HrcU and HrpP are pathogenicity factors in the fire blight pathogen *Erwinia amylovora* required for the type III secretion of DspA/E

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**Abstract**

**Background:** Many Gram-negative bacterial pathogens mediate host-microbe interactions via utilization of the type III secretion (T3S) system. The T3S system is a complex molecular machine consisting of more than 20 proteins. Collectively, these proteins translocate effectors across extracellular space and into the host cytoplasm. Successful translocation requires timely synthesis and allocation of both structural and secreted T3S proteins. Based on amino acid conservation in animal pathogenic bacteria, HrcU and HrpP were examined for their roles in regulation of T3S hierarchy.

**Results:** Both HrcU and HrpP were shown to be required for disease development in an immature pear infection model and respective mutants were unable to induce a hypersensitive response in tobacco. Using in vitro western blot analyses, both proteins were also shown to be required for the secretion of DspA/E, a type 3 effector and an important pathogenicity factor. Via yeast-two hybridization (Y2H), HrpP and HrcU were revealed to exhibit protein-protein binding. Finally, all HrcU and HrpP phenotypes identified were shown to be dependent on a conserved amino acid motif in the cytoplasmic tail of HrcU.

**Conclusions:** Collectively, these data demonstrate roles for HrcU and HrpP in regulating T3S and represent the first attempt in understanding T3S hierarchy in *E. amylovora*.

**Keywords:** Type III secretion system, Secretion hierarchy, Substrate specificity, *Erwinia amylovora*, HrcU, HrpP

**Background**

The type III secretion (T3S) system is a common feature of Gram-negative bacterial pathogens. The T3S system functions to facilitate the translocation of bacterial effector proteins into eukaryotic host cells where they suppress host defense responses, facilitate colonization, and promote disease development [1]. Consequently, T3S has been the focus of intensive research in both animal and plant pathosystems.

The T3S system is a complex proteinaceous machine consisting of more than 20 components. Because the successful translocation of bacterial effectors necessitates a functioning multipartite machine, the production of structural and secreted T3S system components has been assumed to be hierarchical. Recent analyses have confirmed the hierarchical nature of T3S in a few animal and plant pathogens [2–7]. Characterization of this hierarchy has revealed multiple substrate classes. Early substrates are involved in pilus formation while late substrates, like effectors, are secreted after the assembly of a complete T3S system.

An array of factors has been implicated in regulating T3S system hierarchy [8]. Predominately featured are two protein groups 1) YscU/FlhB proteins and 2) YscP/FliK-like proteins. YscU/FlhB proteins include YscU, a T3S protein from *Yersinia* spp. and FlhB, a flagellar protein from *Salmonella* spp., which represent the most characterized regulators of T3S hierarchy [2–5, 9]. YscU/FlhB proteins exhibit four N-terminal transmembrane domains that play a structural role in the inner membrane export apparatus of the T3S system basal body [10, 11]. T3S is completely abolished in *yscU/flhB* null mutants [5, 12, 13]. The C-termini of YscU/FlhB proteins, however, encode a characteristic cytoplasmic domain involved in regulating T3S system hierarchy [3, 5, 9, 12, 14]. This domain is required for conformational changes via autoproteolytic cleavage at
an Asp-Pro-Thr-His (NPTH) motif [4, 9, 15]. The NPTH motif is conserved in all YscU/FlhB homologs and point mutations in the NPTH motif are frequently associated with phenotypes including 1) avirulence, 2) loss of protein-protein interactions and 3) loss of secretion hierarchy [4, 13]. Due to the location of YscU/FlhB proteins at the basal body-cytoplasm interface and due to their role in regulating T3S hierarchy, YscU/FlhB proteins display numerous protein-protein interactions [3, 5, 16, 17]. For example, HrcU from X. campestris has been demonstrated to interact with at least seven other T3S proteins [16, 18–20]. Among these HrcU-interacting proteins are YscP/FliK-like proteins. YscP/FliK-like proteins differ from the YscU/FlhB protein in that they share little amino acid sequence conservation. They are hydrophobic, globular and contain a Pro-X-Leu-Gly C-terminal motif [21]. Mutations affecting YscP/FliK-like proteins frequently compromise the ability of T3S systems to change substrate specificity during hierarchical T3S and consequently are termed T3S substrate specificity switches (T3S4) [8]. T3S4 mutant phenotypes include 1) reduced secretion of late substrates, 2) increased filament length, and sometimes 3) increased secretion of early substrates [6, 16, 22–25]. YscP from Yersinia spp., FliK from flagella, and HpaC from Xanthomonas campestris all represent T3S4 proteins. Both FliK and HpaC have been demonstrated to directly bind the cytoplasmic domains of their cognate YscU/FlhB proteins, and phenotypes associated with NPTH domain mutations are attributed to loss of protein-protein interaction with T3S4 proteins [3, 4, 9, 16, 25].

The Gram-negative plant pathogenic bacterium Erwinia amylovora is the causative agent of fire blight, a disease of rosaceous species including apple and pear. Disease development by E. amylovora requires a functioning T3S system [26]. In E. amylovora, the T3S system is known to secrete at least 12 proteins including the harpins HrpN and HrpW as well as the effector DspA/E (hereafter termed DspE), a pathogenicity factor [27–29]. To date, little is known about how secretion hierarchy is regulated in E. amylovora. While HrpN is required for secretion of translocators HrpN and HrpW, nothing is known about how E. amylovora regulates the substrate specificity of DspE, the most important component of the T3S system for fire blight disease development [29]. In E. amylovora, YscU/FlhB and T3S4 proteins are represented by HrcU (EAM_2905) and HrpP (EAM_2900), respectively. Here, HrcU and HrpP are explored for roles in T3S system regulation in E. amylovora.

Results

HrcU exhibits a conserved NPTH motif required for pathogenicity in E. amylovora

The NPTH motif in YscU/FlhB proteins is the site of autoproteolytic cleavage and conformational change required for protein function [4, 15]. This NPTH motif is conserved in all known YscU/FlhB proteins [3, 5, 30]. Bioinformatic analysis of HrcU from E. amylovora using a dense alignment surface algorithm predicted that, like YscU/FlhB homologs, HrcU encodes four transmembrane domains as well as a cytoplasmic C-terminal tail (Fig. 1a) [10, 31]. Using T-Coffee multiple alignment software, the amino acid sequence of HrcU was compared to multiple homologs in T3S systems of plant and animal bacterial pathogens as well as in the flagellum (Table 1) [32]. The E. amylovora HrcU NPTH motif (HrcUNPTH) was found to be conserved in E. amylovora and in all analyzed homologs (Fig. 1b).

To determine the role of HrcU in disease development, a chromosomal deletion of hrcU was created in E. amylovora Ea1189. Ea1189ΔhrcU was confirmed to be...
nonpathogenic due to a lack of symptom development 6 days post inoculation (dpi) in an immature pear infection model (Fig. 2a). In trans expression of hrcU via the plasmid pRRM1 was able to successfully complement the mutant strain restoring full virulence to Ea1189ΔhrcU (Fig. 2a).

To ascertain the importance of HrcU in E. amylovora, HrcU was subjected to site-directed mutagenesis. The asparagine residue of the NPTH motif is required for YscU/FlhB protein function in assayed homologs [4, 5, 17]. Consequently, the conserved asparagine residue located at position 266 in the amino acid sequence of HrcU was mutated to encode a codon corresponding to alanine. This hrcU mutant allele (HrcU_{N266A}) was cloned into an expression vector creating pRRM2 [33].

To determine the role of HrcU_{N266A} in host-microbe interactions, Ea1189ΔhrcU/pRRM1 and Ea1189ΔhrcU/pRRM2 were inoculated into immature pear fruits. While plasmid-borne hrcU was able to re-establish wild type (WT) virulence levels to Ea1189ΔhrcU, Ea1189ΔhrcU/pRRM2 was unable to restore pathogenicity 6 dpi in immature pear fruits (Fig. 2a). This indicates that HrcU_{NPTH} is required for HrcU function and that HrcU_{NPTH} is necessary to mediate compatible host interactions.

**HrcU_{NPTH} is required for the elicitation of the hypersensitive response**

The hypersensitive response (HR) is a hallmark of incompatible plant-microbe interactions. The HR is characterized by rapid, localized programmed cell-death in response to pathogen-associated proteins frequently represented by T3S system substrates. HR elicitation in E. amylovora requires a functional T3S system [34]. E. amylovora Ea1189 strains were inoculated into Nicotiana benthamiana mesophyll tissue and, 16 h post inoculation (hi), results revealed that E. amylovora Ea1189 requires HrcU, and specifically HrcU_{NPTH}, for HR development (Fig. 2b). While WT Ea1189 and complemented Ea1189ΔhrcU/pRRM1 induced robust HR symptoms in N. benthamiana, Ea1189ΔhrcU and Ea1189ΔhrcU expressing HrcU_{N266A} failed to trigger an incompatible defense response (Fig. 2b). As the HR in response to E. amylovora infection requires T3S, these results suggest that the inability of HrcU_{N266A} to complement Ea1189ΔhrcU is due to the disrupted function of HrcU_{NPTH} in mediating T3S.

**HrpP is required for pathogenicity and hypersensitive response induction**

In YscU/FlhB proteins, the NPTH motif is required for the regulation of T3S hierarchy [3–5, 12]. T3S system hierarchy regulation is mediated via direct and indirect interactions with T3S4 proteins [3, 4, 16, 25]. In E. amylovora, HrpP (EAM_2900) is a predicted T3S4 protein. Bioinformatic analyses of the HrpP amino acid sequence are in accordance with previous observations that T3S4 proteins are poorly conserved.
between species and that, in bacterial plant pathogens, T3S4 proteins are N-terminally truncated relative to homologs in animal pathogenic bacteria and the flagellum (Fig 3a) [8]. Beginning at amino acid position 98 though, HrpP does exhibit a modified Pro-X-Leu-Gly motif that is characteristic of T3S4 with alanine replacing leucine at position 100 (Pro-Glu-Ala-Gly) (Fig 3b) [35].

To establish the role of HrpP in mediating plant-microbe interactions, a chromosomal deletion of HrpP was synthesized, and relevant strains were inoculated into host and non-host plant species. Like Ea1189ΔhrcU strains expressing HrcU_N266A, Ea1189ΔhrpP was non-pathogenic 6 dpi in immature pear fruit and unable to elicit a HR in N. benthamiana (Fig 3c).

HrcU and HrpP interact in E. amylovora
While not all T3S4 proteins have been observed to interact directly with YscU/FlhB counterparts, direct interactions have been recorded between the T3S4 proteins FliK and HpaC [3, 16]. To explore the possibility of HrpP interactions with HrcU in E. amylovora, hrpP and hrcU constructs were cloned into Y2H vectors and assayed in Saccharomyces cerevisiae AH109 via survival on minimal medium and α-galactosidase activity. In the Y2H assay, hrcU alleles featuring the HrcU_N266A point mutation were included along with N-terminal hrcU deletions (HrcU-CT). HrcU-CT constructs were included due to reported transmembrane domain interference with protein-protein interactivity in homologous YscU/FlhB proteins [3, 16]. Alongside HrpP, the T3S protein HrpJ was also screened for the ability to interact with HrcU in yeast as HrpJ is a demonstrated regulator of T3S system hierarchy in E. amylovora and homologs are required for late substrate secretion due to their roles as T3S inner rod proteins [29].

Another example of this occurs in Pseudomonas syringae where HrpJ functions within the bacterial cell to control secretion of translocator proteins such as the harpins HrpZ1 and HrpW1 [36]. In all cases, full-length HrcU encoding N-terminal transmembrane domains were unable to interact with either HrpP or HrpJ (Fig 4). HrpJ exhibited a very weak interaction with both HrcU-CT and HrcU-CT_N266A (Fig 4). Conversely, HrpP interacted strongly with HrcU-CT in Y2H experiments (Fig 4). While the HrcU_NPTH motif was not absolutely required for interactions with HrpP, HrcU-CT_N266A displayed less α-galactosidase activity in the presence of HrpP than did HrcU-CT (Fig 4). This indicates that HrpP does interact with HrcU and that HrcU_NPTH-mediated conformational changes in HrcU affect HrpP binding in Y2H assays. All qualitative Y2H results were assessed quantitatively using image analysis software ImageJ and shown to be statistically significant.

HrcU_NPTH and HrpP are required for the secretion of DspE
The T3S system effector DspE is a pathogenicity factor of E. amylovora and the translocation of DspE is required for fire blight disease development [27, 28, 37, 38]. Mutations affecting the T3S system that result in a loss-of-pathogenicity phenotype are consequently hypothesized to be attributed to decreased DspE translocation by E. amylovora. To determine if HrcU_NPTH and HrpP are involved in regulating DspE secretion, E. amylovora strains were transformed with pLRT201 to express a DspE-CyaA fusion protein and incubated in vitro in hrp-inducing minimal medium (HrpMM) used to mimic conditions of the plant apoplast [39]. Proteins were extracted 48 hpi and subjected to one-dimensional SDS-PAGE separation and western blot analysis using an anti-CyaA antibody. As predicted, an Ea1189 strain harboring native hrcU secreted...
DspE in vitro while Ea1189ΔhrclU failed to secrete any DspE protein (Fig 5a). Likewise, an Ea1189 strain synthesizing HrcUn266A and Ea1189ΔhrpP were also unable to secrete DspE (Fig 5a). Using SDS-PAGE analysis, we also show that Ea1189ΔhrclU complemented with the full-length hrcU on pRRM1 secreted the native DspE protein, while Ea1189ΔhrclU complemented with hrcUn266A on pRRM2 was unable to secrete DspE (Fig 5b). Finally, the wild-type Ea1189 strain containing pRRM2 could still secrete DspE, indicating that HrcUn266A does not exhibit a dominant-negative effect on HrcU (Fig 5b). These results show that HrcU NPTH and HrpP are required for DspE secretion in vitro and suggest that Ea1189ΔhrpP and Ea1189ΔhrclU/pRRM2 are nonpathogenic due to loss of DspE secretion and translocation capability.

Discussion

In this study the roles of HrcU and HrpP in regulating the T3S system in E. amylovora Ea1189 were explored. Using site-directed mutagenesis, phenotypic analyses, Y2H assays and protein visualization, HrcU and HrpP were shown to interact and mediate host-microbe interactions via the regulation of T3S system substrates like the effector DspE.

HrcU and HrpP were both confirmed to be pathogenicity factors in E. amylovora Ea1189. Ea1189ΔhrclU and Ea1189ΔhrpP were both unable to cause disease in immature pear fruits (Fig. 2a and 3c). Likewise, Ea1189ΔhrclU and Ea1189ΔhrpP were also unable to elicit a HR after inoculation into N. benthamiana (Fig 2b and 3c). These results are in agreement with previous observations regarding HrcU in P. syringae and X. campestris [5, 40]. Interestingly, while HrpP in E. amylovora and P. syringae are both required for disease and HR induction, the T3S4 homolog HpaC is not a pathogenicity factor in X. campestris [41, 42].

The important influence of HrcU and HrpP in facilitating disease development is hypothesized to stem from roles in regulating T3S hierarchy. In YscU/FlhB proteins, the regulation of T3S hierarchy hinges on a conserved NPTH amino acid motif [4, 5, 9, 13]. The cytoplasmic C-terminus of HrcU in E. amylovora encodes an NPTH motif (Fig. 1b). Notably, a site-directed mutation of hrcU resulting in the construct HrcUn266A was unable to complement Ea1189ΔhrclU suggesting that the role of HrcU in mediating plant-microbe interactions requires the presence of an asparagine residue at position 266 (Fig. 2). Ea1189ΔhrclU strains expressing HrcUn266A were nonpathogenic and here we report via western blot analysis that HrcU-mediated secretion of DspE was dependent on the integrity of its conserved NPTH motif (Fig. 5). While full-length hrclU was able to complement Ea1189ΔhrclU in trans and restore DspE secretion, hrclUn266A failed to rescue Ea1189ΔhrclU
mutant phenotypes (Fig. 2 and 5). These phenotypes are likely linked as DspE secretion is required for disease development [38]. Collectively, results illustrating the roles of HrcU and HrcU_{N266A} in _E. amylovora_ reinforce data highlighting the importance of the NPTH motif in YscU/FlhB proteins as synonymous mutations in _X. campestris_, enteropathogenic _E. coli_ and _Y. enterocolitica_ also abolish disease development [5, 43, 44].

Notably though, while the NPTH motif is required for T3S-dependent disease development, YscU/FlhB mediated regulation of T3S differs between bacterial species. In _Salmonella_, the flagellar protein FlhB functions to establish hook assembly prior to filament secretion. Consequently, FlhB_{N269A} mutants fail to terminate hook protein secretion and initiate export of flagellin [43]. YscU in turn regulates the secretion of late substrates including translocators and effectors [4, 45].

One of the lesser-described proteins in the YscU/FlhB family is EscU from _Escherichia coli_ EPEC strain E2348/69. EscU is particularly relevant to discussions of _E. amylovora_. Thomassin et al. [46] observed that EscU_{N262A} poorly secretes effectors while differentially regulating the secretion of effector chaperones. While chaperone EspC was secreted at wild-type levels, EspA, EspB and Tir were poorly secreted in vitro. The authors also revealed that Tir-induced actin polymerization was comparably reduced in infected HeLa cells. _E. amylovora_ also utilizes a large consortium of chaperones to regulate effector secretion. Special attention should be given to how chaperones interact with HrcU to regulate secretion hierarchy [38, 47].

HrcU from _X. campestris_ pv. _vesicatoria_ represents the most characterized YscU/FlhB protein in plant pathogenic bacteria. Like YscU, HrcU_{Xcv} inhibits the secretion of late substrates. HrcU_{Xcv} NPTH mutants in turn over-secrete early T3S substrates analogous to increased hook secretion exhibited by FlhB flagellar mutants [5, 9]. Research concerning HrcU_{Xcv} has developed to reveal that while the NPTH motif in YscU/FlhB proteins has been the focus of much attention, additional HrcU domains and amino acid residues play a role in regulating T3S. While this research is the first demonstration of HrcU_{Ea} controlling substrate specificity in _hrp1-T3S_ systems via the NPTH motif, more analyses are required to understand the full scope of HrcU-mediated T3S regulation.

Examinations of the T3S4 protein HrpP in _E. amylovora_ revealed that, while exhibiting a c-terminal P-X-L-G motif, HrpP is diminutive like T3S4 proteins in other plant pathogenic bacteria. Conversely, mammalian bacterial pathogens exhibit T3S4 proteins up 3X in length. While structurally distinct, all T3S4 proteins share some commonalities. Here we present for the first time that HrpP is a pathogenicity factor in _E. amylovora_. Ea1189ΔhrpP was unable to generate disease development on immature pear fruit or induce a HR in _N. benthamiana_ (Fig. 3c). Like HrcU_{N266A}, Ea1189ΔhrpP was also unable to secrete the T3S effector DspE as displayed using an in vitro western blot (Fig. 5).

Other T3S4 proteins, similarly to HrpP in _E. amylovora_, function to promote the secretion of late T3S substrates. Null mutations in YscP from _Y. enterocolitica_, HpaC from _X. campestris_ and HrpP from _P. syringae_ all fail to secrete late T3S substrates [16, 23, 41, 42]. More
notably though, many T3S4 proteins also function to actively suppress the secretion of early T3S substrates and null mutations result in increased secretion of pilus subunits and inner rode proteins. FliK from the flagellum suppresses the secretion of the inner rode-like hook protein FlhB [13, 48]. Mutations in YscP trigger hyper-secretion of the pilin protein YscF and the inner rod protein YscI while HpaC mutants secrete more inner rode protein HrpB2 than wild-type [16, 23, 45].

Conversely, HrpP from *P. syringae pv. tomato* appears to function atypically relative to other know T3S4 proteins. While previously-described T3S4 proteins actively suppress early T3S events, *PstΔhrpP* poorly secretes the early substrate HrpA, a T3S pilus subunit [42]. Consequently, HrpP of *Pst* may be more accurately described as a post-translational activator of T3S as opposed to a T3S4 protein. More experimentation will be required to determine if HrpP of *Ea* also functions as a post-translational activator though it is important to note that HrpP has not been observed to interact with HrcU in *Pst*, while evidence suggests that HrpP binds HrcU as has been reported for other canonical T3S4 proteins [25,49–52].

Using Y2H analysis to explore protein-protein interactions, we demonstrate that HrpP and the cytoplasmic tail of HrcU bind when co-expressed in Saccharomyces cerevisiae (Fig. 4). These results conform to previous observations in other plant and animal pathogenic bacteria. For example, in Salmonella, FlhB binds directly to FliK [50] and in Xanthomonella, HrcU binds HpcC [5, 9]. Notably, YscU from Yersinia has never been shown to interact with YscP indicating potential variability in T3S hierarchy regulation [17]. Despite some variability, to date, all known YscU/FlhB domains are required for T3S function and disease development.

In *E. amylovora*, virulence and DspE secretion assays are consistent with previous observations concerning the functional importance of the HrcUNPTH domain (Fig. 2 and 5). Our Y2H results reveal however that while HrcUNPTH affects HrpP, the domain is not required for binding (Fig. 4). Confirmation of observed HrcU-HrpP interaction data in yeast will require future use of more sensitive and specific techniques such as co-immunoprecipitation assays. In addition, work by Hauner and Buttn [9] also indicates that HrcU exhibits multiple amino acid residues, in addition to the NPTH domain, with functional significance for plant disease outcomes and, in response, a more through mutational analysis will be required to understand the role of HrcU in T3S regulation and interactions with HrpP. Considering that both HrcU and HrpP are pathogenicity factors in *E. amylovora* and as both *Ea1189ΔhrcU* and *Ea1189ΔhrpP* exhibit impaired DspE secretion, it is tempting to speculate that interactions between HrcU and HrpP may be important for their relative roles in pathogenicity.

**Conclusions**

Here we report the first information regarding the roles of HrcU and HrpP in regulating T3S of DspE in *E. amylovora*. Both proteins were shown to be required for pathogenesis in *E. amylovora* *Ea1189*, were required for DspE secretion, and show evidence of inter-protein interactions. Future work should focus on how HrcU and HrpP regulate the secretion of the inner rod protein HrpJ and the needle protein HrpA as well as how regulator copy number influences HrpA secretion. In addition, as effector chaperones are known to be regulated by YscU/FlhB proteins in animal pathogenic bacteria, identifying a role for HrcU in regulating plant pathogenic effector chaperones would be a novel contribution to the plant pathology community.

**Methods**

**Bacterial strains and growth conditions**

Table 2 lists bacterial strains and plasmids used in this study. Unless otherwise referenced, bacterial strains were grown in Luria Bertani (LB) broth supplemented with 50 μg ml⁻¹ ampicillin, 20 μg ml⁻¹ chloramphenicol, 12 μg ml⁻¹ oxytetracycline or 30 μg ml⁻¹ kanamycin where appropriate. All strains were cultured at 28 °C in a shaking incubator.

**DNA manipulation and cloning**

Restriction enzyme digestion, T4 DNA ligation, and PCR amplification of genes were carried out using standard molecular techniques [53]. DNA extraction, PCR purification, plasmid extraction, and isolation of DNA fragments from agarose were performed with related kits (Qiagen, Valencia, CA). The sequences of oligonucleotide primers used in this study are listed in Additional file 1: Table S1. All DNA was sequenced at the Research Technology Support Facility at Michigan State University. Double digestion and directional ligation into pBBR1MCS3 [54] with PCR-generated gene sequences was utilized for mutant strain complementation. Final constructs were transformed into competent *Ea1189* by electroporation and screened on LB agar plates amended with oxytetracycline.

**Bioinformatics**

Lasergene® 7.2.0 software suite was used to manage nucleic and amino acid sequences (DNASTAR, Madison, WI). Genes were annotated in agreement with the *E. amylovora* ATCC 49946 genome [55]. Protein sequence conservation was determined using BLAST programs at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [56]. The sequences of
T3S4 domain-containing proteins were acquired from NCBI, with the accession numbers: ACI16082.1 (Yersinia enterocolitica YscP), CDH77977.1 (Pseudomonas aeruginosa PscP), WP_012228919.1 (Y. pestis FliK), GAO95686.1 (P. syringae HrpP), WP_020830096.1 (Ralstonia solanacearum YscP), and WP_004155345.1 (E. amylovora HrpP). The T3S4 domains were identified using the T3S4 AA sequences described from a previous work [21]. Putative transmembrane domains were predicted using the DAS-Transmembrane Prediction server (http://www.sbc.su.se/~miklos/DAS) [31]. T-Coffee multiple sequence alignment software (http://www.tcoffee.org/homepage.html) was used to create all amino acid sequence alignments [32]. Multiple sequence alignments were visualized using Weblogo 2.8.2 (http://weblogo.berkeley.edu) [57].

### Table 2: Bacterial strains and plasmids and their relevant characteristics

| Strains & Plasmids | Relevant characteristics | Source or reference |
|--------------------|--------------------------|---------------------|
| **Escherichia coli** strain | | |
| DH5α | F- 80lacZ, ΔM15, Δ(lacZYA-argF)U169, endA1, recA1, hsdR17(rK-mK+), deoR, thi-1, supE44, gyrA96, relA1 λ- | Invitrogen, CA, USA |
| Yeast strain | | |
| Saccharomyces cerevisiae AH109 | MATα, trp1-901, leu2-3, 112, his3-200, gal4Δ, gal80Δ, LYS2 :: GAL1 UAS-GAL1 TATA, -His3, GAL2 UAS-GAL2 TATA, ADE2, URA3 :: MEL1 UAS-MEL1 TATA, lacZ | | [61] |
| Envinia amylovora strains | | |
| Ea1189 | Wild type | [62] |
| Ea1189ΔhrcU | hrcU deletion mutant, CmR | This study |
| Ea1189ΔhrpP | hrpP deletion mutant, CmR | This study |
| **Plasmids** | | |
| pBBR1-MCS3 | TcR, broad host-range cloning vector | [54] |
| pGADT7 | LEU2, AmpR, Y2H activation vector | Clontech, CA, USA |
| pGBK7 | TRP1, KmR, Y2H bait vector | Clontech, CA, USA |
| pLRT201 | AmpR, pMJJ20 expressing DspE(1-737)-CyaA | [38] |
| pMJJ20 | AmpR, pWSQ29 containing codons 2 to 406 of CyaA | [63] |
| pRRM1 | TcR, pBBR1-MCS3 containing hrcU | This study |
| pRRM2 | TcR, pBBR1-MCS3 containing hrcU N266A | This study |
| pRRM3 | AmpR, pGADT7 containing hrpP | This study |
| pRRM4 | AmpR, pGADT7 containing hrpP | This study |
| pRRM5 | KmR, pGBK7 containing hrcU | This study |
| pRRM6 | KmR, pGBK7 containing hrcU N266A | This study |
| pRRM7 | KmR, pGBK7 containing hrcU 209-360 | This study |
| pRRM8 | KmR, pGBK7 containing hrcU 209-360, N266A | This study |
| pRRM9 | TcR, pAlter-Ex1 containing hrcU | This study |
| pRRM10 | AmpR, pAlter-Ex1 containing hrcU N266A | This study |
| pAlter-Ex1 | TcR, mutagenesis vector | Promega, WI, USA |
| pKD3 | AmpR, CmR mutagenesis cassette template | [59] |
| pKD46 | AmpR, expresses λ red recombinase | [59] |

*CmR, TcR, AmpR, KmR indicates resistance to chloramphenicol, oxytetracycline, ampicillin and kanamycin*

**Virulence and hypersensitive response assays**

The virulence of *E. amylovora* Ea1189 was assayed using a standard immature pear fruit assay as described previously [58]. In brief, bacterial strains were cultured overnight, washed, and resuspended in 0.5x phosphate buffered saline (PBS) to 1 x 10⁸ to 1 x 10⁹ CFU/ml. Immature pear fruits (*Pyrus communis* L. cv. Bartlett) were surface sterilized with 10% bleach, dried in laminar flow hood, and pricked with a needle prior to application of 2 μl bacterial suspension. Inoculated pears were incubated at 28 °C in humidified chambers. Symptoms were recorded 6 days post inoculation. The experiments were repeated three times with six replications per experiment. To study elicitation of the HR during
 incompatible interactions, *E. amylovora* strains were cultured overnight in LB broth. Bacterial cells were collected via centrifugation and washed twice with 0.5X PBS. Cells were resuspended and adjusted to a final concentration of $1 \times 10^7$ CFU ml$^{-1}$ in 0.5X PBS. 100 µl of cell suspension were in turn infiltrated into 9-week-old *N.* benthamiana leaves using a syringe and HR was observed 16 hpi.

**Mutagenesis**

*E. amylovora* site-directed nonpolar chromosomal mutants were generated using the phage $\lambda$ Red recombinase system previously described [59]. Briefly, *E. amylovora* strain Ea1189 harboring pKD46, encoding recombinases red $\beta$, $\gamma$, and exo, was cultured overnight at 28 °C in a shaking incubator. Strains were reinoculated with 0.1 % L-arabinose in LB broth and cultured for four hours to exponential phase. Cells were made electrocompetent and stored at -80 °C. Homologous recombination fragments encoding acetyltransferase cassettes were generated via polymerase chain reaction (PCR) using the plasmid pKD3 as a template. A PCR purification kit (Qiagen; Valencia, CA) was used to purify recombination fragments before electroformation into competent Ea1189. LB agar amended with chloramphenicol was used to screen putative mutants and single-gene recombinatorial deletion was confirmed using PCR and functional complementation.

Site-directed point-mutations were introduced into HrcU using and as described by the Altered Sites II in vitro Mutagenesis System (Promega; Madison, WI). Briefly, full-length HrcU (NC_013971) was cloned into pAlter-Ex1 via Ncol and NsiI restriction enzyme sites creating pRRM9. Mutagenic oligonucleotide HrcU_N266A (5’-GACCTGCTGCTGGTCGCTCCCA CGCACTATGCG-3’) was designed to convert the asparagine amino acid at position 266 to an alanine residue. pRRM9 and HrcU_N266A primer were denatured and phosphorylated respectively and via PCR pAlter-Ex1 (hrcU_N266A) (pRRM10) was synthesized and transformed into competent *E. coli* cells.

**Yeast two-hybridization**

The bait vector pGBK7T and the prey vector pGADT7 were used for yeast expression and Y2H screening (Clontech, Mountain View, CA, USA). A Frozen-EZ Yeast Transformation II Kit was used to create competent *Saccharomyces cerevisiae* AH109 and for cotransformation of bait and prey (Zymo Research Corporation, Orange, CA, USA). Transformants were selected on minimal SD agar amended with -Ad/His-Leu/-Trp dropout supplement and Mel1 α-galactosidase activity was detected using topically applied X-α-Gal at 4 ug ul$^{-1}$ (Clontech, Mountain View, CA, USA). The intensity of blue color was quantified using ImageJ (http://imagej.nih.gov/ij/download.html). Kendall rank correlation coefficient $r$ tests were performed to determine the statistical significance. Equations are listed below:

**Secretion assays**

Strains were cultured overnight in 50 ml LB broth at 28 °C. Cells were washed twice with 0.5X PBS, and resuspended in 50 ml minimal medium, pH 5.7 [60]. Strains were induced for 48 h with shaking, collected by centrifugation, and the supernatant was filtered using 0.22 µm vacuum filtration (Millipore, Billerica, MA, USA). Filtrate was supplemented with 0.5 mM phenylmethylsulfonyl fluoride and concentrated to approximately 500 µl using 10-kDa Amicon centrifugal filter units (Millipore, Billerica, MA, USA). For ease of detection of secreted DspE protein, we used plasmid pLRT201 which is an expression construct that encodes the first 737 amino acids of DspE fused to an adenylate cyclase (CyaA) reporter [38]. We have previously demonstrated secretion of this DspE1-737-CyaA fusion protein via the T3S system [38]. DspE secretion was examined in the WT *E. amylovora* Ea1189/pLRT201, Ea1189ΔhrcU/pLRT201, Ea1189ΔhrcUΔhrpP/pLRT201 and Ea1189ΔhrcU/pRRM2/pLRT201.

For western blot analysis, proteins were analyzed using anti-CyaA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For protein visualization, proteins were additionally purified to remove biofilm polysaccharides as previously described [29]. Briefly, protein samples were extracted twice with 0.5 volume of water-saturated phenol and precipitated with by the addition of 5 volumes 100 mM ammonium acetate in methanol. After overnight incubated at −20 °C, protein were extracted via centrifugation, resuspended in 50 ul water and reprecipitated in 500 µl of cold acetone. Samples were again incubated overnight at −20 °C and protein pellets were collected by centrifugation at 13,000 g at 4 °C for 30 min and subsequent resuspension in 50 µl 5 % acetic acid supplement with 0.5 mM PMSF. A bicinchoninic acid (BCA) protein assay kit was used to measure protein concentrations and concentrations were:

$t = \frac{n_1-n_4}{\sqrt{2(n+n-1)}}$

$n$ represents sample size

$1/2n(n-1)$ $n_1$ is the number of concordant pairs

$z = \frac{3n+x}{\sqrt{n(n-1)}}$ $n_4$ is the number of discordant pairs

$\sqrt{2(n+n+5)}$
adjusted to 1 μg μl⁻¹. Eight μg of each protein sample were used for western blot analysis.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
All data in support of our findings is contained within this manuscript or included as supplemental figures.

Additional file

Additional file 1: Table S1. Description of data: Sequences of oligonucleotide primers used in this study. (DOCX 109 kb)

Abbreviations
HRS: Hypersensitive response; Hrc: Hypersensitive response and conserved; Hrp: Hypersensitive response and pathogenicity; NPTH: Asparagine-proline-threonine-histidine domain found in YscU/FlhB proteins; T3S: Type III secretion; T3S4: Type III secretion substrate specificity switch.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
RM and GS designed the experiments. RM and QZ conducted the experiments. RM, QZ and GS analyzed the data. RM wrote the manuscript and all authors read, edited and approved the final manuscript.

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