobilized GST and GST fusions of HrcU and mutant derivatives thereof were incubated with E. coli lysates containing C-terminally c-Myc epitope-tagged derivatives of HrcU interaction partners. HpaB–c-Myc and HrcQ–c-Myc co-eluted with GST–HrcU and deletion derivatives thereof but not with GST (Fig. 5b). Significantly reduced amounts of HrpB2–c-Myc were detected in the eluate of GST–HrcUA247–261 when compared with GST–HrcU, GST–HrcUA217–228 and GST–HrcUA243–257 (Fig. 5b). Amino acids 247–261 of HrcU might therefore contribute to the interaction with HrpB2.

The LARxLY motif of HrcU is required for the interaction with HrpB2 and HpaC

In addition to the linker region, we analysed the contribution of the C-terminal LARxLY motif to HrcU function. An HrcU derivative lacking the LARxLY motif (HrcUA312–318) was not proteolytically cleaved and did not complement the hrcU mutant phenotype with respect to T3S and pathogenicity (Figs 6a and S2). Interaction studies revealed that HpaB–c-Myc and HrcQ–c-Myc, but not HrpB2–c-Myc, interacted with GST–HrcUA312–318–c-Myc (Fig. 6b). Furthermore, HpaC–c-Myc did not co-elute with a GST–HrcUA243–257/A312–318 fusion protein (Fig. 6c), suggesting that the LARxLY motif contributes to the interaction of HrcU with both HrpB2 and HpaC.

Amino acid residues A271 and P282 of HrcU contribute to the interaction with HpaC and HrpB2

As it cannot be disproved that deletions and insertions in HrcU lead to conformational changes, we introduced point mutations at amino acid positions 271 (A271N), 282 (P282A) and 308 (V308A) of HrcU. Pentapeptide insertions at these positions led to a loss of HrcU function (see above; Fig. 3b). We also mutated the proline residue at position 312 (P312A) that directly flanks the LARxLY motif and is conserved in YscU family members (Fig. S3). With the exception of HrcUA271N–c-Myc, all HrcU point mutant derivatives were efficiently cleaved (Fig. 6a). Furthermore, all HrcU point mutant derivatives partially complemented the in planta phenotype of strain 85–10AhrCU and restored the wild-type phenotype in strain 85*AhrCU (Fig. 6a and Fig. S2). The finding that HrcUA271N–c-Myc was not efficiently cleaved confirms our hypothesis that cleavage is not essential for HrcU function (see above).

When bacteria were incubated in secretion medium, HrcUA271N–c-Myc, HrcUV308A–c-Myc and HrcUP312A–c-Myc, but not HrcUP282A–c-Myc, partially restored secretion of the putative translocon protein HrpF, the effector protein AvrBs3 and the early substrate HrpB2 in strain 85*AhrCU (Fig. 6a). In vitro interaction studies with GST–HrcU point mutant derivatives revealed that the interaction of HrpB2–c-Myc with GST–HrcUA271N and GST–HrcUP282A was reduced (Fig. 6b). Similar findings were obtained for HpaC–c-Myc, suggesting that the A271N and P282A mutations affect the interaction of HrcU with both HrpB2 and HpaC. The lack of interaction of these derivatives with HpaC and HrpB2 might explain the reduced T3S of early and late substrates (see above).

DISCUSSION

In the present study, we provide experimental evidence that HrcU from Xcv promotes T3S of early and late substrates by different mechanisms and that HrcU cleavage is not essential for protein function. Furthermore, our data suggest that HrcU controls a possible translocation of HrpB2. The analysis of 33 pentapeptide insertion derivatives of HrcU revealed that insertions adjacent to the NPTH motif of HrcU or in the region spanning amino acids 287–327 led to a loss of detectable HrcU cleavage (Fig. 3). Comparison of the predicted structure of HrcUC to solved crystal structures of YscU family members suggests that amino acids 287–327 form two helices that are separated by a β sheet and are located in the vicinity of the NPTH motif (Fig. 3). Insertions in this region might therefore alter the protein conformation in or around the NPTH motif and thus affect the autocatalytic cleavage (Deane et al., 2008; Ferris et al., 2005; Lountos et al., 2009; Wiesand et al., 2009; Zarivach et al., 2008). Cleavage of YscU family members was assumed to be essential for the T3S substrate specificity switch (Björnfot et al., 2009; Fraser et al., 2003; Lavander et al., 2002; Lorenz, 2009; Smith et al.,
Fig. 3. Overview on pentapeptide insertion mutant derivatives of HrcU. (a) Topology model of HrcU. HrcU consists of four transmembrane helices (HrcU_N) (Berger et al., 2010) and a C-terminal cytoplasmic region (HrcU_C) that is presumably cleaved between the asparagine and proline residues of the conserved NPTH motif. Numbers indicate amino acid positions. The right-hand panel shows the predicted tertiary structure of HrcU_C that was generated by the I-TASSER software (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The NPTH loop is shown in red, the LARxLY motif in blue. Arrows and purple regions indicate the positions of amino acids A271 (located on a β sheet) and P282 (located in a disordered region). IM, inner membrane. (b) Overview of the positions of pentapeptide insertions in HrcU_C and their influence on pathogenicity, HrcU cleavage and HrpF secretion. The boxes and letters correspond to single amino acid residues in HrcU_C, and numbers indicate the amino acid positions. The positions and amino acid sequences of pentapeptide insertions are listed below the boxes. In the case of HrcU_Y274 and HrcU_L294, derivatives with two different pentapeptide insertions were identified. HrcU derivatives were
Fig. 3. (cont.) analysed in strains 85-10ΔhrcU (hrpG wt) and 85*ΔhrcU (hrpG*), and bacteria were inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants for complementation studies. Green rectangles indicate pentapeptide insertions that restored the wild-type phenotype with respect to disease symptoms and the HR, respectively. Yellow rectangles indicate a partial complementation by HrcU derivatives whereas pentapeptide insertions that did not complement the hrcU mutant phenotype are indicated by red rectangles. Infection studies were repeated at least three times. Rectangles that are subdivided into triangles indicate phenotypes that varied between full, partial or no complementation of the hrcU mutant phenotype as indicated by the colour of the triangles. For in vitro T3S assays, strains 85*, 85*ΔhrcU and 85*ΔhrcU carrying HrcU or derivatives thereof were incubated in secretion medium and total cell extracts and culture supernatants were analysed by immunoblotting, using an HrpF-specific antibody. Yellow and red rectangles indicate reduced and undetectable levels of HrpF secretion, respectively. Rectangles that are subdivided into triangles indicate secretion patterns that varied between partial and undetectable HrpF secretion. Cleavage of HrcU derivatives was analysed by immunoblot analysis of total cell extracts using a c-Myc epitope-specific antibody. Pentapeptide insertions that did not affect HrcU cleavage are indicated by green rectangles, and insertions that abolished the detection of the C-terminal HrcU cleavage product by red rectangles. WL, water-soaked lesions.

Fig. 4. Analysis of selected HrcU pentapeptide insertion derivatives. (a) Influence of selected pentapeptide insertions on the in vitro secretion of HrpB2, HrpF and AvrBs3. Strains 85* (wt) and 85*ΔhrcU (ΔhrcU) carrying plasmid pBRM (–), HrcU–c-Myc (HrcU) or pentapeptide insertion derivatives thereof as indicated were incubated in secretion medium, and total cell extracts (TE) and culture supernatants (SN) were analysed by immunoblotting using HrpB2-, HrpF- and AvrBs3-specific antibodies, respectively. avrBs3 was ectopically expressed from plasmid pDSF300. (b) Infection studies with Xcv strains carrying selected HrcU pentapeptide insertion derivatives. Strains 85-10 (wt, hrpG wt), 85* (wt, hrpG*), 85-10ΔhrcU (ΔhrcU, hrpG wt) and 85*ΔhrcU (ΔhrcU, hrpG*) carrying plasmid pBRM (–), HrcU–c-Myc (HrcU) or pentapeptide insertions thereof were inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants. Disease symptoms were photographed after 8 days. For better visualization of the HR, leaves were bleached in ethanol 3 days p.i. Dashed lines indicate the infiltrated areas.
Most cleavage-deficient HrcU derivatives, however, partially restored plant reactions and T3S when analysed in hrcU deletion mutants, suggesting that the cleavage event itself is not crucial for HrcU function (Figs 3, 4 and 6). Intriguingly, one of the cleaved HrcU insertion derivatives, HrcU R339, led to a severe reduction in the secretion of late substrates but did not significantly interfere with the secretion of HrpB2 (Fig. 4). We therefore also assume that cleavage alone is not sufficient for HrcU function and that HrcU controls the secretion of early and late substrates by independent mechanisms.

Complementation studies revealed that HrcU function probably depends on the linker region between the transmembrane domain and the NPTH motif, because insertions and deletions in this region abolished protein function (Figs 3 and 5). This is in agreement with the finding that deletions and point mutations in the linker regions of the HrcU homologues FlhB from Salmonella species and EscU from enteropathogenic E. coli interfere with T3S (Fraser et al., 2003; Zarivach et al., 2008). In HrcU, deletion of amino acids 247–261 led to a loss of cleavage and detectable interaction with HrpB2. As amino acids 247–261 are presumably involved in the formation of a β sheet flanking the NPTH loop (Figs 3 and S4), deletion of this region might affect the protein conformation around the NPTH motif. HrcU function presumably also depends on a conserved C-terminal LARxLY motif, which is located on a predicted helix next to the NPTH motif (Fig. 3). Deletion of the LARxLY motif interferes with HrcU cleavage and the efficient interaction of HrcUC with both HrpB2 and HpaC (Fig. 6).

Notably, the interaction between HrcU and HpaC was also affected in the presence of the point mutations A271N
and P282A, respectively. Given that HrcU derivatives with A271N and P282A mutations partially complemented the hrcU mutant phenotype, the interaction between HrcU C and HpaC presumably contributes to but is not essential for HrcU function. A271N and P282A mutations also interfered with the interaction between HrcU and HrpB2, as well as with HrpB2 secretion. This observation is in line with the predicted role of HrcU as a substrate docking site for HrpB2 during the early phase of the T3S process. After the substrate specificity switch, however, the HrcU–HrpB2 interaction is presumably no longer required for HrpB2 secretion, suggesting the presence of alternative docking sites for HrpB2. Thus, a Y318D mutation in HrcU, which presumably mimics the substrate specificity switch, still allows efficient HrpB2 secretion although the interaction with HrpB2 is reduced (Lorenz & Büttner, 2011). In agreement with this model, we show that low levels of HrpB2 are secreted in the presence of an HrcU P265G/Y318D derivative, which does not interact with HrpB2 and HpaC in vitro.

Translocation of HrpB2 1–76–AvrBs3D2 was also observed in hpaC deletion and hrcU mutant strains with mutations in or next to the NPTH motif that led to a loss of HrcU cleavage (Figs 1 and 2). We therefore conclude that the

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**Fig. 6.** The LARxLY motif and amino acids A271 and P282 of HrcU are required for the interaction with HrpB2 and HpaC. (a) The LARxLY motif of HrcU is essential for protein function. Xcv strains 85* (wt) and 85ΔhrcU (ΔhrcU) carrying plasmid pBRM (–), HrcU–c-Myc (HrcU) or derivatives thereof with A271N, P282A, V308A and P312A exchanges or deletions of amino acids 312–318 as indicated were grown in minimal medium (pH 7.0), and total cell extracts (TE) were analysed by immunoblotting using a c-Myc epitope-specific antibody. For T3S assays, bacteria were incubated in secretion medium. TE and culture supernatants (SN) were analysed by immunoblotting, using HrpB2-, HrpF- and AvrBs3-specific antibodies, respectively. *avrBs3* was ectopically expressed from plasmid pDSF300. For infection studies, bacteria were inoculated into leaves of susceptible ECW and resistant ECW-10R pepper plants. Disease symptoms were photographed after 8 days. For better visualization of the HR, leaves were bleached in ethanol 3 days p.i. Dashed lines indicate the infiltrated areas. (b) A271N and P282A mutations and deletion of the LARxLY motif, respectively, abolish the efficient interaction of HrcU with HrpB2. GST and GST fusions of HrcU, HrcUΔ271N, HrcUΔP282A, HrcUΔV308A, HrcUΔP312A and HrcUΔ312–318, respectively, were immobilized on glutathione Sepharose and incubated with *E. coli* lysates containing HrpB2-c-Myc, HrcQ-c-Myc or HpaB-c-Myc. TE and eluted proteins (Eluates) were analysed by immunoblotting, using c-Myc- and GST-specific antibodies. Bands corresponding to GST or GST fusion proteins are marked by asterisks; lower bands presumably represent degradation products. (c) Amino acids A271, P282 and the LARxLY motif contribute to the interaction of HrcU with HpaC. GST and GST fusions of HrcU255–357, HrcU255–357/A271N, HrcU255–357/P282A, HrcU255–357/V308A, HrcU255–357/P312A and HrcU255–357/Δ312–318 were immobilized on glutathione Sepharose and incubated with *E. coli* lysates containing HpaC-c-Myc. TE, and eluates were analysed as described in (b).
HpaC-mediated substrate specificity switch and/or HrcU cleavage suppress the secretion and translocation of HrpB2. Notably, however, translocation of the native HrpB2 protein still needs to be confirmed.

We previously identified translocation signals in the N-terminal regions of the putative translocon proteins HrpF and XopA, which are presumably translocated in the absence of the general T3S chaperone HpaB (Bütter et al., 2004). HrpB2ΔN–AvrBs3ΔA2, however, was not translocated by a hpaB deletion mutant (data not shown). Vice versa, the N-terminal region of HrpB2 did not target the AvrBs3ΔA2 reporter for translocation in hpaC or selected hrcU mutant strains (data not shown). We therefore assume that HpaC and HrcU specifically inhibit the translocation of HrpB2 before the substrate specificity switch. Given the predicted function of HrpB2 as a periplasmic inner rod protein still needs to be confirmed. Notably, however, translocation of the native HrpB2 protein still needs to be confirmed.

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