HrpB7 from *Xanthomonas campestris* pv. *vesicatoria* is an essential component of the type III secretion system and shares features of HrpO/FliJ/YscO family members

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**Abstract**

The Gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* translocates effector proteins via a type III secretion system (T3SS) into eukaryotic cells. The T3SS spans both bacterial membranes and consists of more than 20 proteins, 9 of which are conserved in plant and animal pathogens and constitute the core subunits of the secretion apparatus. T3S in *X. campestris* pv. *vesicatoria* also depends on nonconserved proteins with yet unknown function including HrpB7, which contains predicted N- and C-terminal coiled-coil regions. In the present study, we provide experimental evidence that HrpB7 forms stable oligomeric complexes. Interaction and localisation studies suggest that HrpB7 interacts with inner membrane and predicted cytoplasmic (C) ring components of the T3SS but is dispensable for the assembly of the C ring. Additional interaction partners of HrpB7 include the cytoplasmic adenosinetriphatase HrcN and the T3S chaperone HpaB. The interaction of HrpB7 with T3SS components as well as complex formation by HrpB7 depends on the presence of leucine heptad motifs, which are part of the predicted N- and C-terminal coiled-coil regions. Our data suggest that HrpB7 forms multimeric complexes that associate with the T3SS and might serve as a docking site for the general T3S chaperone HpaB.

**KEYWORDS**

ATPase, C ring, chaperone, coiled coil, effector protein, leucine heptad motifs, plant pathogenic bacterium, type III secretion

1 **INTRODUCTION**

The Gram-negative plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* (Xcv; also designated *X. euvesicatoria* [Jones, Lacy, Bouzar, Stoll, & Schaad, 2004]) is the causal agent of bacterial spot disease on pepper and tomato plants and one of the model organisms for the analysis of bacterial infection strategies (Büttner & Bonas, 2010). Pathogenicity of Xcv depends on the translocation of effector proteins into plant cells where they interfere with essential plant cellular processes and thus promote bacterial proliferation (Büttner, 2016; Büttner & Bonas, 2010; Dean, 2011). Effector protein delivery is mediated by a type III secretion (T3S) system, which is present in many Gram-negative plant and animal pathogenic bacteria and is related to the bacterial flagellum. Both systems are, therefore, referred to as translocation-associated and flagellar T3SS (Abby & Rocha, 2012; Büttner, 2012). The membrane-spanning secretion apparatus of both systems contains at least eight conserved core components, which are known as secretion and cellular translocation (Sct) proteins in animal pathogenic bacteria followed by a letter...
corresponding to the nomenclature of T3SS components from Yersinia spp. (Hueck, 1998).

Structural studies of isolated T3SSs from animal pathogenic bacteria revealed the presence of ring components in both bacterial membranes (Büttner, 2012; Deng et al., 2017). The outer membrane (OM) ring, also termed secretin, consists of members of the SctC protein family and is connected on the extracellular side to a pilus-like structure known as T3S needle in animal and T3S pilus in plant pathogenic bacteria. T3S needles or pili serve as transport channels for effector proteins to the eukaryotic plasma membrane (Büttner, 2012; Deng et al., 2017). On the periplasmic side, the OM ring is in contact with an inner rod structure and the inner membrane (IM) rings, which are assembled by ScfD and ScfT proteins (Büttner, 2012; Deng et al., 2017; Diepold & Wagner, 2014). Embedded in the IM rings is the export apparatus, which presumably forms a transport channel for secreted proteins and consists of members of the SctU, SctV, SctR, SctS, and SctT families (Büttner, 2012; Deng et al., 2017; Lara-Tejero & Galan, 2019). The export apparatus associates with the cytoplasmic adenosinetriphosphatase (ATPase) complex (ScfN and ScfL), which presumably unfolds T3S substrates and is surrounded by a predicted cytoplasmic ring (C ring) or pod-like structures as was described for several T3SSs from animal pathogenic bacteria. C ring or pod-like structures are assembled by members of the SctQ family and are presumably involved in substrate recognition (Diepold et al., 2015; Hu et al., 2015; Lara-Tejero, 2019; Makino et al., 2016; Morita-Ishihara et al., 2006; Späth et al., 2009).

To date, the architecture and mode of action of T3SSs have been intensively studied in animal pathogenic bacteria, whereas much less is known about T3SSs from plant pathogenic bacteria. T3S genes from plant pathogens were designated hypersensitive response and pathogenicity (hrp) because they are essential for disease symptoms on susceptible plants and the induction of the effector-triggered immunity in resistant plants (Büttner & He, 2009; Schmidt & Hensel, 2004; Tampakaki et al., 2010). Effector-triggered immunity depends on the recognition of individual effectors in plants with cognate resistance genes and often leads to the induction of the hypersensitive response (HR), a local cell death at the infection site, which restricts bacterial multiplication (Gill et al., 2015). Hrp-T3SSs from plant pathogenic bacteria have been classified into Hrp1- (in Erwinia spp. and Pseudomonas syringae pathovars) and Hrp2-T3SSs (in Xanthomonas spp. andRalstonia solanacearum) according to similarities in the genetic organisation and regulation of hrp genes (Alfano & Collmer, 1997; Bogdanove et al., 1996; Troisfontaines & Cornelis, 2005). In Xcv, hrp genes are activated when the bacteria enter the plant or are cultivated in special minimal media. hrp gene expression depends on two regulatory proteins, HrpG and HrpX, which are encoded outside the hrp gene cluster (Büttner & Bonas, 2002; Büttner & Bonas, 2010; Wengelnik & Bonas, 1996; Wengelnik et al., 1996). HrpG is an OmpR-type response regulator that perceives an environmental signal via a yet unknown mechanism and activates HrpX, an AraC-type transcriptional regulator (Wengelnik et al., 1996; Wengelnik & Bonas, 1996). HrpX binds to conserved DNA motifs in the promoter regions of most hrp operons and activates hrp gene expression (Koebnik, et al., 2006; Noël et al., 2001).

The hrp gene cluster from Xcv contains eight operons with 25 genes including 11 conserved hrc (hrp conserved), 7 non-conserved hrp, and 6 hpa (hrp associated) genes as well as the effector gene xopF (Büttner & Bonas, 2002). hrc genes encode the core components of the T3SS such as the ATPase, the membrane rings, the predicted C ring, and the ATPase complex. The single-letter nomenclature of Hrc proteins refers to the corresponding Sct proteins from animal pathogens (Bogdanove et al., 1996; Büttner & Bonas, 2002). In contrast to Hrc proteins, Hrp proteins are not widely conserved, and homologues are mainly found within one bacterial species. Hrp proteins from Xcv and other Xanthomonas spp. include the predicted periplasmic inner rod proteins HrpB1 and HrpB2, the pilus protein HrpE, the translocase protein HrpF, and proteins of yet unknown function such as HrpB4, HrpB7, and HrpD6 (Büttner et al., 2002; Hartmann et al., 2012; Hausner et al., 2013; Li et al., 2011; Weber et al., 2005; Weber & Koebnik, 2005). Hrc and Hrp proteins are essential for T3S as structural components, whereas the accessory Hpa (Hrp associated) proteins are often involved in the control of T3S. One example is the T3S chaperone HpaB, which binds to type III effectors and presumably targets them to the T3S ATPase (Büttner et al., 2004; Büttner et al., 2006; Lonjon et al., 2017; Lorenz & Büttner, 2009; Prochaska et al., 2018; Scheibner et al., 2018). HpaB contributes to the translocation of most effector proteins and is, therefore, essential for pathogenicity.

Focus of the present study is the functional characterisation of HrpB7, which is encoded downstream of the ATPase gene hrcN by the seventh gene in the hrpB operon. HrpB7 is conserved in Xanthomonas spp. and shares amino acid sequence identity with HrpD from R. solanacearum. In Xcv, HrpB7 is the only hrp gene product with predicted N- and C-terminal coiled coils and contains features of HrpO/FliJ/YscO family members, which are small α-helical coiled-coil proteins encoded downstream of the T3S-ATPase genes from plant and animal pathogenic bacteria. In the present study, we show that HrpB7 from Xcv is required for secretion and translocation of early and late T3S substrates. HrpB7 forms stable protein complexes even in the absence of the T3SS and interacts with the ATPase and the predicted C ring, suggesting that it is a cytoplasmic structural component of the T3SS. The analysis of HrpB7 mutant derivatives by in vitro and in vivo studies revealed that protein function and complex formation depend on the N- and C-terminal leucine heptad motifs, which are part of the predicted coiled-coil structures of HrpB7 and are presumably involved in protein–protein interactions.

## 2 | RESULTS

### 2.1 | HrpB7 contains leucine heptad motifs and regularly spaced cysteine residues

HrpB7 from Xcv strain 85–10 is a small protein (169 amino acids), encoded downstream of the ATPase gene hrcN by the seventh gene
FIGURE 1  HrpB7 contains leucine heptad motifs and regularly spaced cysteine residues. (a) Schematic representation of the hrp gene cluster and amino acid sequence of HrpB7 (accession number CAJ22064). hrc, hrp, and hpa genes of the hrp gene cluster are represented by red, purple, and yellow arrows, respectively. The direction of transcription of single hrp operons is indicated. The xopF1 operon between hpaE and hrpF, which encodes the effector protein XopF1 and two putative associated chaperones, is replaced by a dashed line. The amino acid sequence of HrpB7 is given below the gene cluster. HrpB7 contains leucine heptad motifs and regularly spaced cysteine residues. (a) Schematic representation of the hrp gene cluster. HrpB7 contains leucine heptad motifs (indicated by red brackets) and five N-terminal leucine heptad motifs, which are shown in bold letters. Numbers refer to amino acid positions. (b) Helical wheel representation of the N-terminal leucine heptad motifs in HrpB7. The letters A–G in the inner circle refer to amino acid positions 1–7 in the heptad motifs (see text), respectively. Amino acids are shown in coloured circles; numbers refer to the amino acid positions. Red circles represent amino acids with nonpolar residues. Blue, green, and purple circles refer to amino acids with polar, positively, and negatively charged amino acids, respectively, as indicated. Cysteine residues are represented by yellow circles. (c) Helical wheel representation of the C-terminal leucine heptad motifs in HrpB7. Amino acids are presented as described in the helical wheel representation of the N-terminal leucine heptad motifs in HrpB7 (b).
neighbouring helices likely stabilise the coiled coil structure (Mason & Arndt, 2004). In the N-terminal two leucine heptad motifs of HrpB7, these criteria are fit when the leucine residues are located at position “a” and the regularly spaced cysteine residues of the (C-X6)5 motif at position “d” of each repeat (Figure 1b). In the C-terminal leucine heptad motif, position “d” is occupied by the nonpolar amino acids alanine, valine, and leucine (Figure 1c). In agreement with the presence of leucine heptad motifs in HrpB7, the analysis of the predicted secondary protein structure by Phyre2 (Kelley, et al., 2015) and SWISS-MODEL (Waterhouse et al., 2018) revealed a predominantly alpha-helical conformation of HrpB7 (Figure S1). Furthermore, coiled-coil structures were predicted for regions spanning amino acids 15 to 50 and 88 to 156 of HrpB7 (prediction by programme coiled coil prediction; https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_lupas.html).
Complementation studies with a nonpolar hrpB7 deletion mutant

Previous transposon mutagenesis approaches showed that a disruption of the hrpB7 gene results in a loss of pathogenicity of Xcv (Bonas et al., 1991; Fenselau & Bonas, 1995). To further assess the contribution of HrpB7 to pathogenicity, we deleted codons 7 to 164 of hrpB7 from the genome of Xcv strain 85–10 and analysed pathogenicity and in planta growth of the resulting mutant strain. In contrast to the wild type, strain 85–10ΔhrpB7 failed to induce water-soaked lesions in susceptible Early Cal Wonder (ECW) pepper plants and was severely affected in in planta growth (Figure 2a,b). Furthermore, strain 85–10ΔhrpB7 did not induce the HR on ECW-10R pepper plants, which contain the Bs1 resistance gene for recognition of the effector protein AvrBs1 (Minsavage et al., 1990; Figure 2a). We observed similar phenotypes with derivatives of strain 85* (85-10hrpG*), which contains a constitutively active version of the regulator HrpG and elicits faster plant reactions than the wild-type strain (Figure 2a; Rossier et al., 1999; Wengelnik et al., 1999). The hrpB7 mutant phenotype was complemented by ectopic expression of hrpB7 under control of the lac promoter, suggesting that it was specifically caused by the absence of hrpB7 and not by a polar effect of the deletion on the expression of the downstream hrcT gene (Figure 2a). This is an important control because hrpB7 from Xanthomonas oryzae pv. oryzicola was reported to contain a promoter for the downstream hrcT gene, which encodes an essential component of the export apparatus (Liu et al., 2014).

We also performed complementation studies with C-terminally c-Myc epitope-tagged derivatives of HrpB7 from Xcv and X. campestris pv. campestris (Xcc; HrpB7Xcc), which share 70% amino acid identity (77% sequence similarity) and show sequence variations in some of the N-terminal leucine and cysteine residues (Figure S1). Both proteins restored pathogenicity in strain 85*ΔhrpB7, suggesting that they were functional (Figure 2c). In contrast to HrpB7Xcv-c-Myc, HrpB7Xcc-c-Myc also fully complemented the phenotype of strain 85–10ΔhrpB7 with respect to disease symptoms and the HR (Figure S2). Given that the ectopic expression of hrpB7Xcc-c-myc in strain 85–10 suppressed the elicitation of plant reactions, we assume that enhanced levels of HrpB7Xcv-c-Myc but not of HrpB7Xcc-c-Myc interfere with pathogenicity in strain 85–10 (Figure S2). Immunoblot analysis showed that all

### Table 1: Characteristics of HrpB7 derivatives with mutations in leucine and cysteine residues

| HrpB7 derivative | Mutations | Epitope | Protein function | Complex formation |
|------------------|-----------|---------|-----------------|-------------------|
| HrpB7C3A        | C43A      | None    | +               | +                 |
|                  |           | c-Myc   | +               | +                 |
| HrpB7C1-2A      | C29,36A   | None    | +               | +                 |
|                  |           | c-Myc   | +               | +                 |
| HrpB7C3A        | C29,36,43A| None    | +               | +                 |
|                  |           | c-Myc   | +               | +                 |
| HrpB7C1-3A      | C29,36,43,50,57A | None | Reduced | Reduced |
|                  |           | c-Myc   | +               | +                 |
| HrpB7C6A        | C90A      | None    | +               | +                 |
|                  |           | c-Myc   | +               | +                 |
| HrpB7C1-6A      | C29,36,43,50,57,90A | None | Reduced | Reduced |
|                  |           | c-Myc   | +               | +                 |
| HrpB7L1S        | L33,40S   | c-Myc   | +               | +                 |
| HrpB7L1G        | L33,40G   | c-Myc   | +               | +                 |
| HrpB7L2S        | L111,118S | c-Myc   | +               | +                 |
|                  |           | Reduced | +               | +                 |
| HrpB7L2G        | L111,118G | c-Myc   | +               | Reduced |
| HrpB7L12S       | L33,40,111,118S | None | +               | –                 |
|                  |           | c-Myc   | +               | –                 |
| HrpB7L12G       | L33,40,111,118G | None | Reduced | –                 |
|                  |           | c-Myc   | –               | –                 |

*Numbers refer to amino acid positions in HrpB7.
Proteins were analysed as untagged or C-terminally c-Myc epitope-tagged derivatives.
Protein function was analysed in strain 85*ΔhrpB7 by infection experiments. + indicates complementation of the hrpB7 mutant phenotype with respect to disease symptoms and HR induction in susceptible and resistant pepper plants, respectively; – indicates no complementation of the hrpB7 mutant phenotype; reduced indicates partial complementation of the hrpB7 mutant phenotype.
Complex formation was analysed by immunoblotting of bacterial protein extracts. + indicates that HrpB7 complexes were detectable; – indicates that no complex formation was detectable; reduced indicates reduced complex formation.
HrpB7 derivatives were stably synthesised (Figures 2d and S2). In addition to the monomeric HrpB7 and HrpB7-c-Myc proteins at the predicted sizes of 18 and 23 kDa, respectively, HrpB7-specific signals were detected at higher molecular sizes, suggesting the presence of HrpB7-containing protein complexes, which were not dissolved by SDS-PAGE (Figures 2d and S2; see below).

### 2.3 HrpB7 is essential for T3S and effector translocation

In addition to infection studies, we investigated the contribution of HrpB7 to the T3S-dependent delivery of the transcription activator-like (TAL) effector protein AvrBs3, which activates expression of the resistance gene Bs3 in AvrBs3-responsive ECW-30R pepper plants (Boch et al., 2009; Römer et al., 2009). Furthermore, we used the N-terminal deletion derivative AvrBs3Δ2, which lacks a native export signal, as a reporter to monitor the translocation of the effector proteins XopC and XopJ. When AvrBs3 and AvrBs3Δ2 fusion proteins were analysed in strain 85*, which naturally lacks the avrBs3 gene, they induced the AvrBs3-specific HR in ECW-30R pepper plants as expected (Minsavage et al., 1990; Noël et al., 2003; Römer et al., 2009; Szurek et al., 2002). No HR induction was observed with strain 85*ΔhrpB7 ΔhrpB7 with (+) or without (−) expression constructs encoding HrpB7 or AvrBs3 as indicated were incubated in secretion medium. TE and culture SN were analysed by immunoblotting using antibodies specific for HrpB7, the translocon protein HrpF, the effector AvrBs3, and the inner membrane ring protein HrcJ, respectively. The upper protein detected by the AvrBs3-specific antibody corresponds to full-length AvrBs3, lower signals likely result from the detection of degradation products. TE, total cell extracts; SN, supernatants.
To study the influence of HrpB7 on in vitro T3S, bacteria were incubated in secretion medium, and total cell extracts and culture supernatants were analysed by immunoblotting. The translocon protein HrpF and the effector protein AvrBs3 (ectopically expressed from a plasmid) were detected in the culture supernatant of strain 85* but not of strain 85*ΔhrpB7 (Figure 3b). In contrast, the predicted IM ring protein HrcJ, which was analysed as lysis control, was only detected in total cell extracts as expected (Figure 3b). We also investigated a possible secretion of HrpB7 in strains 85* and 85*ΔhrpB7 containing hrpB7 or hrpB7-c-myc expression constructs. HrpB7 and HrpB7-c-Myc were detected in total cell extracts but not in the culture supernatants, suggesting that HrpB7 is not secreted under the conditions tested (Figures 3b and S3).

2.4 | HrpB7 forms stable protein complexes and localises to bacterial membranes

As mentioned above, HrpB7 presumably forms complexes corresponding to mono- or heterooligomers in the presence of SDS and...
Furthermore, complex formation was unaffected when also detected upon prolonged boiling or addition of urea (Figure 4a).

β-Cal Wonder was analysed as positive control (Hausner et al., 2017). ECW, Early Localisation studies with HrpB7-PhoA indicate the inoculated areas. (b) Analysis of PhoA activities of HrpB7-PhoA or PhoA fusion proteins as indicated were incubated in secretion medium, and phosphatase activity was analysed as described in Section 4.

FIGURE 5 Localisation studies with HrpB7-PhoAΔ2–120 fusions. (a) HrpB7-PhoAΔ2–120 fusions complement the hrpB7 mutant phenotype. Strains 85* and 85*ΔhrpB7 (ΔhrpB7) with or without (−) expression constructs encoding HrpB7, HrpB7-PhoAΔ2–120 (PhoAΔN), or PhoAΔN-HrpB7 as indicated were infiltrated at a density of 10² CFU ml⁻¹ into leaves of susceptible ECW plants. Disease symptoms were photographed 8 days post inoculation. Dashed lines indicate the inoculated areas. (b) Analysis of PhoA activities of HrpB7-PhoAΔ2–120 fusions. Strains 85* and 85*ΔhrpB7 (ΔhrpB7) with or without (−) expression constructs encoding PhoAΔ2–120 (PhoAΔN) fusion proteins as indicated were incubated in secretion medium, and phosphatase activity was analysed as described in Section 4. Proteins were detected by immunoblotting as described in panel (a). PhoAΔ2–120 fusions are indicated by asterisks. The fusion between the periplasmic predicted lytic transglycosylase HpaH and PhoAΔ2–120 was analysed as positive control (Hausner et al., 2017). ECW, Early Cal Wonder

β-mercaptoethanol (Figure 2d). Complexes were very stable and were also detected upon prolonged boiling or addition of urea (Figure 4a).

Furthermore, complex formation was unaffected when hrpB7 was expressed in Xcv strains 85*Δhrp and 85*ΔhrpΔflog, which lack the complete hrp gene cluster and additionally essential parts of the flagellar T3S gene cluster, respectively (Figure 4b). We conclude that HrpB7 multimersise independently of the T3SS.

Next, we analysed whether HrpB7 associates with the bacterial membranes. For this, strains 85*ΔhrpB7 and 85*Δhrp containing HrpB7-c-Myc were grown in minimal medium under T3S-permissive conditions (pH 5.3), and soluble and membrane fractions were separated by ultracentrifugation. HrpB7-c-Myc was detected in the soluble and the membrane fractions of both strains, suggesting that the association of HrpB7 with the bacterial membranes was independent of the T3SS (Figure 4c). As control, blots were reprobed with an antibody directed against the IM ring component HrcJ, which was mainly detected in the membrane fraction of strain 85*ΔhrpB7 as expected (Figure 4c). Similar results were obtained when bacteria were incubated under T3S nonpermissive conditions (minimal medium, pH 7.0; Figure S4). As HrpB7 lacks predicted transmembrane domains, it is likely not an integral membrane protein. To prove this hypothesis, we incubated the membrane fractions in the presence of 5-M urea and separated integral membrane proteins from membrane-associated proteins by ultracentrifugation. HrpB7 was predominantly detected in the membrane-associated fraction, which is in agreement with the hypothesis that HrpB7 does not insert into the IM or OM (Figure 4d).

Similar results were obtained when bacteria were incubated under T3S nonpermissive conditions (Figure S4).

To further investigate the subcellular localisation of HrpB7, we generated N- and C-terminal fusions of HrpB7 to an N-terminal deletion derivative of the alkaline phosphatase PhoA (PhoAΔ2–120) from Escherichia coli, which is active when located in the periplasm and used as a reporter to analyse protein topology (Berger et al., 2010; Manoil & Beckwith, 1986). HrpB7-PhoAΔ2–120 and PhoAΔ2–120-HrpB7 both complemented the hrpB7 mutant phenotype, suggesting that the PhoAΔ2–120 fusion partner did not interfere with HrpB7 function (Figure 5a). When analysed in Xcv strain 85*ΔphoA, which lacks the native phoA gene (Hausner et al., 2017), no PhoA activity of both fusions was detectable, suggesting that the N- and C-termini of HrpB7 are not located in the periplasm (Figure 5b). Given the absence of predicted transmembrane domains in HrpB7, we conclude that HrpB7 localises to the cytoplasmic side of the IM.

2.5 Cysteine and leucine residues in HrpB7 contribute to protein function and complex formation

To analyse the contribution of single amino acids in HrpB7Xcv to protein function, we exchanged one or several cysteine residues in the N-terminal (C-X₆)₅ motif against alanine and also introduced mutations leading to the substitution of leucine residues at positions 33 and 40 and/or 111 and 118 by serine or glycine (Table 1). The resulting HrpB7 derivatives were analysed as C-terminally c-Myc epitope-tagged derivatives for their ability to complement the hrpB7 mutant phenotype. Exchange of the first three cysteine residues of the (C-X₆)₅ motif or the central cysteine residue at position 90 did not significantly affect HrpB7 function, whereas HrpB7 derivatives with mutations in all five cysteine residues of the (C-X₆)₅ motif did not complement the hrpB7 mutant phenotype (Figure 6a; Table 1). Immunoblot analyses of bacterial protein extracts showed that all HrpB7 derivatives were stably synthesised and that formation of HrpB7-specific complexes was significantly reduced or not detectable for HrpB7C₁₅₅A-C-Myc and HrpB7C₁₆₄A-C-Myc, which was additionally mutated in C90 (Figure 6c; Table 1). When HrpB7 derivatives were analysed as untagged proteins, HrpB7C₁₅₅A and HrpB7C₁₆₄A partially complemented the phenotype of strain
In contrast, HrpB7 derivatives in which the leucine residues in both heptad motifs were exchanged against glycine (HrpB7L12G) only partially complemented the hrpB7 mutant phenotype when analysed as untagged proteins (Figure S6). No complementation was observed when HrpB7L12G was analysed as C-terminally c-Myc epitope-tagged derivative (Figure 6b; Table 1). This is presumably caused by a negative effect of the c-Myc epitope on protein function (see also Figure S2). Immunoblot analysis showed that complex formation was

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**FIGURE 6** Legend on next page.
severely reduced for HrpB7 derivatives carrying substitutions in both leucine heptad motifs, whereas mutations in one motif did not or only slightly interfere with complex formation (Figure 6c). We conclude that both leucine heptad motifs as well as the N-terminal (C-X₆)₅ motif of HrpB7 are required for complex formation and contribute to protein function.

We also investigated whether the leucine heptad motifs contribute to the localisation of HrpB7. Fractionation studies with HrpB7L₁₂G-c-Myc in strain 85ΔhrpB7 grown under T3S-permissive conditions revealed that HrpB7L₁₂G-c-Myc was predominantly detected in the soluble fraction (Figure 6d). This is in contrast to HrpB7-c-Myc, which was equally distributed in the soluble and membrane fraction (see above; Figure 4d) and suggests that the leucine heptad motifs promote the association of HrpB7 with the bacterial membranes.

2.6  HrpB7 interacts with components of the T3SS

Next, we analysed whether HrpB7 interacts with components of the T3SS and performed in vitro glutathione S-transferase (GST) pull-down assays. GST and GST-HrpB7 were immobilised on glutathione sepharose and incubated with bacterial lysates containing C-terminally c-Myc epitope-tagged derivatives of potential interaction partners. When eluted proteins were analysed by immunoblotting, the ATPase HrcN, the IM ring protein HrcD, and the predicted C ring protein HrcQ were detected in the eluate of GST-HrpB7 but not of GST, suggesting that they interact with HrpB7 (Figure 7a,b). Similar results were obtained for a C-terminally c-Myc epitope-tagged derivative of the T3S chaperone HpaB (Figure 7a,b). We also investigated a possible contribution of the leucine heptad motifs of HrpB7 to protein-protein interactions. The results of GST pull-down assays suggest that the leucine heptad motifs contribute to the interaction of HrpB7 with HrcQ, HrcD, and HpaB (Figure 7b). This is in agreement with the predicted role of coiled-coil motifs in protein–protein interactions and supports the hypothesis that coiled-coil motifs contribute to HrpB7 function.

2.7  HrpB7 is dispensable for the formation of the predicted C ring

Given the potential interaction of HrpB7 with HrcQ, we investigated whether HrpB7 is required for the assembly of the predicted C ring. For this, a fusion protein between HrcQ and the superfolder green fluorescent protein (sfGFP) was analysed in Xcv using a modular T3S gene cluster as described previously (Hausner et al., 2019). As expected, HrcQ-sfGFP formed fluorescent foci, which are indicative of the assembly of the T3SS as was previously described for fluorescent reporter fusions containing T3SS components from animal pathogenic bacteria (Diepold et al., 2010; Diepold et al., 2011; Hausner et al., 2019; Li & Sourjik, 2011; Morimoto et al., 2014; Zhang et al., 2017; Figure 7c,d). Foci formation was dependent on the presence of the T3SS and was not detected in the absence of the hrpA to hpaB operons (Hausner et al., 2019; Figure 7c,d). In the absence of HrpB7, foci formation by HrcQ-sfGFP was only slightly reduced, suggesting that HrpB7 is not essential for the assembly of the predicted C ring (Figure 7c,d).

3  DISCUSSION

In this study, we identified HrpB7 as a novel complex-forming component of the T3SS from Xcv. Previous transposon mutagenesis approaches already indicated an essential pathogenicity function of HrpB7 (Bonas et al., 1991; Fenselau & Bonas, 1995). This was confirmed in the present study by the analysis of a hrpB7 deletion mutant and complementation studies (Figure 2). The finding that the phenotype of the Xcv hrpB7 mutant can be complemented by HrpB7 from Xcc points to a conserved function of HrpB7 proteins in...
Xanthomonas spp. (Figure 2). Notably, however, HrpB7 is not widely conserved in Hrp2-T3SSs and shares only 30% amino acid sequence identity (from amino acids 62–165) with HrpD from *R. solanacearum*, which is also encoded by the seventh gene of the *hrpB* operon. In contrast to HrpB7 from *Xcv*, HrpD contributes to but is not essential for pathogenicity of *R. solanacearum* (Van Gijsegem, et al., 2002). The homology of HrpB7 with HrpD is restricted to the central and C-terminal region of HrpB7 including the C-terminal leucine heptad.

**FIGURE 7** HrpB7 interacts with T3S-associated proteins and is dispensable for cytoplasmic ring formation. (a) In vitro interaction studies with HrpB7. GST and GST-HrpB7 were immobilised on glutathione sepharose and incubated with bacterial lysates containing C-terminally c-Myc epitope-tagged derivatives of the ATPase HrcN, the inner membrane ring protein HrcD, the predicted cytoplasmic ring protein HrcQ, and the T3S chaperone HpaB. TE and eluates were analysed by immunoblotting using GST- and c-Myc epitope-specific antibodies as indicated. One representative blot for the detection of GST and GST-HrpB7 is shown. Experiments were performed at least three times with similar results. (b) The leucine heptad motifs of HrpB7 contribute to the interaction with HrcD, HrcQ, and HpaB. GST, GST-HrpB7, and GST-HrpB7L12G were immobilised on glutathione sepharose and incubated with bacterial lysates containing C-terminally c-Myc epitope-tagged derivatives of HrcD, HrcQ, and HpaB. TE and eluates were analysed as described in panel (a). L12G, HrpB7, L12G, HrpB. (c) HrpB7 is dispensable for foci formation by HrcQ-sfGFP. Fluorescent foci were analysed in strain 85Δ*hrpA-hpaB-HAGX* carrying modular level P*hrp-HAGX* expression constructs containing hrcQ-sfgfp and deletions in hrcQ (ΔhrcQ), in the *hrpA* to *hpaB* operons (Δ*hrpA-hpaB*), as well as in hrcQ and *hrpB7* (ΔhrcQΔhrpB7) as indicated. Bacteria were grown in secretion medium, and foci formation was analysed by fluorescence microscopy. The experiment was performed three times with similar results. One representative image for each strain from one experiment is shown. The size bar corresponds to 2 μm. (d) Statistical analysis of HrcQ-sfGFP foci formation. Fluorescent foci were counted in approximately 300 cells per strain in three different transconjugants, and the mean values and standard deviations are shown as percentage of bacterial cells. Asterisks indicate a significant difference between the Δ*hrpA-hpaB* and the hrcQ single-deletion mutant strain or the ΔhrcQΔhrpB7 and the hrcQ single-deletion mutant strain as indicated by brackets with a p value < .05 based on the results of a χ² test. ECW, Early Cal Wonder; GST, glutathione S-transferase; TE, total cell extracts.
motifs, which are part of the predicted coiled-coil structures. HrpB7 is the only hrp gene product from Xcv with predicted N- and C-terminal coiled coils. This protein motif usually consists of two or more α helices winding around each other and is often involved in protein–protein interactions (Delahay & Frankel, 2002; Woolfson, et al., 2012). In agreement with the presence of predicted coiled coils in HrpB7, our in vitro pull-down assays suggest that HrpB7 interacts with T3SS components including the ATPase HrcN, the predicted C ring protein HrcQ, and the IM ring component HrcD (Figure 7). Furthermore, HrpB7 forms stable complexes, probably corresponding to dimers and homo- or heterooligomers, suggesting that it is part of an oligomeric substructure of the T3SS (Figure 4).

Mutations in both N- and C-terminal leucine heptad motifs severely reduced complex formation, thus confirming the predicted contribution of the putative coiled-coil structures to the protein–protein interactions (Figure 6). Given that HrpB7 lacks predicted transmembrane helices and is not secreted and presumably does not localise to the bacterial periplasm, we assume that it associates on the cytoplasmic side with the ATPase complex and the ring structures of the T3SS (Figures 4 and 5).

Notably, complex formation and interaction with the ATPase complex, the C ring and components of the export apparatus were previously reported for T3SS-associated proteins from plant and animal pathogens, which are encoded downstream of the T3S-ATPase genes similarly to HrpB7 (Cherradi et al., 2014; Evans et al., 2006; Fraser et al., 2003; Gazi et al., 2008; Romo-Castillo et al., 2014). Despite a lack of general sequence conservation, these proteins contain predicted coiled coils and are referred to as HrpO/FliJ/YscO protein family (Gazi et al., 2008; Gazi et al., 2009). In addition to HrpB7, the only other characterised member of this protein family from plant pathogenic bacteria is HrpO from P. syringae. HrpO was described as α helical with characteristics of intrinsically disordered proteins and self-associates as was also observed for HrpB7. Furthermore, HrpO interacts with the predicted regulator of the T3S-ATPase, HrpE (Gazi et al., 2008; Uversky, 2013). An interaction with the ATPase complex was also reported for the HrpO/FliJ/YscO family members FliJ from the flagellar T3SS, Spa13 from Shigella flexneri, and EscO (formerly known as Orf15 or EscA) from enteropathogenic E. coli (Cherradi et al., 2014; Evans et al., 2006; Romo-Castillo et al., 2014).

EscO stimulates the activity of the ATPase EscN in vitro in the absence of the negative regulator EscL and enhances EscN hexamerisation (Majewski et al., 2019; Romo-Castillo et al., 2014). A similar positive effect on the ATPase activity was described for FliJ, which promotes hexamerisation of the ATPase Fil of the flagellar T3SS (Ibuki et al., 2011). FIJ and EscO share structural similarity with the α-helical coiled-coil region of the F2F2 ATPase γ subunit, which is part of the central stalk of the ATPase (Ibuki et al., 2011; Majewski et al., 2019).

It was, therefore, suggested that HrpO/FliJ/YscO family members associate with the ATPase complex and are involved in the regulation of the ATPase activity. In agreement with this hypothesis, the crystal structure of the EscN–EscO complex showed that EscO localises to the C-terminal opening of the hexameric EscN pore with the N- and C-terminal coiled-coil regions penetrating the pore (Majewski et al., 2019). The finding that EscO can partially rescue the motility of a fliJ mutant supports the notion that both proteins share a similar function (Romo-Castillo et al., 2014).

It remains to be investigated whether also HrpB7 from Xcv contributes to the assembly of the ATPase complex and to the regulation of its activity as was reported for FliJ and EscO (Ibuki et al., 2011; Majewski et al., 2019). In preliminary in vitro experiments, however, we did not detect a positive influence of HrpB7 on the activity of the purified ATPase HrcN (J. Hausner and D. Büttner, unpublished data). Furthermore, our fluorescence microscopy studies showed that the predicted C ring component HrcQ assembles independently of HrpB7 (Figure 7). Given that a failure in the assembly of the ATPase complex likely results in a loss of C ring formation as shown in Yersinia spp. (Diepold et al., 2010), it is possible that HrpB7 is dispensable for the formation of the ATPase complex. Notably, a similar finding was reported for the HrpO/FliJ/YscO family member YscO from Yersinia spp., suggesting that a role in the assembly of the ATPase does not appear to be a general characteristic function of this protein family (Diepold et al., 2012).

HrpB7 does not only interact with components of the T3SS but also with the T3S chaperone HpaB as revealed by our interaction studies (Figure 7). Notably, an interaction with T3S chaperones was also described for FliJ, Spa13, and InvI, suggesting that HrpO/FliJ/YscO family members are involved in a network of interactions with T3SS components and chaperones (Evans et al., 2004; Evans & Hughes, 2009). In Xcv, the T3S chaperone HpaB is essential for the efficient secretion and translocation of multiple effector proteins and was previously identified as interaction partner of T3SS components including the ATPase, the C ring, and the cytoplasmic domains of components of the export apparatus (Büttner et al., 2004; Büttner et al., 2006; Hartmann & Büttner, 2013; Lonjon et al., 2017; Lorenz & Büttner, 2009; Lorenz et al., 2012; Prochaska et al., 2018). The presence of multiple binding sites for HpaB in the T3SS suggests that HrpB7 does not play an exclusive role in the recruitment of chaperone complexes but might rather be of general importance for T3S. This hypothesis is supported by the finding that HrpB7 is not only essential for the delivery of HpaB-dependent effector proteins but also for the translocation of the HpaB-independent early T3S substrate HrpB2 (Figure 3).

Given the variety of different interaction partners identified for HrpO/FliJ/YscO family members, it seems likely that the observed protein–protein interactions are stabilised by the coiled-coil motifs. We, therefore, analysed the predicted contribution of the putative coiled-coil motifs in HrpB7 to protein–protein interactions and protein function. Characteristic features of coiled coils are repetitive heptad motifs (abcdefg), with hydrophobic amino acids at positions “a” and “d” (Gazi et al., 2009). The presence of unbranched amino acids such as leucine at position “a” as is the case in HrpB7 (Figure 1) might favour the formation of four-stranded parallel coiled coils (Lupas & Gruber, 2005). When we exchanged the leucine residues in the heptad motifs of HrpB7 against glycine, complex formation was severely impaired (Figure 6). Glycine is a known helix breaker and, therefore, likely affects the helical structures within the predicted coiled coils.
X6)5 motif against alanine significantly reduced complex formation against the polar amino acid serine also abolished detectable complex formation (Figure 6). However, HrpB7 derivatives with leucine-to-serine exchanges were partially functional, indicating that complex formation contributes to but is presumably not crucial for HrpB7 function. The observed negative impact of the mutations was strongly reduced when amino acid exchanges were only introduced in either the N- or the C-terminal heptad motif (Figure 6).

We also investigated the role of the five regularly spaced cysteine residues at position “d” of each N-terminal heptad in HrpB7. The exchange of all five cysteine residues in the N-terminal (C-X_d)s motif against alanine significantly reduced complex formation by HrpB7 (Figure 6). This is in agreement with the hypothesis that cysteine residues stabilise the coiled-coil structure by the formation of disulfide bonds and thus presumably contribute to the assembly of intermolecular or intramolecular complexes (Shen et al., 2005; Zhou et al., 1993). Given that alanine still fits the criteria for position “d” of the leucine heptad motif, the C-to-A exchange likely did not completely prevent coiled-coil formation.

In line with this hypothesis is our finding that the C-to-A mut-ation of the leucine heptad motif, the C-to-A mutation impaired but did not abolish HrpB7 function (Figure 6). We conclude that HrpB7 function and complex formation depend on the leucine and cysteine residues in the N- and C-terminal heptad motifs but that single amino acids can be mutated without causing significant detrimental effects. This is in line with the finding that some of the cysteine and leucine residues in the N- and C-terminal heptad motifs are not conserved in HrpB7Xcc, which is functional in Xcv.

Taken together, our study provides the first detailed characterisation of a member of the FliJ/HrpO/YscO family from a Hrp2-T3SS. The experimental results suggest that HrpB7 from Xcv forms an essential oligomeric structure of the T3SS and interacts via coiled coils with the ATPase complex as well as with IM and cytoplasmic rings of the T3SS. In future experiments, we will investigate whether HrpB7 contributes to the assembly of the ATPase complex or additional substructures of the T3SS.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Table S1. E. coli strains were cultivated at 37°C in lysogeny broth medium. Xcv strains were grown at 30°C in nutrient yeast extract glycerol medium (Daniels et al., 1984). For T3SS assays, we used minimum medium A (MA) supplemented with sucrose (10 mM) and casamino acids (0.3%; Ausubel et al., 1996).

4.2 | Plant material and plant infections

For infection studies, bacterial solutions were infiltrated into leaves of the near-isogenic pepper cultivars ECW and ECW-10R with a needle-less syringe (Kousik & Ritchie, 1998; Minsavage et al., 1990). Plants were inoculated with 1 x 10⁶ colony-forming units (CFU) ml⁻¹ in 1-mM MgCl₂ if not stated otherwise. Infected plants were incubated in growth chambers for 16 hr of light at 28°C and 8 hr of darkness at 22°C. Plant reactions were monitored once daily for 10 days postingestion (dpi). The HR was documented after destaining the leaves in 70% ethanol. Infection experiments were performed at least three times with different transconjugants; representative results are shown. In planta growth curves were performed three times as described (Bonas et al., 1991). To monitor translocation of dTALE-2, we used gfp-transgenic N. benthamiana plants (Werner et al., 2011). Infected N. benthamiana plants were incubated for 16 hr of light at 20°C and 8 hr of darkness at 18°C. GFP fluorescence was documented 4 dpi.

4.3 | Generation of Xcv hrpB7 deletion mutant strains

To generate Xcv hrpB7 deletion mutants, DNA fragments flanking hrpB7 and including the first and last 18 nucleotides of the coding sequence were amplified by polymerase chain reaction (PCR) and cloned into the Golden Gate-compatible suicide vector pOOG2 using Bsal and ligase. The resulting construct was transformed into E. coli DH5αpir and transferred into strains 85-10 and 85* by triparental conjugation. Transconjugants were selected as described previously (Huguet, et al., 1998). Double crossovers resulted in hrpB7 deletion mutant strains.

4.4 | Generation of expression constructs

To generate a hrpB7 expression construct, hrpB7 was amplified by PCR from Xcv strain 85-10 and cloned into the BsaI sites of vector pBRM, downstream of a lac promoter and in frame with a C-terminal 3 x c-Myc epitope-encoding sequence. Similarly, hrpB7 was cloned into vector pBRM-Stop, which contains a stop codon upstream of the 3 x c-Myc epitope-encoding sequence, resulting in the synthesis of untagged HrpB7. We also generated a pBRM expression construct encoding HrpB7 from Xcv using primers specific for hrpB7Xcc from strain LMG 568. For the generation of a GST-HrpB7 expression construct, two modules corresponding to hrpB7 and ptac-gst were cloned into vector pBRM-P, resulting in construct PB-P-ptacGST-hrpB7. ptac-gst was amplified by PCR from vector pGEX-2TKM. All PCR amplicons were first subcloned into vector pUC57ΔBsal or alternatively into vector pICh41021 using Sma1 and ligase.

For the exchange of cysteine residues against alanine, 5’ and 3’ regions of hrpB7 were amplified by PCR from plasmid pHrpB7*, in
which the internal BsaI site in hrpB7 had been mutated by PCR. Mutations leading to exchanges of cysteine residues at positions 29, 36, 43, 50, 57, and 90 against alanine were introduced by the primer sequences. Cloning of PCR products into the BsaI sites of vectors pBRM and pBRM-Stop led to the generation of expression constructs encoding HrpB7_{C2A} (HrpB7_{C2A}), HrpB7_{C29,36A} (HrpB7_{C1-2A}), HrpB7_{C29,36,43A} (HrpB7_{C1-3A}), HrpB7_{C29,36,43,50,57A} (HrpB7_{C1-5A}), HrpB7_{C9G} (HrpB7_{C6A}), and HrpB7_{C29,36,43,50,57,90A} (HrpB7_{C1-6A}) with or without C-terminal c-Myc epitope. Primers used for the generation of HrpB7_{C2A} (HrpB7_{C2A}) were designed to introduce three mutations but only resulted in the C43A mutation. HrpB7_{C2A} was, therefore, used as PCR template to generate a hrpB7_{C1-3A} expression construct. Similarly, hrpB7_{C1-3A} was used as template to generate a hrpB7_{C1-5A} expression construct, which was subsequently used as a template to generate hrpB7_{C1-6A}.

For the generation of expression constructs encoding HrpB7 derivatives with exchanged leucine residues, the corresponding mutations were introduced by PCR. For each mutant derivative, the 5'-terminal or in both leucine heptad motifs, which were referred to as L1S (HrpB7_{L33,40S}), L1G (HrpB7_{L33,40G}), L2S (HrpB7_{L111,118S}), L2G (HrpB7_{L111,118G}), L2S (HrpB7_{L33,40,111,118S}), and L12G (HrpB7_{L33,40,111,118G}). hrpB7_{L12G} was amplified by PCR from pBSotophrpB7_{L12G} and cloned together with ptac-gst into vector pBRM-P, thus generating an expression construct that encodes GST-HrpB7_{L12G}. In addition to the generation of hrpB7 expression constructs, we amplified hrcD and cloned the corresponding PCR product in vector pBRM. Generated constructs and primers used in this study are listed in Tables S1 and S2, respectively.

For the construction of PhoA_{A2-120} expression constructs, the phoA_{A2-120} gene fragment from E. coli (GenBank accession number M29668) including an additional linker-encoding sequence (amino acids LSLIHISWPMPG) was amplified using nested forward primers containing the linker-encoding sequence and a phoA-specific reverse primer (see Table S1; Berger et al., 2010). The linker-phoA_{A2-120} gene fragment was subcloned into vector pICH41021 using SmaI and ligase and subsequently ligated with hrpB7 into vector pBRM, resulting in pHrpB7-linker-phoA_{A2-120}. To generate constructs encoding PhoA_{A2-120} as N-terminal fusion partner of HrpB7, the linker-phoA_{A2-120} gene fragment was amplified from construct pICH41021-linker-phoA_{A2-120} using primers that allow cloning of the linker-phoA_{A2-120} fragment into the 5'-cloning site (5'-TATG-3' overhang) of vector pBRM. Ligation of linker-phoA_{A2-120} with hrpB7 into vector pBRM using BsaI and ligase resulted in an expression construct encoding Linker-PhoA_{A2-120}-HrpB7. PhoA_{A2-120} expression constructs were transferred into strain 85*ΔhrpB7 for complementation studies and into strain 85*ΔphoA for the analysis of the phosphatase activity. PhoA_{A2-120} fusion proteins were detected by immunoblotting using a PhoA-specific antibody (Sigma-Aldrich).

### 4.5 Generation of a modular T3S gene cluster construct containing hrcQ-sfgfp and deletions in hrcQ and hrpB7

To introduce a deletion into hrpB7 in the modular T3S gene cluster, hrpB7 was amplified by PCR with primers FS{hrpB7F} and FShrpB7_R using the level −2 construct pAGB194, which contains hrpB7, as template (Hausner et al., 2019). The resulting PCR amplicon corresponding to hrpB7 with a deletion of bp 15 to 429 was cloned into the pUC19 derivative pAGM9121 (Addgene #51833) using BpiI and ligase, thus generating the level −2 construct pAGB772. In a subsequent Golden Gate reaction, the insert of pAGB772 was assembled with modules from additional level −2 constructs pAGB192 (contains hrcL), pAGB193 (contains hrcN), pAGB195 (contains hrcT), and pAGB196 (contains hrcC) in Level −1 vector pAGM1311 (Addgene #47983) using Bsa1 and ligase (Hausner et al., 2019). The assembly of the resulting pAGB778 construct with the additional level −1 construct pAGB197, which contains hrb1 to hrpB4, using BpiI and ligase led to Level 0 construct pAGB784 (corresponding to the hrpa and hrpB operons), which was subsequently cloned into level 1 vector pICH478111 using Bsa1 and ligase (Addgene #48008), thus generating level 1 construct pAGB790. The assembly by BpiI and ligase with additional level 1 constructs pAGB275 (contains hrpc-hrpB operons with a deletion in hrcQ) and pAGB156 (contains hrpF) led to the generation of level M construct pAGB796, which includes the hrp gene cluster with deletions in hrpB7 and hrcQ (Hausner et al., 2019). Construct pAGB796 was assembled with level M construct pAGB322 (contains xopA, hpaH, hrpX, hrpG*, and hrcQ-sfgfp; Hausner et al., 2019) and end-linker pICH79264 in Level P vector pICH75322 using Bsa1 and ligase, thus resulting in the final level P construct pAGB802.

### 4.6 Analysis of in vitro T3S

In vitro T3S assays were performed as described previously (Rossier et al., 1999). Briefly, bacteria were grown overnight in MA medium (pH 7.0) supplemented with sucrose (10 mM) and casamino acids (0.3%) and shifted to MA medium (pH 5.3) containing 50-μg ml⁻¹ bovine serum albumin (BSA) and 10-μM thiamine at an optical density (OD₆₀₀) of 0.15. Cultures were incubated on a rotary shaker for 1.5 hr at 30°C, and bacterial cells and secreted proteins were separated by filtration. Proteins in 2 ml of the culture supernatants were precipitated with trichloroacetic acid and resuspended in 20 μl of Laemmli buffer. Total cell extracts and culture supernatants were analysed by SDS-PAGE and immunoblotting, using antibodies directed against the c-Myc epitope as well as against HrpB7, HrpF, HrcJ, and AvrBs3, respectively (Bonnas et al., unpublished; Büttnér et al., 2002; Knoop et al., 1991; Rossier et al., 2000). Horseradish peroxidase–labelled anti-mouse and anti-goat antibodies were used as secondary antibodies, and binding of antibodies was visualised by enhanced chemiluminescence.
4.7 | GST pull-down assays

For GST pull-down assays, E. coli BL21(DE3) cells containing the expression constructs for the synthesis of GST, GST-HrpB7, GST-HrpB7L12G, and C-terminally c-Myc epitope-tagged potential interaction partners were grown in lysogeny broth medium until an OD₆₀₀ nm of 0.6–0.8. Gene expression, which was driven in all cases by the lac promoter, was induced in the presence of IPTG for 2 hr at 37°C, and after centrifugation bacterial cells were broken with a French press. Cell debris was removed by centrifugation, and soluble GST and GST fusion proteins were immobilised on a glutathione sepharose matrix according to the manufacturer’s instructions (GE Healthcare). After washing of the matrix, immobilised GST and GST-HrpB7 were incubated with bacterial lysates containing the predicted interaction partner for 2 hr at 4°C on an overhead shaker. Unbound proteins were removed by washing, and bound proteins were eluted with Laemmli buffer. Cell lysates and eluted proteins were analysed by SDS-PAGE and immunoblotting, using c-Myc epitope- and GST-specific antibodies.

4.8 | Fractionation experiments with Xcv strains

For the subcellular localisation of HrpB7, bacteria were grown overnight in MA medium (pH 7.0) supplemented with sucrose (10 mM) and casamino acids (0.3%). Bacteria from the overnight culture were used to inoculate 50-ml MA (pH 5.3) medium supplemented with sucrose (10 mM), casamino acids (0.3%), 50-μg/ml BSA, and 10-μM thiamine at an OD₆₀₀ nm of 0.1. The culture was incubated overnight on a shaker. Cells were then harvested by centrifugation, resuspended in 2-ml 50 mM HEPES (2-(4-[2-hydroxyethyl]-1-piperazinylethanesulfonic acid) and lysed with a French press. Cell debris was removed by centrifugation and lysates were centrifuged at 200,000 × g for 90 min at 4°C. The pellet corresponding to the membrane fraction was resuspended in 1-ml 50 mM HEPES buffer. Samples of membrane and soluble fractions were mixed with Laemmli buffer and analysed by immunoblotting using c-Myc epitope- and HrcJ-specific antibodies. For the analysis of membrane-associated proteins, the pellet after ultracentrifugation was resuspended in 1-ml 50 mM HEPES buffer in the presence of 5-M urea and stirred for 1 hr at 4°C. The solution was centrifuged at 200,000 × g for 90 min at 4°C. Samples of the supernatants and the pellets corresponding to the membrane-associated and the integral membrane proteins, respectively, were mixed with Laemmli buffer and analysed by immunoblotting as described above.

4.9 | Phosphatase assays

For the analysis of the PhoA activity, bacteria were resuspended in secretion medium at an OD₆₀₀nm of 0.8 and incubated in the presence of 90-μg/ml X-Phos (5-Bromo-4-chloro-3-indolyl phosphate) on a horizontal shaker for 3 hr at 30°C. The colour change was photographed, and bacterial cell extracts were analysed by immunoblotting using a PhoA-specific antibody (Sigma-Aldrich). The phosphatase assay was performed twice with similar results.

4.10 | Fluorescence microscopy

For the analysis of HrcQ-sfGFP, bacteria were grown overnight in MA medium (pH 7.0) supplemented with sucrose (10 mM) and casamino acids (0.3%). Cells were then resuspended at an OD₆₀₀ nm of 0.15 in MA medium (pH 5.3) supplemented with BSA and thiamine as described above and incubated on a tube rotator at 30°C for 1 hr. Cell suspensions were pipetted on a microscopy slide on top of a pad of 1% agarose dissolved in MA medium (pH 5.3) containing BSA and thiamine as described previously (Hausner et al., 2019). GFP fluorescence was inspected with a confocal laser scanning microscope (Zeiss LSM 780 AxioObserver. Z1) using filter sets for sfGFP (excitation at 485 nm; emission at 510 nm). Experiments were performed with different transconjugants for each strain and repeated twice with similar results.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION
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