Biological Activity of Harpin Produced by *Pantoea stewartii* subsp. *stewartii*

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Pantoea stewartii subsp. *stewartii* causes Stewart's wilt of sweet corn. A hypersensitive response and pathogenicity (Hrp) secretion system is needed to produce water-soaking and wilting symptoms in corn and to cause a hypersensitive response (HR) in tobacco. Sequencing of the *hrp* cluster revealed a putative harpin gene, *hrpN*. The product of this gene was overexpressed in *Escherichia coli* and shown to elicit the HR in tobacco and systemic resistance in radishes. The protein was designated HrpN_{P_{st}}. Like other harpins, it was heat stable and protease sensitive, although it was three- to fourfold less active biologically than *Erwinia amylovora* harpin. We used antibodies to purified HrpN_{P_{st}} to verify that *hrpN* mutants could not produce harpin. This protein was secreted into the culture supernatant and was produced by strains of *P. stewartii* subsp. *indologenes*. In order to determine the importance of HrpN_{P_{st}} in pathogenesis on sweet corn, three *hrpN::Tn5* mutants were compared with the wild-type strain with 50% effective dose, disease severity, response time, and growth rate in planta as parameters. In all tests, HrpN_{P_{st}} was not required for infection, growth, or virulence in corn or endophytic growth in related grasses.

Additional keywords: *Erwinia stewartii*, type III secretion.

The ability of plant-pathogenic bacteria to elicit the hypersensitive response (HR) in resistant or nonhosts is correlated with their ability to cause disease in susceptible hosts. Both of these capabilities are determined by hypersensitive response and pathogenicity (*hrp*) genes (Lindgren 1997), which encode components of a protein secretion pathway, regulatory factors, and a number of secreted effector proteins (Alfano and Collmer 1997; He 1998; Mudgett and Staskawicz 1998). *hrp* genes are conserved in various *Erwinia*, *Pantoea*, *Pseudomonas*, *Xanthomonas*, and *Ralstonia* species that are pathogenic on plants. Sequence similarities between *hrp* genes and genes for type III secretion systems in mammalian pathogens revealed that they are closely related. In animal pathogens, type III secretion systems translocate effector proteins directly into host cells. By analogy, Hrp secretion systems may likewise inject pathogenicity and Avr proteins directly into plant cells.

Under certain conditions, phytopathogenic bacteria can secrete Avr and Hrp outer (Hops) proteins into culture supernatants (Gueneron et al. 2000; Rossier et al. 1999; Van Dijk et al. 1999), but there is only indirect evidence that these proteins are translocated into plant cells via the Hrp pathway (Mudgett and Staskawicz 1999).

In *Erwinia* and *Pantoea* spp., one or two genes in each *hrp* cluster encode a heat-stable, glycine-rich, extracellular protein that can elicit the HR when purified and applied externally to nonhost plant cells. These proteins have been termed harpins. The first harpin was isolated from *Erwinia amylovora* (Wei et al. 1992). A cell-free elicitor preparation (CFEP) obtained after boiling and centrifuging a cell sonicate gave a strong HR when infiltrated into tobacco. The activity of the CFEP was associated with a 44-kDa protein, which was encoded by the *hrpN* gene and called harpin_{Ea} (or HrpN_{Ea}). In strain Ea321, HrpN_{Ea} is needed for the elicitation of the HR on tobacco and for pathogenicity on apples and pears (Wei and Beer 1993; Wei et al. 1992), thus it was initially proposed that HrpN_{Ea} was the main pathogenicity factor of *E. amylovora*. Barny (1995), however, later found that *hrpN* mutants of strain CFPB1430 retained weak but significant pathogenicity and could still elicit a variable HR in tobacco. She attributed these results to different assay conditions and concluded that other necessary effector proteins such as DspE (Bogdanove et al. 1998a; Bogdanove et al. 1998b; Gaudriault et al. 1997) also must be secreted by the Hrp system.

Erwinia chrysanthemi, a wide host-range pathogen that rapidly kills and macerates host tissues, also produces a harpin. Although pectic enzyme production in wild-type bacteria masks the HR, $\Delta pelABCE$ Out⁻ mutants of *Erwinia chrysanthemi* elicit rapid necrosis in tobacco leaves. *hrpN_{Ech}* is required for HR elicitation in tobacco and contributes in a minor way to infectivity on witloof chicory (Bauer et al. 1994; Bauer et al. 1995). In *Erwinia carotovora* subsp. *carotovora*, *rmsA* mutants have been shown to produce a related harpin that is needed for HR elicitation, although its importance in pathogenicity has not been determined (Cui et al. 1996; Mukherjee et al. 1997). Most recently, sequence analysis of the *hrp* cluster of the gall-forming bacterium *Erwinia herbicola* (syn. *Pantoea agglomerans*) pv. *gypsophilae* also has revealed a *hrpN* gene that encodes an active harpin (Mor et al. 2001). Transposon mutagenesis of this gene did not affect pathogenicity but reduced HR elicitation. A second type of harpin, termed HrpW, also is produced by *E. amylovora* (Gaudriault et al. 1998; Kim and Beer 1998). The N terminus of HrpW

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shares properties of harpins and is capable of HR elicitation. The C terminus is related to pectic lyases but lacks enzyme activity. HrpW does not appear to be necessary for pathogenicity or HR elicitation.

In *Pseudomonas syringae* pathovars (He et al. 1993), a harpin is encoded by *hrpZ*. Deletion of *hrpZ* in *P. syringae* pv. *syringae* strain B728A affects virulence on beans only when the bacteria are inoculated onto the surface of leaves (Loniello et al. 1995). In addition to HrpZ, these pathovars also produce a HrpW class harpin (Charkowski et al. 1998) and an Avr-like protein, HopPsyA (HrmA) (Van Dijk et al. 1999). The latter appears to be an important elicitor when wild-type bacteria are infiltrated into tobacco. A similar extracellular HR-elicitor protein, PopA, is secreted by *Ralstonia solanacearum*. *popA* mutants are fully pathogenic on tomatoes and susceptible petunia lines (Arlat et al. 1994). An open reading frame (ORF), *hpa1*, is linked to the *hrp* cluster in *Xanthomonas oryzae* pv. *oryzae* and encodes a harpin-like protein with 26% glycine (Zhu et al. 2000). Mutation of *hpa1* results in the reduction of water-soaking ability.

Harpins exhibit little or no amino acid sequence similarity but have several physical and chemical properties in common. They are protease sensitive, glycine rich, heat stable, acidic, do not contain cysteine, and lack a N-terminal signal sequence (Arlat et al. 1994; He et al. 1993). The elicitor activity does not seem to be confined to any one domain of the protein because various fragments retain activity (Alfano et al. 1996; Arlat et al. 1994; Laby and Beer 1995). *E. amylovora* secretes HrpN_{Ea} in apple seedlings, but the bulk of the secreted protein appears to remain attached to the bacterial cell wall (Perino et al. 1999) and was not observed in association with the host cell wall. Brown et al. (2001) used immunogold labeling to study the release of *P. syringae* pv. *tomato* HrpZ_{Pst} in vitro and in Arabidopsis. In culture, HrpZ_{Pst} was first seen at the base of pili and then became associated with a matrix coating the length of the pilus. In planta, they observed it in the bacterial cytoplasm and inner and outer membranes where labeling extended into the intercellular spaces and onto pili crossing the plant cell wall. In terms of eliciting the HR, the action site for harpin appears to be on the plant cell surface because transgenic *Nicotiana benthamiana* plants expressing HrpZ_{PspH} in the cytoplasm are not affected adversely (Tampakaki and Panopoulos 2000). When a signal peptide is added to the N terminus of the protein so that it is secreted, expression of HrpZ_{PspH} induces necrosis. A protein with a signal peptide that is correlated with harpin sensitivity of pepper tissue has been proposed to be a harpin receptor (Chen et al. 2000). Purified HrpN_{Ea} has been shown to depolarize plant cell membranes in cell suspension cultures (Popham et al. 1995) and leaves (Pike et al. 1998), and HrpZ_{PspH} forms ion-conducting pores in lipid bilayers (Lee et al. 2001) so that pathogens may use them to damage cell membranes in order to release nutrients from host cells early in infection. Alternatively, harpins may aid in the translocation of some Hop and Avr proteins across the plant plasma membrane. The finding that they appear only to have minor effects on virulence, however, means that they may be redundant or this function is optional.

Pantoea stewartii subsp. *stewartii* (syn. *Erwinia stewartii*) has a *hrp-wts* gene cluster that is required for lesion formation and wilting on corn (Coplin et al. 1992a; Coplin et al. 1992b). As reported in an accompanying paper (Frederick et al. 2001),

this cluster is 28 kb and consists of eight complementation groups. The genes in this cluster have sequence homology with *hrp* genes of *E. amylovora* and *E. herbicola* pv. *gypsophylae* and are very similar in genetic organization and regulation. The *hrpN_{Pnss}* gene is located between the *hrpC* and *wtsE* operons and is regulated by HrpS (Frederick et al. 2001). This ORF is predicted to encode a 382 amino acid protein that has 70, 62, 45, and 7% amino acid identity to harpins from *E. herbicola* pv. *gypsophylae*, *E. amylovora*, *E. chrysanthemi*, and *P. syringae* pv. *syringae*, respectively. Wild-type *P. stewartii* subsp. *stewartii* does not cause the HR in tobacco under normal assay conditions when grown on rich media and previously has been considered to be an exception to the rule that gram-negative phytopathogenic bacteria can cause a HR. In this study, however, we demonstrate that *P. stewartii* subsp. *stewartii* can incite a HrpN_{Pnss}-dependent HR on tobacco and *Datura* spp. when bacteria overexpress *hrpS* or are preinduced in a medium that mimics some conditions in the plant apoplast. DM760, the single *hrpN::Tn5* mutant constructed in the accompanying paper (Frederick et al. 2001), was virulent on corn seedlings in water-soaking assays and did not cause a HR on tobacco. In this study, we report the characterization of HrpN_{Pnss}, its biological activity and a quantitative assessment of its importance in the pathogenicity of *P. stewartii* subsp. *stewartii* on sweet corn (*Zea mays* cv. *saccharata* [Sturtevant] Bailey), and in the ability of *P. stewartii* subsp. *stewartii* to multiply in various graminaceous hosts.

RESULTS

P. stewartii subsp. *stewartii* cells and extracts can elicit the HR in tobacco.

P. stewartii subsp. *stewartii* grown on rich media such as Luria-Bertani (LB) or Casamino Acids-peptone-glucose agar or broth did not cause a HR in tobacco under typical assay conditions. Bacterial populations in infiltrated tobacco leaves, however, remained above 5×10^7 CFU/g or at least 12 h, which normally is considered a sufficient cell concentration and time period for most incompatible bacteria to elicit the HR. We hypothesized that the failure of *P. stewartii* subsp. *stewartii* to elicit the HR might be the result of poor expression of the *hrp* genes in tobacco or low activity of the elicitor protein(s).

Two approaches were used to increase *hrp* gene expression and elicitor production. The first involved growing cells in a semi-defined medium that we developed to maximize *hrpJ::lacZ* expression in strain DM711. This inducing medium (IM) was low in inorganic nitrogen and phosphate, contained sucrose and Casamino Acids, and was acidic (pH 5.5). When grown in IM, wild-type strain DC283 frequently gave a typical HR, although this reaction varied from leaf to leaf and plant to plant and ranged from confluent to patchy necrotic areas within the infiltrated tissue. In the second approach, plasmids pES411 and pRF205 were introduced into DC283 to increase expression of the *hrp* genes. pES411 provided extra copies of the entire *hrp* regulatory region (*hrpS*–*hrpXY*–*hrpL*), whereas pRF205 only contained *hrpS* driven by a strong *lacZ* promoter. The transconjugants gave a strong, consistent HR response in tobacco, which was visible as early as 18 h after infiltration with 10^8 CFU/ml. This occurred even when bacteria were grown on rich medium such as LB agar. This HR was much stronger than that observed for DC283 grown in IM.

CFEPs also were active in inducing the HR in tobacco. CFEPs were prepared according to the procedure used for purification of the *E. amylovora* harpin (Wei et al. 1992). DC283 was grown in IM medium ($A_{540} = 0.8$), sonicated in the presence of phenylmethylsulfonyl fluoride (PMSF), boiled, and centrifuged to obtain the soluble fraction. This extract was highly effective in inducing a HR within 18 h. CFEPs also elicited a rapid HR in leaves of 2-month-old *Datura stramonium* L. plants. The elicitor activity was heat stable but appeared to be protease sensitive because we were unable to obtain active preparations without the use of the protease inhibitor PMSF. This suggests that the active elicitor was a polypeptide. CFEPs prepared from *hrpN* mutant DM760(pRF205) and various *hrp* regulatory mutants were inactive, but those from secretion mutants were active in eliciting the HR in tobacco (Frederick et al. 2001).

Production of HrpN_{Pnss} in *Escherichia coli*.

To confirm the protein product of the *hrpN* ORF and to prepare purified HrpN_{Pnss}, we used an *E. coli* T7 expression system. A 1.8-kb *Hind*III fragment containing *hrpN* was cloned

Fig. 1. Genetic map of the *hrpN* region within the *Pantoea stewartii* subsp. *stewartii* *hrp-wts* cluster that was cloned into pDM2530. Boxes delimit the *hrpT*, *hrpV*, *hrpN*, and *wtsE* open reading frames (ORFs), and arrows indicate HrpL-dependent promoters. Transcription of all ORFs is from left to right. Solid circles mark the positions of Tn5 insertions in mutants MA1, DM760, and MA2. Insert cloned into pMA1 and pMA2 is shown below the line.

into vectors pBluescript SK and pT7-7 behind the T7 promoters (Fig. 1). These clones, designated pMA1 and pMA2, respectively, were used to overexpress HrpN_{Pnss} in *E. coli* BL21(DE3) (Studier and Moffatt 1986). T7 polymerase was induced in BL21 by isopropyl- β -D-thiogalactopyranoside (IPTG). CFEPs were then prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Prominent 43-kDa proteins were present in extracts from strains with pMA1 or pMA2, which co-migrated with authentic HrpN_{Ea}. The estimated molecular mass of the *P. stewartii* subsp. *stewartii* protein, as determined by SDS-PAGE, was 43 kDa (Fig. 2). This is slightly higher than the value predicted from the sequence data, which was 39.5 kDa. *E. amylovora*, *E. chrysanthemi*, and *P. syringae* pv. *syringae* harpins similarly have been reported to migrate slower than expected in SDS-PAGE gels (Bauer et al. 1995; He et al. 1993; Wei et al. 1992).

Secretion and biological activity of HrpN_{Pnss}.

Electroelution-purified, acetone-precipitated HrpN_{Pnss} was produced in BL21(DE3), purified by SDS-PAGE (12% gels), electroeluted, and precipitated with acetone. The protein was 85 to 90% homogeneous in Coomassie blue-stained gels (Fig. 2, lane 2) and used to raise antibodies in rabbits. The resulting antiserum reacted with the 43-kDa HrpN_{Pnss} band in Western blots from wild-type strain DC283 but did not react with any proteins in extracts from a Δ *hrp-wts* mutant, DM3020 (Fig. 2), or DM760 *hrpN*::Tn5.

To determine whether HrpN_{Pnss} was secreted into the supernatant, DC283(pRF205) and DM756(pRF205) *hrcC240*::Tn5 were grown overnight in LB at 28°C and the supernatants were concentrated by trichloroacetic acid (TCA) precipitation. HrpN_{Pnss} was detected in the supernatant of the wild-type strain but was barely detectable in the supernatant of the *hrcC*

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) and Western blots of cell-free elicitor preparations (CFEPs) and purified harpins. Lanes M, 1, and 2: Stained with Coomassie brilliant blue. Lane M: Molecular mass markers (#6001LA; Life Technologies Gaithersburg, MD, U.S.A.); lane 1: *Escherichia coli* BL21(DE3/pMA2) CFEP; and lane 2: PAGE-purified HrpN_{Pnss}. Lanes 3–9: Western blot reacted with anti-HrpN_{Pnss} serum. Lane 3: Purified HrpN_{Pnss}; lane 4: DC283(pRF205) wild-type CFEP; lane 5: MA1(pRF205) *hrpN123*::Tn5 CFEP; lane 6: MA2(pRF205) *hrpN254*::Tn5 CFEP; lane 7: DM760(pRF205) *hrpN189*::Tn5 CFEP; lane 8: DM3020(pRF205) Δ *hrp* CFEP; and lane 9: purified HrpN_{Ea}.

secretion mutant (Fig. 3). The relative amounts of harpin in the supernatants and cell pellets were determined by scanning densitometry of the Western blots. About 2.4% of the total HrpN_{Pnss} was present in the supernatant of the wild-type strain, but only 0.04% was present in the supernatant from the *hrcC* mutant. This experiment was repeated with similar results. To control for cell lysis, which may have been responsible for the very low amount of harpin released by the *hrcC* mutant, we developed Western blots with antiserum to *E. coli* AraB, a cytoplasmic enzyme. In *E. coli*, this antiserum cross-reacts strongly with a 45-kDa cytoplasmic protein known as "Band X" (De Gier et al. 1996). This unknown protein commonly is used as a control for cell lysis in secretion experiments. The anti-AraB serum cross-reacted with a 45-kDa cytoplasmic protein in total cell extracts from *P. stewartii* subsp. *stewartii*, which probably is the same as Band X, and with a

Fig. 3. Western blot of proteins from the supernatants and cell pellets of *Pantoea stewartii* subsp. *stewartii* wild-type strain DC283 and *hrcC* mutant DM756 reacted with antisera to HrpN_{Pnss} (top row) and *Escherichia coli* AraB (bottom row). Bands X and Y were used as controls for cell lysis. They are *P. stewartii* subsp. *stewartii* cytoplasmic and secreted proteins, respectively, which cross-react with AraB antiserum.

Fig. 4. Comparison of biological activities of HrpN_{Pnss} and HrpN_{Ea} in tobacco leaves at various dosages. Percent hypersensitive response (HR) was determined as the proportion of the infiltrated area of the leaf that developed visible necrosis at 24 h. Each treatment was replicated on three different tobacco leaves. Standard errors were 3 to 22% for HrpN_{Ea} and 16 to 56% for HrpN_{Pnss}. Percent HR for the two harpins was significantly different ($P = 0.05$) at concentrations above 10 µg/ml. ■ = HrpN_{Ea}; ● = HrpN_{Pnss}.

36-kDa protein, which we termed "Band Y." Band X was not detected in *P. stewartii* subsp. *stewartii* supernatants, whereas Band Y clearly was secreted (Fig. 3). These results indicate that almost all of the harpin present in DC283 supernatants was secreted in a *hrp*-dependent manner.

The CFEs prepared from *E. coli* BL21(DE3/pMA2) and *E. coli* BL21(DE3/pMA1) caused a typical HR in tobacco leaves within 18 h, whereas those prepared from *E. coli* BL21(DE3/pT7-7) and *E. coli* BL21(DE3/pBluescript SK) did not produce any necrosis. In a comparison of the activities of HrpN_{Pnss} and HrpN_{Ea} (similarly produced from BL21[DE3/pMA6]) in the same tobacco plants, 44 µg of HrpN_{Pnss} per ml was needed to cause 50% HR but only 12 µg of HrpN_{Ea} per ml produced the same result (Fig. 4). In another independent experiment, 49 µg of HrpN_{Pnss} per ml was needed to cause 50% HR in tobacco leaf panels, whereas only 14 µg of HrpN_{Ea} per ml incited the same amount of necrosis. Therefore, the HR elicitor from *E. amylovora* was considered to be three- to fourfold more active than that from *P. stewartii* subsp. *stewartii*. The difference in activities between the two harpins within each experiment was statistically significant.

We also tested the effect of HrpN_{Pnss} and HrpN_{Ea} on the pH response of tobacco suspension-cultured cells and on the cell

Fig. 5. Time-course of pH/K⁺ changes induced by HrpN_{Ea} and HrpN_{Pnss} at 6 µg/ml and by HrpN_{Pnss} with K252a in tobacco cell suspension cultures. At 1-h intervals, the pH was measured and aliquots were removed for analysis by atomic absorption spectrophotometry. The experiment was repeated once, with similar results. Buffer-treated control values were subtracted from all points. Data are means ± standard deviation (SD). ■ = HrpN_{Ea}; ● = HrpN_{Pnss}; ▲ = HrpN_{Pnss} + K252a.

membrane potentials of tobacco leaves. At 6 μg per ml, HrpN_{Pnss} and HrpN_{Ea} induced a quick, sharp pH increase immediately after application to cell suspensions, with a slow decline for 2 h and leveling for the subsequent 3 h (Fig. 5). HrpN_{Ea}, however, caused higher pH/K⁺ changes than HrpN_{Pnss}. With HrpN_{Pnss} (Fig. 5) and HrpN_{Ea} (data not shown), these responses were blocked completely by the protein kinase inhibitor K252a, suggesting that protein phosphorylation is required for initiation of the pH change. Tissue from the lowest fully expanded leaf was removed and used for membrane potential measurements. HrpN_{Ea} produced more membrane depolarization in tobacco leaf tissue than HrpN_{Pnss} when tested at 20 μg per ml (Fig. 6). HrpN_{Ea} depolarization was faster, closer to the diffusion potential, and/or more sustained than that produced by HrpN_{Pnss}. When the next highest leaf on the same plant was infiltrated, 20 μg of HrpN_{Ea} per ml caused necrosis, but concentrations of up to 80 μg of HrpN_{Pnss} per ml did not. This further suggests that, despite the high sequence homology between the two harpins, qualitative differences exist between them.

A radish bioassay (Han et al. 2000) developed previously to assess the induction of systemic resistance by bacterial biocontrol agents was used to determine whether HrpN_{Pnss} was able to induce systemic resistance in plants. Radish seedling roots were treated with HrpN_{Pnss} and 2,6-dichloroisonicotinic acid (INA), a known SAR inducer. After 7 days, the foliage was challenged by inoculation with *Xanthomonas campestris* pv. *amoraciae* strain 704b, a pathogen of radish. The leaves were then rated for severity of bacterial spot symptoms after 6 to 8 days. HrpN_{Pnss} treatment resulted in a 41% reduction in disease severity ratings over the inoculated control plants (Table 1). This was comparable to the amount of disease control caused by INA treatment, which resulted in a 48% reduction in disease severity.

Mutagenesis of *hrpN*.

In the accompanying paper (Frederick et al. 2001) on the organization of the *hrp-wts* cluster in *P. stewartii* subsp. *stewartii*,

iii, we report that a single *hrpN::Tn5* mutant, DM760, could not elicit the HR on tobacco but was fully pathogenic on sweet corn. Although this result suggests that *hrpN* might not be required for pathogenesis, more *hrpN::Tn5* mutants were needed to decrease the possibility that the pathogenicity of DM760 was the result of the production of a truncated HrpN_{Pnss} molecule that was still active biologically. Two additional Tn5 insertions into *hrpN* were isolated and marker exchanged into the genome of wild-type strain DC283 to produce strains MA1 and MA2 (Fig. 1). Marker exchange of the transposon mutations was confirmed by Southern blotting. Nucleotide sequencing placed the respective insertion mutations in DM760, MA1, and MA2 at amino acids 189, 123, and 254 of the HrpN_{Pnss} peptide, respectively. To discern whether the mutants might produce any truncated HrpN_{Pnss} proteins, the CFEPs from the mutant and wild-type strains containing pRF205 were assayed for HrpN_{Pnss} in Western blots (Fig. 2). The CFEPs from the mutants did not contain any cross-reacting proteins, whereas a protein ladder, typical of harpin-degradation products, was observed in the parent strain. Whereas this result suggests that truncated proteins are not produced, we do not know whether the N terminus of HrpN_{Pnss} contains any epitopes that our antiserum would recognize. In tobacco leaves, the mutants failed to elicit any necrosis at 2.5×10^8 cells per ml, even when *hrp* gene expression was increased by the introduction of pRF205 *lacp-hrpS*. In contrast, DC283(pRF205) caused a strong HR within 18 h.

To complement the *hrpN* mutants for HR elicitation, we used cosmid pES411 and a derivative thereof containing the *hrpN189::Tn5* insertion, which had been used to construct DM760. The phenotype of the *hrpN::Tn5* mutants, with and without the plasmids, was tested in tobacco. Panels of leaves were infiltrated with 2.5×10^8 CFU/ml of DC283(pES411), MA1(pES411), MA1(pES411 *hrpN189::Tn5*), MA2(pES411), MA2(pES411 *hrpN189::Tn5*), DM760(pES411), and DM760(pES411 *hrpN189::Tn5*). Only DC283(pES411), MA1(pES411), MA2(pES411), and DM760(pES411) produced a ne-

Fig. 6. Depolarization of tobacco leaf cell membrane potentials by **A**, HrpN_{Ea} and **B**, HrpN_{Pnss}. Segments were cut from the same leaf and measured in parallel. Arrow indicates the time at which the bathing solution was switched to a solution containing 20 μg of HrpN_{Ea} or HrpN_{Pnss} per ml. Numbers denote millivolts. Breaks are points at which a cell died and a neighboring mesophyll cell was impaled. The experiment was repeated once, with similar results.

Table 1. Comparison of the efficacy of HrpN_{Pnss} and 2,6-dichloroisonicotinic acid (INA) in the suppression of bacterial leaf spot of radish by induction of systemic resistance

Treatment ^x	<i>Xanthomonas campestris</i> pv. <i>amoraciae</i>	Mean disease severity ^y
Control	–	1.0
Control	+	3.9
HrpN _{Pnss}	+	2.7
INA	+	2.5
LSD _{0.05} ^z		0.7

^x Roots were treated 3 days after germination with 200 μl of autoclaved 0.25-strength Hoagland's solution (control), with 200 μl of a 50 $\mu\text{g}/\text{ml}$ solution of INA or with 200 μl of a 0.5 mg/ml solution of purified HrpN_{Pnss}. Plants were challenge inoculated on the foliage with a 10^8 CFU/ml suspension of *X. campestris* pv. *amoraciae* 7 days following treatment with the SAR-inducing agents.

^y Values represent the mean disease severity ratings for two experiments. True leaves (five replicates of five plants each per experiment) were rated for disease symptoms 6–8 days following challenge inoculation with *X. campestris* pv. *amoraciae*. Disease scale: 1 = symptomless; 2 = fewer than three lesions/leaf; 3 = more than three lesions/leaf, less than 25% leaf area affected by disease; 4 = lesions covering 25–50% of the leaf surface area; 5 = lesions covering more than 50% of the leaf surface area; and 6 = one or more leaves dead.

^z Least significant difference ($P = 0.05$) according to Fisher's method.

crotic response. Likewise, when the CFEPs from the above strains were tested, only the CFEPs from the latter four strains gave a HR or reacted with anti-HrpN_{Pnss} serum (data not shown). These results indicate that the *hrpN* gene is required for harpin production and elicitation of the HR by *P. stewartii* subsp. *stewartii*.

HR⁻ phenotype of *hrpN*_{Pnss} mutants was complemented by *hrpN*_{Ea}

Weak cross-reactions were observed between an anti-HrpN_{Pnss} serum and HrpN_{Ea} and between an anti-HrpN_{Ea} serum and HrpN_{Pnss} (data not shown). These results along with sequence homologies suggest that the two harpins might be functionally interchangeable. To test this idea, we electroporated plasmid pCPP1012, which contains *hrpN* from *E. amylovora*, into the *hrpN* mutant MA1. This plasmid enabled MA1 to cause a HR on tobacco at 5×10^8 CFU/ml. When a CFEP from the transformant was infiltrated into tobacco leaves, it caused a strong HR in tobacco leaves within 18 h and reacted strongly with anti-HrpN_{Ea} serum in Western blots (data not shown). This indicates that *P. stewartii* subsp. *stewartii* can synthesize and secrete a heterologous harpin.

HrpN_{Pnss}-deficient mutants of *P. stewartii* subsp. *stewartii* were fully pathogenic on corn.

The pathogenicity of mutants MA1, MA2, and DM760 on corn was compared with that of wild-type strain DC283 with respect to 50% effective dose (ED₅₀), disease severity, and response time. In two experiments, the average ED₅₀ for DC283 was 18 ± 2 CFU/plant and the ED₅₀ for DM760, MA1, and MA2 were 26 ± 1 , 22 ± 10 , and 24 ± 5 CFU/plant, respectively, and were not significantly different ($P = 0.05$). Disease severity ratings for DC283 and the mutants were 4.2 to 4.6 ± 1.2 , whereas the rating for DM3020 Δ *hrp-wts* was 1.1 ± 0.4 . The differences between the mutants and wild type for disease severity ratings and response times (data not shown) likewise were not statistically significant at $P = 0.05$. These results show that mutations in *hrpN* did not affect the pathogenicity of *P. stewartii* subsp. *stewartii* because the mutants were neither quantitatively nor qualitatively different from the wild-type controls. To measure the ability of the *hrpN* mutants to grow and persist in planta, 7-day-old corn seedlings were inoculated with DC283, MA1, and MA2 grown in IM (pH 5.5). Bacterial populations were determined 0 to 192 h after inoculation. There was not a significant difference between the wild type and mutants in their ability to grow in planta. Populations increased up to 48 h, with doubling times of 7, 7, and 6 h for DC283, MA1, and MA2, respectively. They then leveled off. Similar results were obtained with DM760 in a separate study (data not shown).

Host-range studies.

P. stewartii subsp. *stewartii* is able to colonize a number of North American grass hosts (Poos 1940). This suggests that it is an endophyte or a minor pathogen of these grasses. Because *P. stewartii* subsp. *stewartii* does not occur in South America, it probably did not coevolve with corn and may have adapted to this host after the latter was introduced into North America. It is therefore possible that *hrpN* is needed to colonize alternative hosts. The endophytic growth of wild-type strain DC356 and the *hrpN* mutant MA1 was examined in grass species *Sor-*

ghum halepense L. (Johnsongrass), *Sorghum vulgare* cv. Rio (Rio sorghum), *Pennisetum glaucum* L. (pearl millet), *Sorghum vulgare* cv. Atlas (Atlas sorghum), *Digitaria* sp. (large crabgrass), *Alopecurus pratensis* L. (green foxtail), *Panicum virgatum* L. (switch grass), *Panicum capillare* L. (witch grass), *Triticum spelta* L. (Champ spelt), *Avena sativa* L. (Garland oats), and *Panicum miliaceum* L. (broom corn millet). The average initial bacterial population was $1.2 \pm 0.6 \times 10^6$ CFU/g. In most cases, the population remained constant or increased slightly after 15 days. The populations in Garland oats and broom corn millet, however, increased to 3 to 4×10^7 CFU/g after 9 days. Although many grasses supported high endophytic populations of the wild-type strain and *hrpN* mutant ($>10^5$ CFU/g), no symptoms were observed on any of the inoculated plants. DC356 growth did not differ ($P = 0.05$) from that of MA1 in any of the hosts.

Production of harpin by *P. stewartii* subsp. *indologenes*.

Three strains of *P. stewartii* subsp. *indologenes* (Mergaert et al. 1993), which are closely related to *P. stewartii* subsp. *stewartii*, were tested for HR elicitation and harpin production. All three strains produced a HR on tobacco, and CFEPs prepared from two of the strains produced cross-reactions with HrpN_{Pnss} antiserum in dot blots. The two positive strains, NCPPB 2275 and NCPPB 2280, were isolated from *Pennisetum americanum* and *Setaria italica*, respectively, and were formerly classified as *E. herbicola*. The negative strain NCPPB 1845, isolated from pineapple, was formerly *Erwinia ananas* (Mergaert et al. 1993).

DISCUSSION

HrpN_{Ea} was the first protein shown to traffic a Hrp secretion pathway and the first bacterial protein demonstrated to elicit the HR when applied exogenously to plants (Wei et al. 1992). Even though this discovery stimulated much research on type III secretion systems and led to the commercial application of harpin as a SAR inducer (Dong et al. 1999; Strobel et al. 1996), the role of harpins in pathogenesis is still uncertain. Because they are associated with most Hrp secretion systems, they probably benefit the pathogen, otherwise they would not be so conserved. Paradoxically, except in *E. amylovora*, harpin does not appear to be required for pathogenicity, although in some pathogens loss of *hrpN* or *hrpZ* can reduce symptoms and infectivity. In most cases, purified harpins have no visible adverse effects on cells of susceptible plant hosts. They can, however, cause membrane damage and therefore could be involved in stimulating the release of nutrients from host cells. This might be essential under certain inoculation conditions or, possibly, for epiphytic growth. Another currently favored hypothesis is that harpins aid in the transfer of some effector proteins across plant-plasma membranes. Again this role is speculative because some essential, secreted Dsp/Hop proteins such as DspE in *E. amylovora* and *E. herbicola* pv. *gypsophila* and WtsE in *P. stewartii* subsp. *stewartii* (Barny 1995; Bogdanove et al. 1998a; Bogdanove et al. 1998b; Frederick et al. 2001; Mor et al. 2001) seem to function well, without the aid of harpins. Therefore, either harpin is purely optional, or else these bacteria have other harpin-like proteins that serve the same function. The role of harpins in the elicitation of the nonhost HR is likewise unclear. Alfano et

al. (1996) questioned whether sufficient HrpZ_{Pss} is secreted into the apoplast by *P. syringae* pv. *syringae* to cause visible symptoms in nonhosts. Consequently, the necrosis obtained with this purified harpin may be the result of testing abnormally high concentrations. This raises the question as to whether the typical HR test in tobacco, which is used to identify plant-pathogenic bacteria, might sometimes be an artifact if one tests very high bacterial concentrations or grows inocula in apoplast-mimicking media to overexpress harpin, as we need to do with *P. stewartii* subsp. *stewartii*. Again, the fact that the loss of harpin frequently eliminates or reduces the HR could mean either that it acts as a major elicitor or it is required specifically for the delivery of yet unknown Avr proteins that are recognized by tobacco.

Sequencing the *hrp-wts* cluster in *P. stewartii* subsp. *stewartii* revealed a *hrpN* gene located between the secretion and Dsp/Wts regions (Frederick et al. 2001). The predicted product of this gene is rich in glycine, lacks cysteine, and does not have a signal peptide. The results of this study confirm that the *hrpN* product is indeed a harpin, with properties similar to those produced by *Erwinia* spp. When purified, the protein was able to induce HR in tobacco and *Datura* spp. and was effective in inducing systemic protection in radishes to bacterial leaf spot. Antiserum to PAGE-purified HrpN_{Pss} was used to detect harpin production and show that it is secreted into culture supernatants. *hrpN::Tn5* mutants did not produce detectable harpin and did not elicit a HR in tobacco. These mutations were not polar on downstream genes, so the fact that the *hrpN* mutants retained very little, if any, ability to cause the HR indicates that HrpN_{Pss} is responsible for the majority of the elicitor activity present in cells and CFEPs in our HR assays. HrpN_{Pss} production and HR elicitation could be restored by complementation with cosmid pES411, but not if it contained a *hrpN::Tn5* insertion, thereby confirming the association between genotype, HR phenotype, and harpin production. HrpN_{Ea} also was able to complement the HR⁻ phenotype of *hrpN* mutants, indicating that the two harpins are functionally equivalent. In addition, the anti-HrpN_{Pss} serum cross-reacted with HrpN from *E. amylovora*, *P. stewartii* subsp. *indologenes*, and *E. herbicola* pv. *gypsophila* (Mor et al. 2001).

P. stewartii subsp. *stewartii* and other *Pantoea* spp. do not elicit the HR in tobacco if inoculum is grown on rich medium. This could be because i) bacterial cells die quickly after infiltration into tobacco tissue, ii) HrpN_{Pss} is unstable in tobacco tissue, iii) *hrp* genes are expressed poorly in tobacco, iv) or HrpN_{Pss} is less active on tobacco than other harpins. Failure of the bacteria to grow is probably not a factor because populations in tobacco remained above 5×10^7 CFU/g for at least 12 h, which is a sufficient cell concentration and time for most pathogens to elicit the HR. Because preinduced cells did cause the HR, it is not likely that HrpN_{Pss} is especially unstable in tobacco. Therefore, inadequate *hrp* gene expression and lower biological activity are the most likely explanations. When a constitutive *HrpS* plasmid was used to increase *hrp* gene expression and boost HrpN_{Pss} production, it enabled *P. stewartii* subsp. *stewartii* to cause the HR in tobacco. We also showed that HrpN_{Pss} has three- to fourfold less biological activity than HrpN_{Ea} in tobacco necrosis assays and equivalent concentrations of HrpN_{Pss} are considerably less effective in causing membrane depolarization in tobacco leaf segments and pH/K⁺ changes in the supernatants of tobacco suspension-

cultured cells than HrpN_{Ea}. Therefore, it will be interesting to compare *hrp* gene expression in corn and tobacco, test the effects of HrpN_{Pss} on corn cells, and learn whether *P. stewartii* subsp. *stewartii* could induce the HR in tobacco if the *hrp* genes are expressed at the same level as they are in corn.

Often the loss of an *avr* gene results in very subtle effects on pathogenicity that can be detected only by the use of low inoculum concentrations, certain hosts, or natural entry routes. For this reason, we carefully measured the effects of *hrpN* mutations on the virulence and infectivity of *P. stewartii* subsp. *stewartii* with a number of different evaluation criteria, inoculation techniques, and hosts. We were still unable to show even minor effects of a *hrpN* mutation on the ED₅₀, response time, symptom severity, or growth rate of *P. stewartii* subsp. *stewartii* in sweet corn and did not see any reduction in the ability of bacteria to grow endophytically in various grasses. In this respect, *P. stewartii* subsp. *stewartii* differs from *E. amylovora*. Because several domains of HrpN and HrpZ can elicit the HR, we attempted to show that our *hrpN::Tn5* mutants did not produce truncated products that might remain functional for virulence. Although we could not detect any truncated harpin fragments, we do not know whether our polyclonal antiserum recognizes the N terminus of HrpN_{Pss}, thus this remains a possibility. It is more likely that the action mechanism of harpin in compatible interactions is very different from that by which it induces a HR in tobacco. Therefore, intact HrpN_{Pss} would probably be needed in corn if, in fact, it is required at all for pathogenicity of *P. stewartii* subsp. *stewartii* on that host. Consequently, the results of this study indicate that HrpN_{Pss} has a very minor role, if any, in the virulence of *P. stewartii* subsp. *stewartii* to sweet corn and maize and is not required to deliver essential Dsp/Hop proteins such as WtsE to host cells. Only one other harpin has been described for another member of the *Pantoea* group (Mor et al. 2001) and likewise is not essential for pathogenicity.

The pathogenic *Pantoea* spp., which most likely have evolved from endophytic "herbicola" strains by the acquisition of pathogenicity islands via horizontal gene exchange, may carry only a minimal complement of type III secretion genes and their effector proteins, in order to remain compatible with a wide range of hosts. This could be why *P. stewartii* subsp. *stewartii* does not naturally elicit the nonhost HR, and why we found very many genes for effector proteins and other harpins within the *hrp-wts* cluster. This study is, to our knowledge, the first assessment of the role of harpin in the virulence of a pathogen of monocots and raises the possibility that harpin may be more important in pathogenesis on dicots than on monocots. The *hrp* cluster in *P. stewartii* subsp. *stewartii* is very similar to that in *E. herbicola* pv. *gypsophila*. Thus, *P. stewartii* subsp. *stewartii* may have received it relatively recently from a pathogen of dicots, providing a reason why *hrpN* is still present.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacteria and plasmids used in this study are listed in Table 2. All *P. stewartii* subsp. *stewartii* strains were derived from DC283 or DC356, which are spontaneous nalidixic acid-(Nal^r) and rifampicin-resistant (Rif^r) mutants, respectively, of wild-type strain SS104 (Coplin et al. 1981). Culture media,

bacterial growth, and mating conditions for *P. stewartii* subsp. *stewartii* have been described previously (Coplin 1978; Coplin et al. 1986). The following antibiotics were used in selective media, per ml: 20 µg of tetracycline, 20 µg of kanamycin, 100 µg of ampicillin, 20 µg of nalidixic acid, and 50 µg of rifampicin. IM, which is optimal for the expression of *hrp* genes, consisted of 100 mM 2-(*N*-morpholino)ethane-sulfonic acid (MES; Sigma Chemical, St. Louis, MO, U.S.A.), 2 mM (NH₄)₂SO₄, 0.1% Casamino Acids (Difco, Detroit, MI, U.S.A.), 1 mM potassium phosphate (pH 7.2), 1% sucrose, and 1 mM MgSO₄. The medium was adjusted to pH 5.5 with NaOH and autoclaved for 20 min. Plasmids were mobilized from *E. coli* HB101 into *P. stewartii* subsp. *stewartii* by pRK2013::Tn7 (Coplin et al. 1986) or introduced by electroporation with a Gene Pulsar (Model 1652076; Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's protocol. Purified HrpN_{EA} and anti-HrpN_{EA} antibodies were a gift from S. V. Beer.

General DNA manipulations and sequence information.

Plasmid DNA isolation, agarose gel electrophoresis, restriction analysis, transformation, ligation, Southern hybridization, and random primer labeling were performed according to standard protocols (Ausubel et al. 1995; Sambrook et al. 1989). Nonradioactive Southern blots were performed with

the Photogene Nucleic Acid Detection System, version 2.0, as described by the manufacturer (Life Technologies, Gaithersburg, MD, U.S.A.) except that Western blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI, U.S.A.) was used to detect hybridizing bands.

HrpN_{PNS} purification and immunoblotting.

E. coli BL21(DE3/pMA2) cultures were grown overnight to stationary phase in 100 ml of Terrific broth containing 200 µg of ampicillin per ml (Sambrook et al. 1989), at 37°C, with 0.4 mM IPTG (Studier and Moffatt 1986). Cells were disrupted by sonication (Model MS2T; Tekmar, Cincinnati, OH, U.S.A.), with a 40% duty cycle and an output control setting of 4, in the presence of 1 mM PMSF. The sonicate was heated at 100°C for 10 min, then centrifuged to remove the denatured proteins (Wei et al. 1992). PMSF was included in all subsequent steps and storage buffers. The resulting CFEP contained partially purified HrpN_{PNS}. To further purify the HrpN_{PNS} for antibody preparation, the CFEP was separated on 8% preparative SDS-PAGE gels (Laemmli 1970). The 43-kDa HrpN_{PNS} band was visualized with ice-cold 0.25 M KCl (Hager and Burgess 1980). The protein was electroeluted from gel slices for 4 h at 200 V in Tris-glycine-SDS buffer (25 mM Tris, pH 8.3; 192 mM glycine; and 0.1% SDS) with an Elutrap apparatus (Schleicher & Schull, Keene, NH, U.S.A.). SDS was re-

Table 2. Bacterial strains and plasmids

Bacterial strains or plasmid	Relevant characteristics ^x	Reference or source
<i>Pantoea stewartii</i> subsp. <i>stewartii</i>		
DC283	SS104 Nal ^r	Coplin et al. 1981
DC356	SS104 Rif ^r	Coplin et al. 1992a; Coplin et al. 1992b
DM760	DC283 <i>hrpN189::Tn5</i>	Frederick et al. 2001
DM3020	DC283 <i>Δhrp-wts</i>	Coplin et al. 1992b
MA1	DC283 <i>hrpN123::Tn5</i>	This study
MA2	DC283 <i>hrpN254::Tn5</i>	This study
DM711	DC283 <i>hrpJ79::Tn3HoHoI</i>	Frederick et al. 1993
DM756	DC283 <i>hrcC240::Tn5</i>	Frederick et al. 2001
<i>Pantoea stewartii</i> subsp. <i>indologenes</i>		
NCPBP 1845	Wild type	Mergaert et al. 1993
NCPBP 2275	Wild type	Mergaert et al. 1993
NCPBP 2280	Wild type	Mergaert et al. 1993
<i>Escherichia coli</i>		
HB101	<i>thr leu thi recA hsdR hsdM pro</i>	Boyer and Roulland-Dussoix 1969
SM17-1λpir	λpir lysogen of S17-1 <i>thi pro recA hsdR⁻ hsdM⁺ RP4-2-Tc::Mu Sm^r Tp^r</i>	Miller and Mekalanos 1988
BL21(DE3)	<i>hsdS gal (λI_{ts} 857 indI S_{am}7 nin5 lacUV5-T7 gene I)</i>	Studier and Moffatt 1986
Plasmids		
pMA1	1.8-kb <i>HindIII</i> fragment in pBluescript (SK)	This study
pMA2	1.8-kb <i>HindIII</i> fragment in pT7-7	This study
pMA6	1.3-kb <i>HindIII</i> fragment from pCPP1012 containing <i>hrpN_{EA}</i> in pBluescript (SK)	This study
pRK2013::Tn7	ColE1 <i>mob⁺ Sm^r Sp^r Tp^r kan::Tn7</i>	D. Dean ^y
pDM2530	3-kb <i>HindIII</i> - <i>BamHI</i> <i>hrpN⁺</i> fragment of pES411 in pGP704	This study
pCPP1012	4.2-kb <i>BamHI</i> fragment containing <i>hrpN_{EA}</i> from pCPP430 in pBluescript KS ⁻	S. V. Beer ^z
pES411	<i>hrp⁺</i> genomic clone in pLAFR3, containing <i>wtsF-hrpL</i>	Coplin et al. 1986
pRF205	1.8-kb <i>HindIII</i> fragment containing <i>hrpS⁺</i> in pVK100	Frederick et al. 1993
pT7-7	T7 expression vector, Ap ^r	Tabor and Richardson 1985
pBluescript	Ap ^r , KS, and SK	Stratagene, La Jolla, CA, U.S.A.
pGP704	<i>oriV_{RSK} mob_{RP4} Ap^r</i> , suicide vector	Miller and Mekalanos 1988

^x Nal^r, Rif^r, Tc^r, Sm^r, Tp^r, Ap^r, Sp^r: resistant to nalidixic acid, rifampicin, tetracycline, streptomycin, trimethoprim, ampicillin, and spectinomycin, respectively.

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^z Department of Plant Pathology, Cornell University, Ithaca, NY, U.S.A.

moved by precipitating the protein with 80% acetone at -20°C (Hager and Burgess 1980) and resuspending it in 50 mM potassium phosphate, pH 6.5. Protein concentration was determined according to Bradford (1976). The amount of HrpN_{Pnss} and HrpN_{Ea} in the CFEPs used to compare biological activities in tobacco was quantitated by scanning the clearly visible bands of these proteins on their Coomassie blue-stained PAGE gels with a densitometer (Model GS300; Hoefer Scientific, San Francisco, CA, U.S.A.) and comparing peak heights with known amounts of purified HrpN_{Pnss} as a standard.

To compare the biological activities of HrpN_{Ea} and HrpN_{Pnss}, known amounts of the two proteins were infiltrated into panels of tobacco leaves and the proportion of the infiltrated area showing necrosis (percent HR) after 24 h was recorded. Each of three leaves received all dosages of both harpins in two independent experiments. These data (percent HR) were transformed to probit form, and a nonparametric Student's *t* test was used to analyze the transformed data. Within each experiment, the amount of HrpN_{Ea} needed to cause 50% HR was compared to the amount of HrpN_{Pnss} needed to incite 50% necrosis in tobacco leaves.

To extract HrpN_{Pnss} from *P. stewartii* subsp. *stewartii* strains containing pES411, cells were grown in 100 ml of LB (with appropriate antibiotics), at 28°C , to an A_{540} of 0.8. Cells were pelleted and washed in 10 ml of IM, resuspended in 100 ml of the same medium, and incubated, with shaking overnight. To extract from strain DC283(pRF205), the bacteria were grown overnight in LB, at 28°C . CFEPs were prepared as noted earlier. To obtain extracellular HrpN_{Pnss}, culture supernatants (200 ml of LB) were centrifuged at $3,840 \times g$ for 10 min, boiled for 10 min, cooled to room temperature, and mixed with an equal volume of 10% TCA. The mixture was held on ice for 15 min and centrifuged for 10 min at $20,400 \times g$. The pellet was then washed with cold 70% ethanol and dissolved in 50 mM potassium phosphate buffer (pH 6.5).

For Western blotting, CFEPs were electrophoresed and electroblotted on ImmunoSelect nitrocellulose membrane (Life Technologies) (Sambrook et al. 1989). The filter was probed with anti-HrpN_{Pnss} or anti-HrpN_{Ea} serum, and immunodetection of the bands was performed with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody using a Protoblot II AP System Kit (Promega) according to the manufacturer's instructions. For dot blots, 8 μl of each sample was spotted onto ImmunoSelect membranes and developed in the same way as above. Rabbit antiserum to *E. coli* AraB was used as a control for cell lysis in secretion experiments.

Production of anti-HrpN_{Pnss} antibodies.

HrpN_{Pnss} antibodies were produced in two adult white New Zealand rabbits (3 to 4 kg of body weight) in response to injections with HrpN_{Pnss}. Electroelution-purified, acetone-precipitated HrpN_{Pnss} (1.5 μg per μl) dissolved in 50 mM potassium phosphate buffer, pH 6.5, was used for this purpose. For primary immunization, 400 μg of the antigen was emulsified in 2 ml of a 1:1 mixture of Freund's complete adjuvant and phosphate buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 ; and 1.4 mM KH_2PO_4 , pH 7.4) and two 0.5 ml of aliquots of the emulsion were injected subcutaneously into the hips of each rabbit. Booster injections of 200 and 100 μg were administered after 4 and 6 weeks, respectively. Eight days after the second booster injection, the rabbits were bled

from the marginal vein of the ear and small samples of blood were collected. Immunoglobulin G (IgG) titer was measured by direct enzyme-linked immunosorbent assay, and the antiserum was harvested after 8 weeks by heart puncture. To partially purify the IgG, 18 ml of antiserum was precipitated with concentrated ammonium sulfate, resuspended in 2 ml of PBS buffer, and dialyzed against PBS containing 0.2 mM PMSF to remove excess salt.

HR assay in tobacco plants.

Tobacco plants (*Nicotiana tabacum* L. cv. Wisconsin) were grown in a greenhouse and transferred to a controlled environment chamber several days before use. The chamber was maintained at 28°C , 90% relative humidity, and a cycle of 16 h light–8 h dark. To assay for HR elicitation, bacteria were grown in Casamino Acids-peptone-glucose broth, transferred to IM liquid medium, grown for approximately 6 h, then pelleted and resuspended in 10 mM phosphate buffer, pH 7.0, at a concentration of 5×10^8 cells per ml ($A_{540} = 0.52$). Tobacco leaves were inoculated by pricking them with a dissecting needle and forcing inoculum into the wound with the open end of a 3-ml disposable plastic transfer pipette pressed against the upper leaf surface. The margins of the water-soaked infiltrated areas were marked, and the plants were rated for HR development at 24 h. The percent HR for different strains was calculated as the proportion of the infiltrated area showing necrosis.

Assay for pH change and K^+ efflux.

Tobacco suspension-cultured cells derived from *N. tabacum* L. Hicks (obtained from M. Atkinson, University of Wisconsin) were maintained as described previously (Atkinson et al. 1990). Four days after transfer to fresh medium, 0.1 g of cells per ml were collected by filtration and resuspended in 5 ml of standard assay medium (175 mM mannitol; 0.5 mM K_2SO_4 ; 0.5 mM CaCl_2 ; and 0.5 mM MES, adjusted to pH 6.0, with NaOH). Cell suspensions were equilibrated in the assay medium in 30-ml beakers at 27°C on a rotatory shaker at 150 rpm for 2 h. HrpN_{Ea} or HrpN_{Pnss} (final concentration 6 μg per ml) or assay medium was added to tobacco suspension cells after equilibration. pH measurements were recorded every hour for 6 h after treatment, and 200 μl of aliquots were taken for K^+ measurements and analyzed in an atomic absorption spectrophotometer. Two experiments with three repetitions of each treatment were performed.

Electrophysiology.

Tobacco plants (*N. tabacum* cv. Samsun NN) were grown under fluorescent lights (250 $\mu\text{E} \times \text{m} \times \text{s}$; 14-h photoperiod at approximately 28 to 30°C). The lowest fully expanded leaf was used for electrophysiological measurements approximately 8 to 9 weeks after planting. The next higher leaf was inoculated for symptoms, as described above. Membrane potentials were measured with glass microelectrodes in leaf segments, as described by Pavlovkin et al. (1986). At least one cell from each of two separate plants was measured in each type of treatment. Segments were placed in a Plexiglas measuring chamber and held in the darkness. The tissue was perfused continuously by gravity flow with $1\times$ buffer (1 mM KCl; 1 mM $\text{Ca}[\text{NO}_3]_2$; 0.25 mM MgSO_4 ; and NaH_2PO_4 plus Na_2HPO_4 to give a Na^+ concentration of 1 mM, pH 5.7) (Higinbotham et al. 1964). Spongy mesophyll cells were impaled through a cut

edge of the segments. When a stable potential had been reached, the perfusion solution was switched to one containing HrpN_{Ea} or HrpN_{Pnss} at a final concentration of 20 µg per ml. Measurements were performed in parallel with segments from the same leaf to reduce variability (Pike et al. 1998). Two measurements were performed with each harpin.

Systemic resistance assays in radishes.

Radish seeds (*Raphanus sativus* L. cv. Fuego) were grown in plastic pouches, as described by Han et al. (2000). The roots of 3-day-old seedlings were treated with 200 µl of a 50 µg per ml solution of INA or 200 µl of a 0.5 mg per ml solution of purified HrpN_{Pnss}. Plants were challenge inoculated on the foliage with a 10⁸ CFU/ml suspension of *X. campestris* pv. *amoraciae* strain 704b at 7 days following treatment with INA or HrpN_{Pnss}. Bacterial spot symptoms were rated, as described in Table 1.

Transposon mutagenesis and marker exchange.

E. coli S17-1 λ pir(pDM2530) was mutagenized with λ Tn5, as described by Dolph et al. (1988). Two plasmids that had insertions in the 1.8-kb *Hind*III fragment were identified. Both of these plasmids were mobilized into DC283 and selected for Km^r. The pDM2530::Tn5 plasmid could not replicate in DC283 and integrated into the chromosome by a single cross-over. Transconjugants were grown in LB–Km broth for 2 days, then plated for single colonies and screened for loss of Ap^r, indicating excision of the integrated plasmid.

Inoculation of corn seedlings and grasses.

Sweet corn seedlings (*Z. mays* L. cv. Earliking) were grown at 28°C and 16 h daylight in 50.8 × 25.4 × 6.35-cm flats containing soil–vermiculite–peat moss (1:1:1). In order to assay the virulence of *P. stewartii* subsp. *stewartii* strains, three different inoculation procedures were used to inoculate 8-day-old seedlings with DC283 and *hrpN* mutants. In the “pseudostem inoculation” method, 5.0 µl of inoculum (1,250 CFU/plant) were pipetted onto the cut ends of decapitated 8-day-old seedlings, and plants were rated at 10 days after inoculation on a scale of 1 to 5: 1 = no symptoms, 2 = scattered lesions, 3 = slight wilting, 4 = severe wilt, and 5 = dead. The data were used to calculate ED₅₀, response time, and growth rate. ED₅₀ values were determined graphically by plotting dose (colony-forming units) versus the probit of the proportion of the inoculated plants showing disease symptoms at 11 days after inoculation. A paired Student's *t* test was used to analyze ED₅₀ data, and a nonparametric Student's *t* test was used to analyze response time data.

A number of grasses related to corn were tested for their ability to support the multiplication of wild-type strain DC356, and 11 species that supported bacterial growth to ≥10⁵ CFU/g were selected for further study. All grasses were grown in flats, as described above. Bacterial inoculum was grown overnight in 5 ml of LB at 28°C, then pelleted and resuspended in 0.01 M potassium phosphate buffer (pH 7.2). Suspensions were adjusted to A₅₄₀ = 0.57. Twenty plants were inoculated for each strain–host combination. Stems were punctured with the eyelet end of a #4 sewing needle dipped in the bacterial suspension. The eyelet held approximately 1 µl of inoculum. At 0, 3, 9, and 15 days after inoculation, populations of the mutant and wild-type strains were determined. Two entire

plants per sample were homogenized in 5 to 20 ml of 0.85% saline using a Tissumizer (Tekmar), and then appropriate dilutions were plated on selective media plates. Two samples per host per interval were taken, and CFU/g of fresh weight was determined. The population dynamics of the wild-type strain DC356, MA1, and MA2 at different time intervals for each experiment were compared with a paired Student's *t* test ($n_1 + n_2 - 3$ degrees of freedom; $P = 0.05$).

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