The C-Terminal Half of the HrpN Virulence Protein of the Fire Blight Pathogen *Erwinia amylovora* Is Essential for Its Secretion and for Its Virulence and Avirulence Activities

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The HrpN (harpin) protein of the fire blight pathogen *Erwinia amylovora* is an essential virulence factor secreted via the bacterial type III secretion system. HrpN also has avirulence activity when delivered to tobacco by *E. amylovora* and has defense elicitor activity when applied to plants as a cell-free protein extract. Here, we characterize a series of random mutations in *hrpN* that altered the predicted amino acid sequence of the protein. Amino acid substitutions and deletions in the highly conserved, C-terminal portion of HrpN disrupted the virulence and avirulence activities of the protein. Several of these mutations produced a dominant-negative effect on *E. amylovora* avirulence on tobacco. None of the mutations clearly separated the virulence and avirulence activities of HrpN. Some C-terminal mutations abolished secretion of HrpN by *E. amylovora*. The results indicate that the C-terminal half of HrpN is essential for its secretion by *E. amylovora*, for its virulence activity on apple and pear, and for its avirulence activity on tobacco. In contrast, the C-terminal half of HrpN was not required for cell-free elicitor activity. This suggests that the N-terminal and C-terminal halves of HrpN mediate cell-free elicitor activity and avirulence activity, respectively.

Additional keywords: DspA/E, HrpW, HrpJ, hypersensitive response.

Fire blight is a serious, centuries-old disease of apple and pear caused by the bacterium *Erwinia amylovora* (Arthur 1885; Burrill 1880; Denning 1794). Infection is typically first observed in flowers and tender shoots (van der Zwet and Keil 1979) and, once inside the plant, the bacteria move systemically and may infect all parts of a tree (Bogs et al. 1998). Blighted trees have brown to black, shriveled leaves, twigs, and fruit; may exhibit bacterial ooze; and often develop cankers on branches and the trunk (van der Zwet and Keil 1979). Control measures for fire blight are limited, and include antibiotic sprays at flowering, pruning of blighted branches, and the

use of fire-blight-tolerant rootstocks and cultivars (Sobiczewski et al. 1997).

Pathogenesis by *E. amylovora* is complex and involves a number of components (Oh and Beer 2005). One indispensable contributor to the virulence of *E. amylovora* is the bacterial type III secretion system (T3SS) (Kim et al. 1997). The T3SS is a protein secretion and translocation system that delivers bacterial proteins to host cells and is employed by many plant and animal pathogens, including species of *Ralstonia*, *Xanthomonas*, *Erwinia*, *Yersinia*, *Shigella*, and *Salmonella* and enteropathogenic strains of *Escherichia coli* (Journet et al. 2005). In *Erwinia amylovora*, several proteins secreted via the T3SS are known to contribute to the pathogen's virulence or avirulence activities, including DspA/E (Bogdanove et al. 1998; Gaudriault et al. 1997), AvrRpt2_{EA} (Zhao et al. 2006), HrpJ (Nissinen et al. 2007), and HrpN (Bogdanove et al. 1996; Wei et al. 1992).

HrpN of *E. amylovora* is the founder protein of the “harpin” class of proteins secreted by the T3SS, which elicit cell death and defense responses when infiltrated into the extracellular spaces of plants (He et al. 1994; Wei et al. 1992). The ability of externally applied harpins to elicit plant defense responses sets these proteins apart from other proteins secreted by the T3SS, most of which seem to function following introduction to the host cell cytoplasm (Mudgett 2005). In infected apple tissues, HrpN has been detected inside of, secreted from, and associated with *E. amylovora* cells, but not inside cells of infected apple seedlings, based on in situ immunolocalization experiments (Perino et al. 1999). On the other hand, a recent study using a HrpN-cyaA fusion demonstrated that small amounts of HrpN may be translocated into the cells of tobacco leaves infiltrated with *E. amylovora* (Bocsanczy et al. 2008).

The plant defense responses triggered by externally applied HrpN have been studied extensively. When leaves of *Arabidopsis* plants are infiltrated with HrpN, microscopic cell death develops and systemic acquired resistance (SAR) is induced (Peng et al. 2003). The stimulation of SAR by HrpN requires *NDRI* and *EDSI*, suggesting that HrpN triggers SAR via resistance (*R*)-gene-mediated signaling pathways. HrpN induces SAR in *Arabidopsis* through the *NPRI/NIM1*-dependent, salicylic acid (SA) signaling pathways (Dong et al. 1999). Cell physiological studies indicate that HrpN elicits Ca⁺⁺ influx (Blume et al. 2000; Pike et al. 1998), K⁺ efflux (El-Maarouf et al. 2001; Popham et al. 1995), and an oxidative burst (Chandra and Low 1997; Desikan et al. 1996) in nonhost plant cells.

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These patterns are similar to those observed during the hypersensitive response (HR) (Atkinson et al. 1990). In tobacco cells, cell death elicitation by HrpN involves interference with normal mitochondrial function and ATP synthesis (Xie and Chen 2000).

Crop protection products and protocols have been developed based on the ability of HrpN to elicit plant defenses by direct application. Postharvest treatment of apple fruit with HrpN protein results in improved control of blue mold (de Capdeville et al. 2003), and postharvest application of HrpN to Hami melon fruit induces local and systemic resistance to *Trichothecium roseum* (Yang et al. 2005). This occurs by induction of defenses in the fruit, not by contact of HrpN with the pathogen directly (de Capdeville et al. 2002; Yang et al. 2005). Externally applied HrpN also induces insect resistance and enhances plant growth through an ethylene-dependent mechanism (Dong et al. 2004), and induces drought tolerance in *Arabidopsis* through an abscisic acid (ABA)-dependent signaling mechanism (Dong et al. 2005).

In contrast with its role in plant defense, less is known about the virulence activity of HrpN. The HrpN homolog from *E. chrysanthemi* is essential for full virulence on witloof chicory, and the protein may be important during lesion initiation (Bauer et al. 1995). North American *E. amylovora* “fruit tree” and “raspberry” strains can be distinguished by amino acid differences in their HrpN N-terminal regions, although the possible relevance of these variations to host specificity is unclear (Jock and Geider 2004). In *E. amylovora*, DspA/E and HrpN act together to trigger oxidative stress responses in pear, and this appears to be related to the disease process (Venisse et al. 2003). In addition, it has been shown that *E. amylovora* HrpN plays a critical role in translocation of DspA/E to the interior of plant cells (Bocsanczy et al. 2008).

In this study, we performed random, in vitro, polymerase chain reaction (PCR)-based mutagenesis of the *hrpN* gene and used an in trans complementation system to assess the effects of the mutations on HrpN protein expression, secretion, virulence on pear and apple fruit, avirulence activity on tobacco, and cell-free elicitor activity when infiltrated into tobacco leaves. The results demonstrate that the C-terminal half of HrpN is essential for all of the tested activities of HrpN except for cell-free elicitor activity.

RESULTS

Generation of a series of random mutations in *hrpN*.

Alterations to the 1.2-kb coding sequence of *hrpN* were generated using mutagenic PCR. Thirty-four different mutated *hrpN* genes were identified by sequencing 120 candidate clones. Although the conditions of the mutagenic PCR favored the creation of single-point mutations, some of the products had multiple-point mutations. In all, 13 clones were selected for further characterization; these clones represent a fairly uniform distribution of lesions across the *hrpN* coding sequence and they were predicted to encode 13 distinct mutant forms of HrpN. The DNA lesions and codon alterations for each mutant *hrpN* gene examined in this study are listed (Table 1). They included eight single-amino-acid substitutions (*hrpN*^{G25R}, *hrpN*^{G108D}, *hrpN*^{N111D}, *hrpN*^{L218P}, *hrpN*^{L257S}, *hrpN*^{G266S}, *hrpN*^{A385T}, and *hrpN*^{D390G}), two double-amino-acid substitutions (*hrpN*^{S11A,M63T} and *hrpN*^{S347G,M395T}), two clones with premature stop codons (*hrpN*^{Q189*} and *hrpN*^{Q255*}), and one amino acid substitution combined with a premature stop codon (*hrpN*^{S99P,Q159*}). Three additional unique mutant genes (*hrpN*^{S347G}, *hrpN*^{M395T}, and *hrpN*^{G266S,S347G,M395T}) that separated or combined some of the randomly generated mutations were subsequently created via site-directed mutagenesis.

Mutations in the *hrpN* gene affected virulence functions of *E. amylovora*.

To complement the *hrpN* deletion mutant of *E. amylovora* strain Ea273 (*Ea273ΔhrpN*), we generated vector p1575A, which contains the *hrpN* gene under control of its native promoter. An engineered *AvrII* restriction site near the 5' end and a native *AflIII* site at the 3' end of the *hrpN* gene in p1575A allowed swapping of mutated *hrpN* genes into the complementation vector. p1575A encodes wild-type HrpN protein. *Ea273ΔhrpN*(p1575A) exhibited virulence comparable with Ea273 in immature pear fruit and apple seedlings (Fig. 1) (data not shown). Each of the 16 mutated *hrpN* genes described above was swapped with the wild-type *hrpN* gene in p1575A and introduced into *Ea273ΔhrpN*.

Control strains and *Ea273ΔhrpN* strains carrying each of the 16 mutant *hrpN* genes were tested for virulence activity by inoculating immature apple and pear fruit and monitoring development of necrosis and bacterial ooze. Ea273 induced severe symptoms on pear fruit at 4 days postinoculation (dpi) and on apple fruit at 14 dpi (Fig. 1). In contrast, *Ea273ΔhrpN* did not trigger disease symptoms on apple or pear fruit. The virulence of *Ea273ΔhrpN* was restored in these assays when it carried p1575A (*Ea273ΔhrpN*(p1575A)). *Ea273ΔhrpN* carrying the empty vector pBluescript II KS+ (*Ea273ΔhrpN*(pBluescript II KS+)) was not virulent.

Of the 16 mutated *hrpN* genes, 10 complemented *Ea273ΔhrpN* for virulence: *hrpN*^{S11A,M63T}, *hrpN*^{G25R}, *hrpN*^{G108D}, *hrpN*^{N111D}, *hrpN*^{L218P}, *hrpN*^{G266S}, *hrpN*^{S347G}, *hrpN*^{A385T}, *hrpN*^{D390G}, and *hrpN*^{M395T} (Fig. 1). A mutated *hrpN* gene encoding a double-amino-acid substitution (*hrpN*^{S347G,M395T}) did not complement the necrogenic activity of *Ea273ΔhrpN*, although bacterial ooze was produced at the inoculation sites on pear. Five mutated *hrpN* genes completely failed to restore virulence activity to *Ea273ΔhrpN* (Fig. 1). These included a single-amino-acid substitution (*hrpN*^{L257S}), a triple-amino-acid substitution (*hrpN*^{G266S,S347G,M395T}), and three predicted C-terminal deletions of HrpN, (*hrpN*^{S99P,Q159*}, *hrpN*^{Q189*}, and *hrpN*^{Q255*}).

Mutations in the *hrpN* gene affected *hrpN*-dependent avirulence activity of *E. amylovora*.

The *hrpN* gene contributes to *E. amylovora* avirulence on tobacco, a nonhost plant. Therefore, we determined whether the mutations in *hrpN* affected *E. amylovora* avirulence activity. Tobacco leaves were infiltrated with bacterial suspensions of 5×10^6 CFU ml⁻¹ and observations of macroscopic tissue collapse typical of the HR were made 24 h postinoculation (hpi). At this relatively low inoculum level, Ea273 induced an HR

Table 1. Molecular nature of the lesions in each mutant *hrpN* gene examined in this study^a

Mutant <i>hrpN</i> gene	Codon changes
<i>hrpN</i> ^{G25R}	ggg > agg
<i>hrpN</i> ^{G108D}	ggt > gat
<i>hrpN</i> ^{N111D}	aac > gac
<i>hrpN</i> ^{L218P}	ctt > cct
<i>hrpN</i> ^{L257S}	tta > tca
<i>hrpN</i> ^{G266S}	ggt > agt
<i>hrpN</i> ^{S347G}	agt > ggt
<i>hrpN</i> ^{A385T}	gcc > acc
<i>hrpN</i> ^{D390G}	gat > ggt
<i>hrpN</i> ^{M395T}	atg > acg
<i>hrpN</i> ^{S11A,M63T}	tca > gca, atg > acg
<i>hrpN</i> ^{S347G,M395T}	agt > ggt, atg > acg
<i>hrpN</i> ^{G266S,S347G,M395T}	ggt > agt, agt > ggt, atg > acg
<i>hrpN</i> ^{Q189*}	cag > tag
<i>hrpN</i> ^{Q255*}	cag > tag
<i>hrpN</i> ^{S99P,Q159*}	tcg > ccg, cag > tag

^a Amino acids indicated in single-letter code; asterisk indicates stop codon.

while Ea273 Δ *hrpN* did not (Fig. 1). In addition, leaf panels infiltrated with Ea273 Δ *hrpN*(p1575A) developed an HR at 24 hpi while those infiltrated with Ea273 Δ *hrpN*(pBluescript II KS+) did not, indicating that the wild-type *hrpN* gene in p1575A complemented Ea273 Δ *hrpN* for avirulence on tobacco (Fig. 1). Therefore, assaying the HR at this inoculum density allowed assessment of the contribution of mutant *hrpN* genes to *E. amylovora* avirulence on tobacco.

Mutated *hrpN* genes that fully restored *hrpN*-dependent avirulence to Ea273 Δ *hrpN* included *hrpN*^{S11A,M63T}, *hrpN*^{G25R}, *hrpN*^{G108D}, *hrpN*^{N111D}, and *hrpN*^{L218P} (Fig. 1). Mutant *hrpN* genes that partially restored *hrpN*-dependent avirulence to Ea273 Δ *hrpN* included *hrpN*^{G266S}, *hrpN*^{S347G}, *hrpN*^{A385T}, *hrpN*^{D390G}, *hrpN*^{M395T}, and *hrpN*^{S347G,M395T}. Ea273 Δ *hrpN* strains carrying these mutant *hrpN* genes were scored as partially complemented for *hrpN*-dependent avirulence because they consistently caused patchy collapse of <50% of the infiltrated area. In contrast, inoculations with Ea273 and complemented

Ea273 Δ *hrpN* strains caused the collapse of the entire infiltrated area. Finally, five mutant *hrpN* genes failed to complement Ea273 Δ *hrpN*, including *hrpN*^{L257S}, *hrpN*^{G266S,S347G,M395T}, *hrpN*^{S99P,Q159*}, *hrpN*^{Q189*}, and *hrpN*^{Q255*}.

Five mutated *hrpN* genes blocked the *hrpN*-independent HR.

HrpN is one of several proteins that contribute to *E. amylovora* avirulence on tobacco; *hrpN* mutant strains can induce an HR on tobacco when inoculum concentrations are sufficiently high. When infiltrated into tobacco at 1×10^8 CFU ml⁻¹, Ea273, Ea273 Δ *hrpN*, and Ea273 Δ *hrpN*(p1575A) all caused an HR (Fig. 2A). With this inoculum density, the specific contribution of *hrpN* to the HR was not discernable; thus, the HR caused by Ea273 Δ *hrpN* at 1×10^8 CFU ml⁻¹ represented *hrpN*-independent *E. amylovora* avirulence on tobacco. The T3SS-

Fig. 1. Tests for complementation of Ea273 Δ *hrpN* virulence and avirulence activity by mutant *hrpN* genes. Disease symptoms on immature pear and apple fruit as well as the hypersensitive response (HR) on tobacco are shown for each *Erwinia amylovora* strain. For the HR assay, tobacco leaf panels were infiltrated with 5×10^6 CFU ml⁻¹ of *E. amylovora* strains. The positive and negative controls shown in the first five columns are mock-inoculated (CK), wild-type *E. amylovora* strain Ea273 (WT), *hrpN* deletion strain Ea273 Δ *hrpN* (Δ *hrpN*), control empty vector strain Ea273 Δ *hrpN* (pBluescript II KS+) (EV), and Ea273 Δ *hrpN* complemented with p1575A, the plasmid bearing the wild-type *hrpN* gene (HrpN). The remaining columns show Ea273 Δ *hrpN* strains carrying the various mutated *hrpN* genes swapped into p1575A. Column headings identify the predicted amino acid changes encoded by each mutant *hrpN* gene.

Fig. 2. Dominant negative effect of certain mutated *hrpN* genes on the hypersensitive response (HR) on tobacco. **A**, Five mutant *hrpN* genes suppressed the *hrpN*-independent HR on tobacco. Tobacco leaf panels were infiltrated with 1×10^8 CFU ml⁻¹ of the following strains: *hrpN* deletion strain Ea273 Δ *hrpN* (Δ); wild-type Ea273 (WT); type III secretion system mutant Ea273-K178 (K178); Ea273 Δ *hrpN* carrying p1575A, which encodes wild-type HrpN (Δ (HrpN)); and Ea273 Δ *hrpN* carrying mutated *hrpN* genes swapped into p1575A (labeled with the predicted amino acid changes in HrpN in parenthesis). **B**, Avirulence activity of wild-type Ea273 is not affected by carrying plasmids encoding mutated forms of *hrpN*. Tobacco leaf panels were infiltrated with 1×10^7 CFU ml⁻¹ of the following: wild-type Ea273 (WT); wild-type Ea273 carrying the empty vector pBluescript II KS+ (WT(EV)); wild-type Ea273 carrying p1575A (WT(HrpN)); and wild-type Ea273 carrying mutated *hrpN* genes swapped into p1575A (these strains are labeled with predicted amino acid changes in HrpN in parenthesis). **C**, Avirulence activity of wild-type Ea273 is not affected by co-infiltration with two Ea273 Δ *hrpN* strains carrying plasmids encoding mutated forms of HrpN. Tobacco leaf panels were either infiltrated with control single strains or co-infiltrated with Ea273 (WT) plus each of four different mutant strains. Strain designations are the same as in panel A. Final inoculum density of each strain was 1×10^8 CFU ml⁻¹.

defective strain Ea273-K178 failed to cause an HR at 1×10^8 CFU ml⁻¹ (Fig. 2A), indicating that the *hrpN*-independent HR induced by Ea273Δ*hrpN* required the T3SS and was most likely caused by proteins secreted by the T3SS.

Ea273Δ*hrpN* strains carrying 11 selected mutant *hrpN* genes were tested for *hrpN*-independent, HR-inducing activity. The genes were selected because they partially or completely failed to restore *hrpN*-dependent avirulence to Ea273Δ*hrpN* on tobacco (Fig. 1). Of the 11 strains, 6 caused complete tissue collapse; thus, the mutant *hrpN* genes that they carried had no effect on the *hrpN*-independent HR. These included *hrpN*^{G266S}, *hrpN*^{S347G}, *hrpN*^{A385T}, *hrpN*^{D390G}, *hrpN*^{M395T}, and *hrpN*^{S347G,M395T} (Fig. 2A). These were the same six mutant *hrpN* genes that partially restored *hrpN*-dependent avirulence to Ea273Δ*hrpN* (Fig. 1).

In contrast, when infiltrated at 1×10^8 CFU ml⁻¹, five mutant *hrpN* genes produced mild chlorosis but virtually no collapse of the infiltrated area (Fig. 2A). These were the five mutant *hrpN* genes that failed to restore *hrpN*-dependent avirulence to Ea273Δ*hrpN*: *hrpN*^{L257S}, *hrpN*^{G266S,S347G,M395T}, *hrpN*^{S99P,Q159*}, *hrpN*^{Q189*}, and *hrpN*^{Q255*}. Thus, these five mutant *hrpN* genes blocked the *hrpN*-independent HR caused by Ea273Δ*hrpN*.

We were interested in whether these five *hrpN* mutant genes could interfere with the avirulence activity of wild-type Ea273. Therefore, tobacco leaves were infiltrated with Ea273 strains carrying the five plasmid-borne *hrpN* mutations. Whether these bacterial strains were delivered at 1×10^8 CFU ml⁻¹ (data not shown) or at a lower inoculum density of 1×10^7 CFU ml⁻¹, no blocking of the HR was apparent (Fig. 2B). Similarly, when wild-type Ea273 was coinoculated with Ea273Δ*hrpN* strains carrying two of these mutant *hrpN* genes, there was no observable reduction in the HR (Fig. 2C).

Expression and secretion of HrpN mutant proteins in *E. amylovora*.

The inability of some of the mutated *hrpN* genes to complement the virulence and avirulence activities of Ea273Δ*hrpN* might be based on instability of the encoded protein, lack of secretion of the protein, or, specifically, loss of HrpN virulence or avirulence functions. To explore the first two possibilities, we used Western blot analyses to determine whether the mutant HrpN proteins were expressed in and secreted by Ea273Δ*hrpN*. Ea273Δ*hrpN* strains carrying *hrpN*^{L257S}, *hrpN*^{S99P,Q159*}, *hrpN*^{Q189*}, *hrpN*^{Q255*}, *hrpN*^{G266S,S347G,M395T}, *hrpN*^{G266S}, and

Fig. 3. Expression and secretion of HrpN proteins in *Erwinia amylovora* cells. *E. amylovora* strains were grown in *hrp*-inducing medium at 18°C for 26 h and the cell pellets and supernatants were collected separately. HrpN protein was detected by Western blotting using an HrpN polyclonal antibody. Upper blot: HrpN proteins in the cell pellets. Lower blot: HrpN proteins secreted into the supernatant. For both panels, lanes were loaded from left to right as follows: control wild-type strain Ea273 (WT); type III secretion system mutant Ea273-K178; *hrpN* deletion strain Ea273Δ*hrpN*; Ea273Δ*hrpN* complemented with p1575A, the plasmid bearing the wild-type *hrpN* gene (HrpN); remaining lanes, Ea273Δ*hrpN* carrying mutant *hrpN* genes swapped into p1575A. Labels above lanes indicate the predicted mutant HrpN proteins encoded. Migration points of molecular weight size markers are indicated.

hrpN^{S347G,M395T} as well as control strains were grown in *hrp*-inducing liquid medium, and the proteins from cell lysates and culture supernatants were analyzed separately by probing blots with an HrpN polyclonal antibody (Wei et al. 1992).

As expected, a 44-kDa band corresponding to wild-type HrpN was detected in cell lysates from Ea273, Ea273-K178, and Ea273Δ*hrpN*(p1575A) (Fig. 3). HrpN proteins of the expected sizes were also detected in cell lysates of Ea273Δ*hrpN* strains carrying plasmids encoding six of the seven mutated HrpN proteins: HrpN^{L257S}, HrpN^{Q189*} (18 kDa), HrpN^{Q255*} (25 kDa), HrpN^{G266S,S347G,M395T}, HrpN^{G266S}, and HrpN^{S347G,M395T} (Fig. 3). However, no protein corresponding to HrpN^{S99P,Q159*} (15 kDa) was detected in cell lysates of Ea273Δ*hrpN* carrying *hrpN*^{S99P,Q159*}, indicating that HrpN^{S99P,Q159*} was not produced or was not stable in Ea273Δ*hrpN* or was not detected by the antibody.

We next examined proteins in the culture supernatants to determine whether expressed HrpN mutant proteins were secreted from Ea273Δ*hrpN* (Fig. 3). As expected, HrpN protein was secreted into the culture medium by Ea273 and Ea273Δ*hrpN* (p1575A) but not by the T3SS mutant strain Ea273-K178 or by Ea273Δ*hrpN*. Bands corresponding to mutant proteins HrpN^{Q255*}, HrpN^{G266S,S347G,M395T}, HrpN^{G266S}, and HrpN^{S347G,M395T} were detected in the culture supernatants, indicating that they were secreted from Ea273Δ*hrpN* cells. However, HrpN^{L257S} and HrpN^{Q189*} were not detected in culture supernatants (Fig. 3); thus, these mutant proteins were produced in Ea273Δ*hrpN* cells but they were not secreted. HrpN^{S99P,Q159*} was not observed in the supernatant, which was expected because the protein was not detected in the cell lysates of Ea273Δ*hrpN* carrying *hrpN*^{S99P,Q159*}.

Mutant *hrpN* genes that blocked the *hrpN*-independent HR did not affect secretion of DspA/E, HrpW, or HrpJ.

In addition to HrpN, Ea273 secretes several other proteins in a T3SS-dependent manner. One possible explanation for the block of the *hrpN*-independent HR might be a general disruption of T3SS function or interference with the secretion of particular proteins by the T3SS. Therefore, we tested the effect

of selected mutated *hrpN* genes on the secretion of HrpW (Gaudriault et al. 1998; Kim and Beer 1998), HrpJ, and DspA/E from Ea273Δ*hrpN*.

E. amylovora strains were grown under *hrp*-inducing conditions, and protein blots of the supernatant fractions were probed with polyclonal antibodies raised against HrpJ, HrpW, and DspA/E (Fig. 4). As expected, HrpJ, HrpW, and DspA/E were detected in the supernatant fraction from Ea273 and not from the T3SS mutant Ea273-K178. The *hrpN* deletion strain Ea273Δ*hrpN* secreted all three proteins. Ea273Δ*hrpN* strains carrying plasmids encoding wild-type HrpN or mutant proteins HrpN^{L257S}, HrpN^{S99P,Q159*}, HrpN^{Q189*}, HrpN^{Q255*}, and HrpN^{G266S,S347G,M395T} secreted HrpJ, HrpW, and DspA/E into the supernatants. Thus, the mutated *hrpN* genes that disrupted the *hrpN*-independent HR did not block the secretion of HrpJ, HrpW, or DspA/E in culture.

Cell-free elicitor activity of mutant HrpN proteins.

Harpin proteins are characterized by the ability to induce collapse reminiscent of the HR in nonhost leaves when infiltrated as cell-free elicitor preparations (CFEP) (Wei et al. 1992). Therefore, we investigated whether mutated *hrpN* genes that blocked the HR in tobacco encoded proteins with altered cell-free elicitor activity. Mutant genes *hrpN*^{G266S,S347G,M395T}, *hrpN*^{L257S}, *hrpN*^{Q255*}, *hrpN*^{Q189*}, and *hrpN*^{S99P,Q159*} were cloned into the T7 expression vector p1084A and introduced into *Escherichia coli*. The empty vector pBluescript II KS+ served as a negative control.

Strains were induced for F protein expression and CFEP were prepared and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5A). Induced protein bands of the expected size of 44 kDa were present in CFEP of *E. coli* carrying *hrpN*, *hrpN*^{G266S,S347G,M395T}, and *hrpN*^{L257S}, while bands of the predicted sizes of 25 and 18 kDa were present in CFEP of *E. coli* carrying *hrpN*^{Q255*} and *hrpN*^{Q189*}, respectively. However, the expected 15-kDa band was not observed in CFEP from the *E. coli* strain carrying *hrpN*^{S99P,Q159*}. Repair of the S99P mutation by site-directed mutagenesis (leaving only the Q159* mutation) did not enable

Fig. 4. Secretion of DspA/E, HrpJ, and HrpW by *Erwinia amylovora* strains. *E. amylovora* strains were grown in *hrp*-inducing medium and supernatants were collected and analyzed by Western blotting. DspA/E, HrpJ, and HrpW were detected using polyclonal antisera.

expression of this protein in *E. coli* (data not shown). However, it is possible that HrpN^{S99P,Q159*} and HrpN^{Q159*} may have formed inclusion bodies in *E. coli* and, therefore, may have been lost during CFEP production.

CFEP from *E. coli* strains carrying *hrpN*, *hrpN*^{G266S,S347G,M395T}, *hrpN*^{L257S}, *hrpN*^{Q255*}, *hrpN*^{Q189*}, *hrpN*^{S99P,Q159*}, and control empty vector pBluescript II KS+ were infiltrated into tobacco leaves and the responses were assessed 24 h later (Fig. 5B). CFEP from *E. coli* carrying pBluescript II KS+ had no elicitor activity, as expected. Similarly, CFEP from *E. coli* carrying *hrpN*^{S99P,Q159*} did not cause collapse. In contrast, tobacco leaf panels collapsed after infiltration with CFEP from *E. coli* strains carrying *hrpN*, *hrpN*^{G266S,S347G,M395T}, *hrpN*^{L257S}, *hrpN*^{Q255*}, and *hrpN*^{Q189*}. Thus, HrpN, HrpN^{G266S,S347G,M395T}, HrpN^{L257S}, HrpN^{Q255*}, and HrpN^{Q189*} had cell-free elicitor ac-

tivity. CFEP dilution series experiments revealed no differences in elicitor activity between HrpN and the mutant proteins (data not shown). It was not possible to determine whether HrpN^{S99P,Q159*} had cell-free elicitor activity because this protein was not observed in CFEP from *E. coli* carrying *hrpN*^{S99P,Q159*}.

DISCUSSION

This study represents a structure-function analysis of HrpN, a critically important virulence protein of *Erwinia amylovora*. The mutations that altered HrpN function are summarized in Figure 6. Our results indicate that the C-terminal half of HrpN plays a specific role in its secretion by the T3SS and in its virulence and avirulence activities. Among the secreted mutant HrpN proteins, there was a general correlation between loss of avirulence activity and loss of virulence activity; no mutations were found that abolished one without affecting the other. This suggests that the virulence and avirulence activities of HrpN may not be functionally separable.

In contrast, the cell-free elicitor activity of HrpN was separable from its avirulence activity: the N-terminal half of the protein was sufficient for cell-free elicitor activity while the C-terminal half was essential for avirulence activity. Our results are consistent with the findings of Shrestha and associates (2008), who reported that the N-terminal portion of *E. pyrifoliae* HrpN has elicitor activity whereas its C-terminal half does not. In our study, mutant HrpN proteins HrpN^{Q255*} and HrpN^{G266S,S347G,M395T} had cell-free elicitor activity (Fig. 5) but did not trigger an HR when delivered by *E. amylovora* (Figs. 1 and 2). It is possible that these mutant HrpN proteins were unstable when secreted by *E. amylovora* into the extracellular space. Alternatively, the quantities of HrpN^{Q255*} and HrpN^{G266S,S347G,M395T} secreted into the extracellular space by Ea273Δ*hrpN* may have been insufficient to trigger cell death. A third possibility is that tobacco plants recognize HrpN by different mechanisms when it is delivered by *E. amylovora* as opposed to being infiltrated as a cell-free elicitor protein.

The key role of the C-terminal half of HrpN in its biological activities is especially interesting because that region of the protein is highly conserved among various phytopathogenic bacterial species. An amino acid alignment of *E. amylovora* HrpN with the HrpN proteins from seven closely related bacterial species is shown, along with predicted amino acid changes in HrpN described in this study (Fig. 7). All the mutations that perturbed HrpN biological function caused alterations or deletions in the highly conserved C-terminal half of HrpN. Therefore, this study reveals a correlation between the structural conservation of the C-terminal half of HrpN, its secretion via the T3SS, and its virulence and avirulence activities.

It was surprising to discover that HrpN amino acid residues 189 to 255 play a role in its secretion by the T3SS. This finding runs contrary to several studies indicating that the C-terminal portion of T3SS substrates is dispensable for their secretion (Anderson and Schneewind 1997; Schesser et al. 1996; Sory et al. 1995). These studies used translational fusions of N-terminal substrate fragments with reporter proteins. T3SS substrate recognition appears to be mediated by the substrate N-terminal portion or the 5' end of the mRNA encoding the T3SS substrate (Galan and Wolf-Watz 2006). Our study does not rule out that the N-terminus of HrpN is involved in its secretion. Rather, our results positively indicate that portions of the C-terminal half of HrpN are required for its secretion by *E. amylovora*. The HrpN^{L257S} protein may be blocked in secretion due to disruption of the normal conformation of that region. Alterations and deletions in the HrpN C-terminus may result in a misfolded HrpN protein that cannot pass through the T3SS

Fig. 5. Cell-death-inducing activity of cell-free elicitor preparations (CFEPs) from *Escherichia coli* strains expressing wild-type and selected mutant forms of HrpN. **A**, A T7 RNA polymerase system was used to express proteins in DH5α(pGP1-2) carrying the following: empty vector pBluescript II KS+ (EV); p1084A, the plasmid bearing the wild-type *hrpN* gene (HrpN); and five mutant forms of *hrpN* swapped into p1084A (the CFEPs are identified by the predicted amino acid changes to HrpN). CFEP (30 µl) from each strain was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie blue staining. Asterisks (*) indicate the HrpN protein bands; chevron (<) at 15 kDa indicates expected location of HrpN^{S99P,Q159*}. **B**, Tobacco leaf at 24 h after infiltration with 40 µl ml⁻¹ of CFEP protein from each strain. CFEPs are labeled as in panel A.

or that aggregates into inclusion bodies inside the *E. amylovora* cell. Alternatively, the C-terminal half of HrpN could associate with a critical, unknown chaperone protein that mediates HrpN secretion via the T3SS. In fact, it was reported recently that HrpN secretion is severely reduced in a *hrpJ* mutant strain of Ea273 (Nissinen et al. 2007). Finally, it is possible that some mutant HrpN proteins are secreted through the T3SS but remain tightly associated with the *E. amylovora* cell surface and, therefore, do not move freely into the culture supernatant.

Five mutant *hrpN* genes had a dominant-negative effect on the *hrpN*-independent HR despite having variable effects on the HrpN protein (Figs. 2 and 3). HrpN^{Q255*} and HrpN^{G266S,S347G,M395T} were expressed and secreted; HrpN^{Q189*} and HrpN^{L257S} were expressed but not secreted; HrpN^{S99P,Q159*} was not detectable. A general block of the T3SS cannot explain this phenomenon because Ea273Δ*hrpN* strains carrying dominant-negative mutant *hrpN* genes secreted DspA/E, HrpW, and HrpJ (Fig. 4) and induced chlorosis of the infiltrated area (Fig. 2), whereas the T3SS mutant Ea273-K178 did neither. One possibility is that the mutant forms of HrpN act inside the *E. amylovora* cell to interfere with the secretion of one or more essential proteins that facilitate the HR; HrpN^{S99P,Q159*} may accomplish this despite being expressed at a very low level, or not being fully translated, or being unstable. Another possibility, at least for the secreted proteins HrpN^{Q255*} and HrpN^{G266S,S347G,M395T}, is that they interfere with the translocation of effectors into the plant cell. It has been shown that HrpN facilitates the translocation of DspA/E into host cells (Bocsanczy et al. 2008); therefore, it is conceivable that the dominant-negative effect is related to the translocation function of HrpN. Finally, it is possible that HrpN^{Q255*} and HrpN^{G266S,S347G,M395T} actively interfere with the plant defense signaling pathways to block the HR.

MATERIALS AND METHODS

Generation of mutations in the *hrpN* gene.

Standard molecular biological techniques were followed (Sambrook and Russell 2001). QIAprep and QIAquick kits (Qiagen, Valencia, CA, U.S.A.) were used for plasmid isolation and gel purification. DNA sequencing was conducted at The Pennsylvania State University Huck Institute Nucleic Acid Facility and at the Cornell University Biotechnology Program DNA Sequencing Facility. Nucleotide and amino acid alignments were compared using ClustalX (Thompson et al. 1997) and ClustalW2 (Larkin et al. 2007).

To facilitate cloning steps, an *AvrII* restriction site was created 20 bp downstream of the ATG in the *E. amylovora* *hrpN* gene in pCPP1084 (Wei et al. 1992), creating p1084A. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used according to the manufacturer's instructions, with forward primer *hrpN*-for (5'-GAGTCTGAATACAAGTGGCCTAGG-3'), reverse primer *hrpN*-Bam (5'-GGATCCTTAAGCCGCGCCAGCTTGCCCAAGTGCCAATT-3'), and p1084A as the PCR template. Underlined nucleotides indicate changes from the wild-type sequence. Creation of the *AvrII* site in p1084A did not alter the encoded protein. *EcoRI* and *BamHI* sites were added to flank the *hrpN* coding sequence using forward primer *hrpN*-*Eco* (5'-GAATTCATGAGTCTGAATACAAGTGGCCTAGG-3'), reverse primer *hrpN*-*Bam* (5'-GGATCCTTAAGCCGCGCCAGCTTGCCCAAGTGCCAATT-3'), and p1084A as the PCR template. Underlined nucleotides indicate differences from the wild-type sequence to maintain the engineered *AvrII* site; *EcoRI* and *BamHI* sites are indicated in bold italics. The PCR product was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, U.S.A.), amplified,

and then the *hrpN* coding sequence was excised with *EcoRI* and *BamHI* and ligated into similarly cut pGADT7 (Clontech, Mountain View, CA, U.S.A.). The resulting plasmid, pGAD-*hrpN*A, served as the template for generating mutations in the *hrpN* gene via low-fidelity PCR.

Primers for mutagenesis were *hrpN*-*Eco* and *hrpN*-*Bam*; 35- to 45-cycle PCR reactions were performed with 60°C annealing. PCR products were cloned into pCR2.1-TOPO, amplified, and sequenced to identify mutations in the *hrpN* coding sequence. Thirteen different randomly generated mutant

	1	403	Vir	HR	HR block	Exp	Sec	CFE
wild-type HrpN			+	+	-	+	+	+
S99P Q159*			-	-	+	-	-	-
Q189*			-	-	+	+	-	+
Q255*			-	-	+	+	+	+
L257S			-	-	+	+	-	+
M395T S347G G266S			-	-	+	+	+	+
M395T S347G			+/-	+/-	-	+	+	
G266S			+	+/-	-	+	+	
S347G			+	+/-	-			
A385T			+	+/-	-			
D390G			+	+/-	-			
M395T			+	+/-	-			

Fig. 6. Schematic summary of mutant HrpN proteins examined that had a functional defect. Plus sign (+) indicates that an activity was observed; minus sign (-) indicates that an activity was not observed; plus/minus sign (+/-) indicates that partial activity was observed. Activities described are: virulence activity on immature apple and pear fruit (Vir); HrpN-dependent avirulence on tobacco leaves (HR); blocking the HrpN-independent HR caused by an *hrpN* deletion mutant strain of *Erwinia amylovora* (HR block); protein expression in *E. amylovora* (Exp); secretion from *E. amylovora* cells into culture medium (Sec); cell-free elicitor activity on tobacco leaves (CFE); blank spaces = not tested.

hrpN genes were selected for study of biological function. Subsequently, three additional mutant *hrpN* genes (variations of 2 of the previously selected 13 clones) were produced via site-directed mutagenesis. Two single mutations, *hrpN*^{S347G} and *hrpN*^{M395T}, were introduced separately into the wild-type *hrpN* sequence of template p1084A using the following primers: forward primer 5'-GACGGAATGACACCAGCCGGTATGGAGCAG-3' and reverse primer 5'-CTGCTCCATACCGGCTGGTGTCATTCCGTC-3' for *hrpN*^{S347G}; and forward primer 5'-GATGCCATTAACAATACGGCACTTGGCAAGCTG-3' and reverse primer 5'-CAGCTTGCCAAGTGCCGTATTGTTAATGTCATC-3' for *hrpN*^{M395T}. The triple mutant *hrpN*^{G266S,S347G,M395T} was created by introducing the mutation *hrpN*^{G266S} into mutant gene *hrpN*^{S347G,M395T} using forward primer 5'-GTGGGTACCGGTATCAGTATGAAAGCGGGC-3' and reverse primer 5'-GC

CCGCTTTCATACATGATACCGGTACCCAC-3'. Changes from the wild-type *hrpN* sequence are underlined in each primer.

Bacterial strains and plasmids.

Wild-type Ea273 and mutant derivatives thereof were used in this study. Ea273 (ATCC 49946) is a strain from New York that is highly virulent on immature pear fruit and causes a strong HR on tobacco. Ea273-K178 is a T3SS-deficient *hrcN* mutant (Bocsanczy et al. 2008) that was used as a negative control in secretion tests and some virulence and avirulence assays. To examine effects of mutated *hrpN* genes in trans in Ea273, we created the *hrpN* deletion strain Ea273Δ*hrpN*. Ea273Δ*hrpN* has a deletion of 1,002 bp of the *hrpN* sequence, beginning 149 bases upstream of the start codon and extending through the first 853 bp of the coding sequence, which repre-

Fig. 7. Amino acid sequence alignment of HrpN proteins from eight different plant-pathogenic bacterial species. Sequences were aligned using ClustalW. *E. a.* = *Erwinia amylovora* (accession number Q01099); *E. p.* = *E. pyrifoliae* (accession number CAE25425); *P. a.* = *Pantoea agglomerans* pv. *gypsophila* (formerly *E. herbicola* pv. *gypsophila*; accession number AAF76342); *P. s.* = *Pantoea stewartii* subsp. *stewartii* (formerly *E. stewartii*; accession number AAG01466); *Pe. a.* = *Pectobacterium atrosepticum* (formerly *E. carotovora* subsp. *atroseptica*; accession number AAS20361); *Pe. b.* = *Pectobacterium beta-vascularum* (formerly *E. carotovora* subsp. *betavascularum*; accession number ABD22989); *Pe. ca.* = *Pectobacterium carotovorum* subsp. *carotovorum* (formerly *E. carotovora* subsp. *carotovora*; accession number AAQ73910); *Pe. ch.* = *Pectobacterium chrysanthemi* (formerly *E. chrysanthemi*; accession number AAC31978). The amino acid substitutions for the *E. amylovora* HrpN mutant proteins reported in this work are indicated above the alignment; mutations resulting in an early stop are indicated by asterisks (*). Residues that are identical in all eight of the wild-type proteins are shown as white letters on black background.

sents a null mutation of *hrpN*. Using the known sequence of the Hrp cluster of Ea321, primers were designed to amplify a 422-bp noncoding region upstream of *hrpN*, and a 456-bp region that included 356 bp of *hrpN* and 100 bp of noncoding sequence downstream of *hrpN*. Primers for the upstream region were (forward) 5'-GCTCTAGAGCGTCCCAGGCGCAT CAGTTAGTCA-3' and (reverse) 5'-GGAATTCCGCCGGGAT CAATCAGCCAGGTG-3'; primers for the downstream region were (forward) 5'-GGAATTCCCGTTCTTTCGTCAATAAAG GCG-3' and (reverse) 5'-CGGGATCCCGGGTAACCTTGAT GTATTGCGTGG-3'. The upstream reverse and downstream forward primers included *EcoRI* restriction sites, the upstream forward primer included an *XbaI* site, and the downstream reverse primer included a *BamHI* restriction site to facilitate later insertion into the suicide vector, pKNG101 (Kaniga et al. 1991).

The upstream and downstream fragments were double-digested with *EcoRI* and *XbaI*, and *EcoRI* and *BamHI*, respectively, and ligated into pBluescript KS+ cut with *EcoRI* and *BamHI*. The newly joined fragments were excised from the intermediate vector with *BamHI* and *XbaI* and were ligated into pKNG101. pKNG101 was purified from day-old cultures of *Escherichia coli* strain G534 using the Quantum Prep midiprep kit (BioRad, Hercules, CA, U.S.A.), concentrated using ethanol precipitation, and digested with *XbaI* and *BamHI*. The pKNG101 plasmid containing the *hrpN* region was transformed into chemically competent *E. coli* SM10λpir (G412) cells, and cells were screened for plasmid uptake by plating on streptomycin. The presence of the inserted fragment of the correct size was confirmed by PCR amplification with the outer primers used in creating the insert, as well as by midiprep and digestion with *BamHI* and *XbaI*.

E. coli containing the vector and the insert of the correct size were conjugated to rifampicin-resistant Ea273 using a protocol described by Bogdanove and colleagues (1996). Transconjugants of Ea273 were identified by plating cells on media containing rifampicin and streptomycin. Transconjugants were incubated in Luria-Bertani (LB) with rifampicin for 1 h to allow for a double crossover between the vector and genomic sequences. Recombinant Ea273 isolates were identified by rifampicin resistance, streptomycin susceptibility, and PCR amplification of a band of approximately 1 kb in length by outer primers as opposed to the approximately 2-kb length of the intact sequence. Recombinants had an appearance characteristic to *Erwinia amylovora* on MM2CU (minimal medium with copper) (Bereswill et al. 1998).

For in trans complementation studies with Ea273Δ*hrpN*, a vector that includes the *hrp* box upstream of the *hrpN* coding region was generated. A DNA fragment including the *E. amylovora* *hrpN* gene coding region and 175-bp upstream of the *hrpN* start codon was amplified by 30 PCR cycles at 60°C annealing with forward primer 5'-GCTCTAGACATTCACCT GGCTGATTGATC-3' with an *XbaI* site and reverse primer 5'-GCGGATCCAAGCTTAAGCCGCGCCAG-3' with a *BamHI* site (restriction sites indicated in bold italics), using Ea273 genomic DNA as the template. A 1,391-bp PCR product, including 4 bp after the stop codon, was ligated into the *XbaI* and *BamHI* sites of pBluescript II KS(+) to create pCPP1575.

The *hrpN* *HindIII* insert in p1084A, containing the engineered *AvrII* site in the *hrpN* coding sequence, was exchanged with the *hrpN* *HindIII* fragment in pCPP1575 to create p1575A. After confirming the integrity of p1575A by DNA sequencing, the previously identified *hrpN* mutant coding sequences were excised from pCR2.1-TOPO with *AvrII* and *AflIII* and swapped into *AvrII/AflIII*-cut p1575A. Sequencing of the cloned p1575A derivatives verified the presence of the expected *hrpN* gene mutations.

Bacteria were grown on LB or modified Lennox medium (Jensen et al. 2003) at 28 to 30°C for *E. amylovora* or LB at 37°C for *Escherichia coli*. The antibiotics kanamycin (50 μg ml⁻¹), rifampicin (10 μg ml⁻¹), and carbenicillin (100 μg ml⁻¹) were used as appropriate. Plasmids were introduced into bacteria by heat shock or electroporation.

Virulence assays.

Immature 'Gala' apple fruit were surface disinfested with 70% ethanol, halved, and placed cut-side down on wet filter paper in petri dishes. Apple halves were inoculated by pricking the skin of the fruit with a sterile tack dipped into confluent bacterial cultures growing on petri plates. Sterile water was used for mock inoculations. The fruit were maintained on wet filter paper in unsealed petri dishes in a growth chamber (22°C, 75% relative humidity [RH], 90 μmol m⁻² s⁻¹ light for 10 h day⁻¹) for up to 21 days. Four apple halves from different fruit were tested per strain, and the experiments were conducted at least twice. Virulence assays were also done in immature 'Bartlett' pear fruit as previously described (Oh et al. 2005). Briefly, 5 μl of bacterial suspension at 2 × 10⁸ CFU ml⁻¹ (optical density at 600 nm [OD₆₀₀] = 0.2) were dropped into holes in the pear fruits, and the fruit was kept on wet paper towels in a box sealed with plastic wrap and maintained at 25°C for 6 days in the dark. Experiments were repeated at least five times, using three pear halves per bacterial strain in each experiment. Fruit were photographed at 4 days (pear) or 14 days (apple) after inoculation.

Avirulence assays.

Tobacco plants (*Nicotiana tabacum* L. cv. Glurk) plants were grown at 21°C, 40% RH, with light at 100 μmol m⁻² s⁻¹ for 10 h day⁻¹. Approximately 6 weeks after planting, leaf panels were infiltrated as previously described (Wei et al. 1992). After infiltration, plants were maintained under their normal growth conditions and responses assessed 24 h later. HR photographs were taken at 72 h after infiltration. For *hrpN*-dependent HR tests, leaves were infiltrated with *E. amylovora* strains at 5 × 10⁶ CFU ml⁻¹. For tests of the *hrpN*-independent HR, inoculum density of 1 × 10⁸ CFU ml⁻¹ was used. Experiments were repeated at least four times, with six or eight plants being infiltrated with each strain for each experiment.

To test for dominant effects of *hrpN* mutations on the HR produced by wild-type *Erwinia amylovora*, selected p1575A-derived plasmids carrying *hrpN* mutations were electroporated into Ea273. Tobacco leaf panels were infiltrated with the resulting strains at 1 × 10⁷ CFU ml⁻¹ and 1 × 10⁸ CFU ml⁻¹ and the development of the HR was monitored as described above. In addition, mixed strain inoculations were performed in which wild-type Ea273 was mixed with Ea273Δ*hrpN* strains carrying selected mutant *hrpN* genes. For these experiments, the bacterial inoculum contained both strains at 1 × 10⁸ CFU ml⁻¹. Experiments were repeated twice, with four to six plants used per experiment.

Immunoblotting of bacterial proteins.

To detect proteins secreted from *E. amylovora*, bacterial strains were grown in 100 ml of *hrp*-inducing medium (Huynh et al. 1989) with mannitol as the carbon source and supplemented with antibiotics necessary to maintain the plasmids and without rifampicin. Cultures were grown for approximately 26 h at 18°C until the initial OD₆₀₀ of 0.2 reached 0.75. Preparation of proteins from the cell pellet and supernatant was carried out as described (Ham et al. 1998) with the following modifications. All steps were carried out at 4°C or on ice. Following initial centrifugation, the upper 70 ml of supernatant liquid was carefully removed to fresh tubes without disturbing

the cell pellet. The remaining culture solution was discarded and cell pellets were suspended in 10 ml of phosphate-buffered saline. The cell suspension (100 μ l) was mixed with 35 μ l of 4 $\times$ loading buffer (Laemmli 1970) and then frozen at -20°C . The reserved supernatant fraction was centrifuged for 40 min at $17,200 \times g$ and the upper 45 ml were transferred to fresh tubes. Then, 100 mM phenylmethanesulfonyl fluoride (PMSF) and 25% (wt/vol) trichloroacetic acid (TCA) were added to final concentrations of 0.5 mM and 9.5%, respectively, and proteins were precipitated at -20°C overnight. Samples were centrifuged at $17,200 \times g$ for 40 min and the resulting protein pellets were washed with acetone, dissolved in 100 μ l of 1 $\times$ loading buffer plus 3 μ l Tris-HCl (pH 8), and then frozen at -20°C . Ea273 and Ea273-K178 were used as positive and negative secretion controls, respectively.

For detection of HrpN, protein samples from the pellets (12.5 μ l) and supernatants (25 μ l) were loaded onto 12% SDS-PAGE gels; for detection of HrpJ, HrpW, and DspA/E, 25 μ l of supernatant preps were loaded on 7.5% gels. Precision Plus protein markers (BioRad) were included in each gel. Proteins were transferred to Hybond ECL Plus membranes (GE Healthcare, Piscataway, NJ, U.S.A.) using a Mini Trans-Blot Cell (BioRad). Polyclonal primary antibodies were used at the following dilutions: anti-HrpN, 1:10,000 (Wei et al. 1992); anti-DspA/E, 1:200 (Bogdonove et al. 1998); anti-HrpJ, 1:7,500 (Bocsanczy et al. 2008); and anti-HrpW, 1:7,500 (Bocsanczy et al. 2008). HRP-conjugated, donkey anti-rabbit secondary antibody (AbCam Inc, Cambridge, MA, U.S.A.) was used at 1:2,500 dilution. Bands were detected using the ECL Plus kit (GE Healthcare) and CL-Xposure film (Pierce Biotechnology, Rockford, IL, U.S.A.) according to the manufacturers' instructions.

Production and testing of cell-free elicitors.

HrpN proteins were overexpressed in *Escherichia coli* using a T7 RNA polymerase system (Tabor and Richardson 1985). DH5 α (pGP1-2) cells were transformed with empty vector pBluescript II KS(+), p1084A, or p1084A derivatives carrying mutant *hrpN* genes. LB broth (50 ml) containing appropriate antibiotics was inoculated with overnight bacterial cultures and grown at 30°C until the initial OD₆₀₀ of 0.2 reached 1.0. After heat-induction at 42°C for 20 min, cultures were further grown at 40°C for 1 h. Subsequent steps were conducted at 4°C or on ice. Cells were pelleted, suspended in 5 ml of buffer (5 mM potassium phosphate buffer [pH 6.5] and 0.5 mM PMSF), and lysed with a VirSonic100 sonicator (VirTis, Gardiner, NY, U.S.A.). After centrifuging at $12,000 \times g$ for 1 h, the recovered clarified supernatants were boiled for 10 min, centrifuged at $12,000 \times g$ for 20 min, and transferred to fresh tubes. Total protein in the CFEP was quantified using the Bradford assay with bovine serum albumin as the standard, and SDS-PAGE analysis was used to verify induced expression of protein products of the predicted size for each strain. CFEP (30 μ l) was loaded on each lane of 12 and 15% gels and visualized by Coomassie Blue staining. Tobacco leaf panels were infiltrated with 40 μ g of CFEP per milliliter for each bacterial strain.

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LITERATURE CITED

- Anderson, D. M., and Schneewind, O. 1997. A mRNA Signal for the Type III Secretion of Yop Proteins by *Yersinia enterocolitica*. *Science* 278:1140-1143.
- Arthur, J. C. 1885. Proof that bacteria are the direct cause of the disease in trees known as pear blight. *Am. Assoc. Adv. Sci. Proc.* 34:294-298.
- Atkinson, M. M., Keppler, L. D., Orlandi, E. W., Baker, C. J., and Mischke, C. F. 1990. Involvement of plasma membrane calcium influx in bacterial induction of the K^+/H^+ and hypersensitive responses in tobacco. *Plant Physiol.* 92:215-221.
- Bauer, D. W., Wei, Z. M., Beer, S. V., and Collmer, A. 1995. *Erwinia chrysanthemi* harpin_{Ech}: An elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. *Mol. Plant-Microbe Interact.* 8:484-491.
- Bereswill, S., Jock, S., Bellemann, P., and Geider, K. 1998. Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. *Plant Dis.* 82:158-164.
- Blume, B., Nurnberger, T., Nass, N., and Scheel, D. 2000. Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell* 12:1425-1440.
- Bocsanczy, A. M., Nissinen, R. M., Oh, C.-S., and Beer, S. V. 2008. HrpN of *Erwinia amylovora* functions in the translocation of DspA/E into plant cells. *Mol. Plant Pathol.* 9:425-434.
- Bogdanove, A. J., Wei, Z. M., Zhao, L. P., and Beer, S. V. 1996. *Erwinia amylovora* secretes harpin via a type III pathway and contains a homolog of *yopN* of *Yersinia* spp. *J. Bacteriol.* 178:1720-1730.
- Bogdanove, A. J., Kim, J. F., Wei, Z. M., Kolchinsky, P., Charkowski, A. O., Conlin, A. K., Collmer, A., and Beer, S. V. 1998. Homology and functional similarity of an *hrp*-linked pathogenicity locus, *dspEF*, of *Erwinia amylovora* and the avirulence locus *avrE* of *Pseudomonas syringae* pathovar tomato. *Proc. Natl. Acad. Sci. U.S.A.* 95:1325-1330.
- Bogs, J., Bruchmuller, I., Erbar, C., and Geider, K. 1998. Colonization of host plants by the fire blight pathogen *Erwinia amylovora* marked with genes for bioluminescence and fluorescence. *Phytopathology* 88:416-421.
- Burrill, T. J. 1880. Anthrax of fruit trees; or the so-called fire blight of pear, and twig blight of apple trees. *Am. Assoc. Adv. Sci. Proc.* 29:583-597.
- Chandra, S., and Low, P. S. 1997. Measurement of Ca^{2+} fluxes during elicitation of the oxidative burst in aequorin-transformed tobacco cells. *J. Biol. Chem.* 272:28274-28280.
- de Capdeville, G., Wilson, C. L., Beer, S. V., and Aist, J. R. 2002. Alternative disease control agents induce resistance to blue mold in harvested 'Red Delicious' apple fruit. *Phytopathology* 92:900-908.
- de Capdeville, G., Beer, S. V., Watkins, C. B., Wilson, C. L., Tedeschi, L. O., and Aist, J. R. 2003. Pre- and post-harvest harpin treatments of apples induce resistance to blue mold. *Plant Dis.* 87:39-44.
- Denning, W. 1794. On the decay of apple trees. *Trans. Soc. Promotion Agric. Arts Manufact. Inst. State N. Y.* 1:219-222.
- Desikan, R., Hancock, J. T., Coffey, M. J., and Neill, S. J. 1996. Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase-like enzyme. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 382:213-217.
- Dong, H. S., Delaney, T. P., Bauer, D. W., and Beer, S. V. 1999. Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *Plant J.* 20:207-215.
- Dong, H. P., Peng, J. L., Bao, Z. L., Meng, X. D., Bonasera, J. M., Chen, G. Y., Beer, S. V., and Dong, H. S. 2004. Downstream divergence of the ethylene signaling pathway for harpin-stimulated *Arabidopsis* growth and insect defense. *Plant Physiol.* 136:3628-3638.
- Dong, H. P., Yu, H. Q., Bao, Z. L., Guo, X. J., Peng, J. L., Yao, Z., Chen, G. Y., Qu, S. P., and Dong, H. S. 2005. The ABI2-dependent abscisic acid signalling controls HrpN-induced drought tolerance in *Arabidopsis*. *Planta* 221:313-327.
- El-Maarouf, H., Barny, M. A., Rona, J. P., and Bouteau, F. 2001. Harpin, a hypersensitive response elicitor from *Erwinia amylovora*, regulates ion channel activities in *Arabidopsis thaliana* suspension cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 497:82-84.
- Galan, J. E., and Wolf-Watz, H. 2006. Protein delivery into eukaryotic cells by type III secretion machines. *Nature* 444:567-573.
- Gaudriault, S., Malandrin, L., Paulin, J. P., and Barny, M. A. 1997. DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Mol. Microbiol.* 26:1057-1069.

- Gaudriault, S., Brisset, M. N., and Barny, M. A. 1998. HrpW of *Erwinia amylovora*, a new Hrp-secreted protein. FEBS (Fed. Eur. Biochem. Soc.) Lett. 428:224-228.
- Ham, J. H., Bauer, D. W., Fouts, D. E., and Collmer, A. 1998. A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. Proc. Natl. Acad. Sci. U.S.A. 95:10206-10211.
- He, S. Y., Bauer, D. W., Collmer, A., and Beer, S. V. 1994. Hypersensitive response elicited by *Erwinia amylovora* harpin requires active plant metabolism. Mol. Plant-Microbe Interact. 7:289-292.
- Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean—regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.
- Jensen, P. J., Rytter, J., Detwiler, E. A., Travis, J. W., and McNellis, T. W. 2003. Rootstock effects on gene expression patterns in apple tree scions. Plant Mol. Biol. 53:493-511.
- Jock, S., and Geider, K. 2004. Molecular differentiation of *Erwinia amylovora* strains from North America and of two Asian pear pathogens by analyses of PFGE patterns and *hrpN* genes. Environ. Microbiol. 6:480-490.
- Journet, L., Hughes, K. T., and Cornelis, G. R. 2005. Type III secretion: A secretory pathway serving both motility and virulence (review). Mol. Membrane Biol. 22:41-50.
- Kaniga, K., Delor, I., and Cornelis, G. R. 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: Inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene 109:137-141.
- Kim, J. F., and Beer, S. V. 1998. HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class. J. Bacteriol. 180:5203-5210.
- Kim, J. F., Wei, Z. M., and Beer, S. V. 1997. The *hrpA* and *hrpC* operons of *Erwinia amylovora* encode components of a type III pathway that secretes harpin. J. Bacteriol. 179:1690-1697.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature 227:680-685.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948.
- Mudgett, M. B. 2005. New insights to the function of phytopathogenic bacterial type III effectors in plants. Annu. Rev. Plant Biol. 56:509-531.
- Nissinen, R. M., Ytterberg, A. J., Bogdanove, A. J., Van Wijk, K. J., and Beer, S. V. 2007. Analyses of the secretomes of *Erwinia amylovora* and selected *hrp* mutants reveal novel type III secreted proteins and an effect of HrpJ on extracellular harpin levels. Mol. Plant Pathol. 8:55-67.
- Oh, C. S., and Beer, S. V. 2005. Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 253:185-192.
- Oh, C. S., Kim, J. F., and Beer, S. V. 2005. The Hrp pathogenicity island of *Erwinia amylovora* and identification of three novel genes required for systemic infection. Mol. Plant Pathol. 6:125-138.
- Peng, J. L., Dong, H. S., Dong, H. P., Delaney, T. P., Bonasera, J. M., and Beer, S. V. 2003. Harpin-elicited hypersensitive cell death and pathogen resistance require the *NDR1* and *EDS1* genes. Physiol. Mol. Plant Pathol. 62:317-326.
- Perino, C., Gaudriault, S., Vian, B., and Barny, M. A. 1999. Visualization of harpin secretion in planta during infection of apple seedlings by *Erwinia amylovora*. Cell. Microbiol. 1:131-141.
- Pike, S. M., Adam, A. L., Pu, X. A., Hoyos, M. E., Laby, R., Beer, S. V., and Novacky, A. 1998. Effects of *Erwinia amylovora* harpin on tobacco leaf cell membranes are related to leaf necrosis and electrolyte leakage and distinct from perturbations caused by inoculated *E. amylovora*. Physiol. Mol. Plant Pathol. 53:39-60.
- Popham, P. L., Pike, S. M., and Novacky, A. 1995. The effect of harpin from *Erwinia amylovora* on the plasmalemma of suspension-cultured tobacco cells. Physiol. Mol. Plant Pathol. 47:39-50.
- Sambrook, J., and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Schesser, K., Frithz-Lindsten, E., and Wolf-Watz, H. 1996. Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. J. Bacteriol. 178:7227-7233.
- Shrestha, R., Park, D. H., Cho, J. M., Cho, S., Wilson, C., Hwang, I., Hur, J. H., and Lim, C. K. 2008. Genetic organization of the *hrp* genes cluster in *Erwinia pyrifoliae* and characterization of HR active domains in HrpN(Ep) protein by mutational analysis. Mol. Cells 25:30-42.
- Sobiczewski, P., Deckers, T., and Pulawska, J. 1997. Fire Blight (*Erwinia amylovora*): Some Aspects of Epidemiology and Control. Research Institute of Pomology and Floriculture, Skierniewice, Poland.
- Sory, M., Boland, A., Lambermont, I., and Cornelis, G. R. 1995. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. Proc. Natl. Acad. Sci. U.S.A. 92:11998-12002.
- Tabor, S., and Richardson, C. C. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. U.S.A. 82:1074-1078.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876-4882.
- van der Zwet, T., and Keil, H. L. 1979. Fire blight: A bacterial disease of rosaceous plants. In: Agriculture Handbook 510. United States Department of Agriculture, Washington, D.C.
- Venisse, J. S., Barny, M. A., Paulin, J. P., and Brisset, M. N. 2003. Involvement of three pathogenicity factors of *Erwinia amylovora* in the oxidative stress associated with compatible interaction in pear. FEBS (Fed. Eur. Biochem. Soc.) Lett. 537:198-202.
- Wei, Z. M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88.
- Xie, Z. X., and Chen, Z. X. 2000. Harpin-induced hypersensitive cell death is associated with altered mitochondrial functions in tobacco cells. Mol. Plant-Microbe Interact. 13:183-190.
- Yang, B., Shiping, T., Jie, Z., and Yonghong, G. 2005. Harpin induces local and systemic resistance against *Trichothecium roseum* in harvested Hami melons. Postharvest Biol. Technol. 38:183-187.
- Zhao, Y. F., He, S. Y., and Sundin, G. W. 2006. The *Erwinia amylovora* *avrRpt2(EA)* gene contributes to virulence on pear and *AvrRpt2(EA)* is recognized by *Arabidopsis* RPS2 when expressed in *Pseudomonas syringae*. Mol. Plant-Microbe Interact. 19:644-654.