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Characterization of the Hypersensitive Response Eliciting Protein, Harpin$_{Es}$, of *Erwinia* (*Pantoea*) *stewartii* and its Role in Determining the Pathogenicity of the Bacterium on Corn

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Abstract: Using *hrpN* of *Erwinia amylovora* as a radio-active probe, *hrpN* of *Erwinia stewartii* was located within its *wts* cluster. This gene was cloned and mutagenized with Tn*5* to get mutants MA1 and MA2. These *hrpN* mutants were verified by Southern blotting, Western blotting, sequencing and hypersensitive response to make sure that the Tn5 insertions were in the open reading frame of the *hrpN*$_{Es}$ gene and no truncated harpin$_{Es}$ was produced by these mutants. In order to assess the role of the *hrpN*$_{Es}$ gene product i.e., harpin$_{Es}$ in the pathogenicity of the bacterium, mutants MA1 *hrpN* 123 :: Tn*5*, MA2 *hrpN* 254 :: Tn*5* and DM760 *hrpN* 189 :: Tn*5* were compared to the wild-type strain DC 283 using ED50, disease severity, growth rate in planta and response time as pathogenicity parameters. The ED50 of the mutants was not significantly different (p=0.05) from that of the wild-type. The differences between the response times for mutants and the wild-type were not statistically significant (p=0.05) either. The mutants grew and caused as severe disease in the corn seedlings as the wild-type did. The results indicated that the mutations in the *hrpN* did not affect the pathogenicity of *E. stewartii* in any way i.e. the mutants were neither qualitatively nor quantitatively different from the wild-type controls.

Key words: *Erwinia stewartii*, *Pantoea stewartii*, *hrpN* gene, type III secretion

INTRODUCTION

The invasion of plants by pathogens results in either an incompatible interaction in a resistant host or non-host leading to the elicitation of defense responses such as the Hypersensitive Response (HR), or a compatible interaction leading to disease development. The ability of plant pathogenic bacteria to elicit the HR in resistant host is correlated with their ability to cause disease in susceptible host. Both of these capabilities are controlled by *hrp* genes (for hypersensitive response and pathogenicity)[1,2]. Typical *hrp* mutants are pleiotropically defective in planta; they do not elicit HR on non-host and they can not multiply and cause disease in host plants. In general, *hrp* genes clusters are large and consist of many complementation groups. Usually, one gene in the cluster encodes a heat-stable, protease-sensitive and glycine-rich protein called harpin, which has been found in *P. syringae* pv. *syringae, Erwinia amylovora* and *E. chrysanthemi* and which is required for elicitation of HR in tobacco and other non-host plants[3]. Harpin also contributes to the pathogenicity of *P. syringae* pv. *syringae* and *E. chrysanthemi,* but *E. amylovora* is the only bacterium in which it is required for pathogenicity. The remaining genes in *hrp* clusters are needed for secretion of exrtracellualr proteins, *hrp* gene regulation and possible disease specific extracellular pathogenicity factors.

hrp genes have been found in all genera of Gram-negative plant pathogens except Agrobacterium. Many of the genes in the *hrp* clusters are conserved and some are even functionally interchangeable[2,4]. Most of the conserved *hrp* genes encode proteins that constitute a Sec-independent secretion pathway, termed type III. This pathway is present and is involved in the secretion of extracellular pathogenicity proteins in bacterial pathogens of both animals and plants but is generally not found in their non-pathogenic counterparts[5-8]. Examples of animal pathogens that have type III secretion genes homologus to *hrp* genes are *Yersinia pestis, Shigella flexneri* and *Salmonella typhimurinum*[9-11].

The first proteinaceous elicitor of the HR was isolated from *E. amylovora*[3]. 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

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associated with a 44 kD protein, termed harpin$_{Ea}$ This was purified by HPLC and the amino terminus was sequenced. To find the structural gene encoding harpin$_{Ea}$, an oligonucleotide probe corresponding to the ninth to fifteenth amino acid residues was hybridized to the *hrp* gene cluster. The corresponding gene was located in the middle of the *hrp* cluster and designated *hrpN*$_{Ea}$ Later on, harpin was reported to be produced by several other plant pathogenic bacteria i.e. *P. syringae* pv. *syringae* 61[12], *P. syringae* pv. tomato, *P. syringae* pv. *glycinae*[13] and *E. chrysanthemi* EC16[14].

Harpins, despite differences in their amino acid sequences, have several physical and chemical properties in common; they are protease-sensitive, have open structures (i.e., contain no cysteine), are glycine-rich, heat-stable and acidic in nature[12,15]. However, they seem to be quite different from each other regarding the length of the peptide needed for HR elicitation and the translocation recognition signal of the protein. The N-terminal 128 amino acids of harpin$_{Ea}$ are needed for elicitor activity and the C-terminus is responsible for secretion[16]. In contrast, the elicitor activity of harpin$_{Ech}$ and harpin$_{Pss}$ is not confined to any one region of the protein[17]. In addition, harpin$_{Pss}$ has two directly repeated sequences in its C-terminus, which are lacking in harpin$_{Ea}$[12]. Secretion targeting signals also appear to differ. *hrpN* mutants of *E. amylovora* complemented with *hrpN*$_{Ech}$ and vice versa, could synthesize the heterologous harpin but were unable to secrete it to the exterior[18].

E. stewartii also has a *hrp*-like gene cluster, termed *wts* (water soaking), which is required for lesion formation and wilting on corn[19]. This cluster is 28 kb in size and consists of seven complementation groups. The *wts* genes of *E. stewartii* hybridize with *hrp* genes of *E. amyovora* and *P. syringae* pv *phaseolicola* and some *wts* subclones functionally complement certain *hrp* mutants of *E. amylovora*[20,21]. This suggests that many of the *wts* genes are structurally and functionally similar to *hrp* genes *E. amylovora*. However, wild-type *E. stewartii* was not known to cause an HR in tobacco under normal assay conditions, so we did not originally call them *hrp* genes. Nevertheless, we have been able to demonstrate that *E. stewartii* can indeed incite an HR on tobacco and Datura when we engineer increased expression of the *wts* regulon by putting *wtsA* under the control of a constitutive promoter[20]. Under a proposal to revise *hrp* nomenclature, the *wts* genes were renamed as *hrp*[22]. Since *hrpN* gene of *E. amylovora* which encodes harpin, a pathogenicity determinant, is located in the part of the *hrp* cluster that collinearly hybridizes with the *wts* cluster of *E. stewartii*, we hoped to find a *hrpN*$_{Ea}$ homolog in the *wts* cluster and wanted to investigate if this homolog would encode proteins with harpin-like functions.

In this research, we report the characterization of harpin$_{Es}$ and its role in the pathogenicity of *E. stewartii* on corn.

MATERIALS AND METHODS

Bacterial strains, plasmids and media: The present research were started a few years back but were completed and concluded in 2001. Bacteria and plasmids used in this study are listed in Table 1. All *E. stewartii* strains were derived from DC 283 or DC 356, which are spontaneous nalidixic-acid-resistant (Nal^r) and rifampicin-resistant (Rif^r) mutants of wild-type strain SS 104[23], respectively. Culture media, growth of bacteria and mating conditions for *E. stewartii* have been described previously[19,24]. The following antibiotics were used in selective media in the amounts indicated (μg mL^{-1}); tetracycline 20, kanamycin 20, ampicillin 100, nalidixic acid 20 and rifampicin 50. Inducing Medium (IM), which is optimal for expression of *wts* genes, consisted of 100 mM 2-[N-Morpholino]ethanesulfonic acid (MES; Sigma Chemical, St. Louis), 2 mM $(NH_4)_2SO_4$, 0.1%

Table 1: Bacterial strains and plasmids

Bacterial strains /plasmids	Relevant characteristics[a]	Reference or source
Bacterial strains: *Erwinia* (*Pantoea*) *stewartii*		
DC 283	SS 104 Nal^r	[23]
DM 760	DC 283 *hrpN* 189:: Tn*5*	[20]
MA 1	DC 283 *hrpN* 123:: Tn*5*	This study
MA 2	DC 283 *hrpN* 254:: Tn*5*	This study
DM 3020	DC 283 Δ *wts*	Majerczak[b]
SM 17-1λpir	Λpir lysogen of S17-1 *thi pro recA* ^{r} *hsdR*$^-$ *hsdM*$^+$RP4-2-Tc::Mu Sm^r Tp^r	Beer[c]
BL 21 (DE3)	*hsdS* gal (λcIts 857 *indI* Sam7 min5 *lac*UV5-T7 gene I	[32]
Plasmids:		
pMA2	1.8 kb *Hind*III fragment cloned in pT7-7,	This study
pDM 2530	Ap^r	Majerczak[b]
pCPP 430	3 kb *Hind*III/*BamH*I *hrpN*$^+$ fragment of pES411 cloned in pGP704, AP^r	Beer[c]
pES 411	Sp^r, *hrp* gene cluster of *Erwinia amylovora* Ea321	Coplin[b]
pRF 205	*wts*$^+$ clone (*wtsA,C,I,D,F,N* and *E*) in pVK100 from *E. stewartii* chromosome 1.8 kb *HindIII* fragment (having *wtsA*) from pES1044 in pVK100	Frederick[b]
pT7-7	Cloning vector, Ap^r	Tabor[d]

[a] Nal^r, Rif^r, Tc^r, Sm^r, Tp^r, Ap^r, Sp^r : resistant to naladixic acid, rifampicin, tetracycline, streptomycin, trimethoprim, ampicillin and spectinomycin, respectively

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casamino acids (Difco, Detroit, MI), 1 mM potassium phosphate (pH 7.2), 1% sucrose and 1 mM $MgSO_4$. The pH of the medium was adjusted to 5.5 with NaOH and then it was autoclaved for 20 min. Plasmids were mobilized from *E. coli* HB 101 into *E. stewartii* by pRK 2013::Tn*7*[19] or introduced by electroporation using a BIO-RAD Gene Pulsar™ (Model 1652076) according to manufacturer's protocol.

Transposon mutagenesis and marker-exchange: Our lab previously isolated a Tn*5* insertion mutation near the center of the putative *hrpN* ORF and marker-exchanged it into the wild-type strain DC283 to produce strain DM760[20]. To create some more Tn*5* insertion mutations, *E. coli* S17-1 λ*pir* (pDM2530) was mutagenized with λTn*5* as described by Dolph *et al.*[25]. Two plasmids were identified that had insertions in the 1.8 kb *Hind*III fragment. Both of these plasmids were mobilized into DC 283 and selected for Km^r. The pDM2530::Tn*5* plasmid could not replicate in DC283 and integrated into the chromosome by a single cross-over. To allow for the excision of the integrated plasmid to occur, the Ap^rKm^r transconjugants were grown in LB-Km broth for two days. The resulting new *hrpN* mutants were named as MA1 and MA2.

Harpin$_{Es}$ purification: *E. coli* BL21(DE3/pMA2) cultures were grown overnight to stationary phase in 100 mL Terrific Broth containing 200 μg mL^{-1} ampicillin[26] at 37°C. Cells were disrupted by sonication (sonicater model MS2T, Jewell Electrical Instruments, Inc.) using 40% duty cycle and output control of 4, the sonicate was heated at 100°C for 10 min and then it was centrifuged to remove the denatured proteins[3]. The resulting Cell-Free Elicitor Preparation (CFEP) contained partially purified harpin$_{Es}$. To further purify harpin$_{Es}$, the CFEP was separated on 8% preparative SDS-PAGE gels[27]. The 43 kDa harpin$_{Es}$ band was visualized with ice-cold 0.25 M KCl[28]. The protein was electroeluted from gel slices for 4 h at 200 V in Tris/glycine/SDS (25 mM Tris, pH 8.3; 192 mM glycine; 0.1% SDS) buffer using an Elutrap apparatus (Schlecher and Schull). SDS was removed by precipitating the protein with 80% acetone at -20°C[10] and then resuspending it in 50 mM potassium phosphate, pH 6.5. Protein concentration was determined according to Bradford [29]. To extract harpin$_{Es}$ from *E. stewartii* strains containing pES411, the cells were grown in 100 mL 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. For extraction from strain DC283(pRF205), the bacteria were grown overnight in LB at 28°C. CFEPs were prepared as above.

Production of anti-harpin$_{Es}$ antibodies: Harpin$_{Es}$ antibodies were produced in two adult white New Zealand rabbits (3-4 kg body weight) in response to injection with harpin$_{Es}$[30]. Electroelution-purified, acetone-precipitated harpin$_{Es}$ (1.5 μg μL^{-1}) suspended 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 mixture (1:1) of Freund's complete adjuvant and PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4. $7H_2O$ and 1.4 mM KH_2PO_4, pH 7.4) and two 0.5 mL aliquots of the emulsion were injected subcutaneously into the hips of each rabbit. Four weeks later, two booster injections (200 and 100 μg antigen respectively/rabbit emulsified in 2 mL 1:1 mixture of incomplete Freund's adjuvant and PBS), at 2 week intervals were delivered intramuscularly in the hips of each rabbit. Eight days after the second booster injection, the rabbits were bled form the marginal vein of the ear and small samples of blood were collected. IgG titer was measured by direct ELISA[30] and the antiserum was harvested after 8 weeks by heart puncture. To partially purify the IgG, 18 mL antiserum was precipitated with concentrated ammonium sulfate[30], resuspended in 2 mL of PBS buffer and dialyzed against PBS containing 0.2 mM Phenyl Methyl Sulfonyl Fluoride (PMSF) to remove excess salt. The reactivity of the antiserum was confirmed by reaction with harpin$_{Es}$ in Western blots.

Western blotting: For western blotting, CFEPs were electrophoresed and electroblotted on an ImmunoSelect (GIBCO BRL) nitrocellulose membrane[26]. The filter was probed with anti-harpin$_{Es}$ serum and immunodetection of the bands was performed with rabbit alkaline phosphate-conjugated secondary antibody (Protoblot^R II AP system kit) according to the manufacturer's (Promega) protocol.

Inoculation of corn seedlings: Sweet corn seedling (Earliking) were grown at 28°C and 16 h daylight in 20x10x2.5 flats containing soil:vermiculite:peatmoss (1:1:1). In order to assay the virulence of *E. stewartii* strains, 8-day-old seedlings were inoculated with DC283 and *hrpN* mutants, using three different inoculation procedures[19,31]. The data obtained were used for calculating ED_{50}, response time, disease severity and doubling times for the mutants and the wild-type. A paired t test was used for the analysis of ED_{50} data and a non-parametric t test was used to analyze the response time data.

HR assay in tobacco plants: Tobacco plants (*Nicotiana tabacum* L. var.Wisconsin) were grown in a greenhouse and then transferred to a controlled environment chamber

several days before use. The chamber was maintained at 28°C, 90% relative humidity, 16 h light and 8 h dark cycle. Bacteria were prepared by pelleting and resuspending overnight cultures in 10 mM phosphate buffer at a concentration of $5x10^8$ cells mL^{-1} (A540=0.52). Tobacco leaves were inoculated by pricking them with a dissecting needle and then forcing inoculum into the wound using the open end of a 3 mL disposable plastic transfer pipet pressed against the lower leaf surface[14]. The margins of the water-soaked infiltrated areas were marked and the plants were rated for HR development at 24 h. The percent of HR for different strains was calculated as the proportion of the infiltrated area showing necrosis.

RESULTS AND DISCUSSION

Expression of *hrpN* in *E. coli*: A 1.3 kb *Hind*III fragment from cosmid pCPP430, containing *hrpN* of *E. amylovora*, was used to probe Southern blots of cosmid pESS 411 DNA (*Erwinia stewartii*) restricted with *Hind*III, *Bam*HI and *Kpn*I. The smallest pESS 411 fragment strongly hybridizing with the probe was a 1.8 kb *Hind*III fragment located in the region of the *wts* cluster which was previously shown to be collinear with the $hrpN_{Ea}$. The nucleotide sequence of the entire 1.8 kb *Hind*III fragment was determined. A 1,146 bp open reading frame was identified by homology with *E. amylovora* and *E. chrysanthemi hrpN* genes and designated $hrpN_{Es}$. To confirm the production of $harpin_{Es}$ by its predicted open reading frame and determine its molecular size, the 1.8 kb *Hind*III fragment containing *hrpN* was cloned into vector pT7-7 behind the T7 promotor. This clone, designated pMA2, was used to express $harpin_{Es}$ in *E. coli* BL21(DE3)[32]. The estimated molecular mass of this protein, as determined by SDS-PAGE, was 44 kDa, which agrees with sequence data.

Purification of $harpin_{Es}$: The electro-eluted, acetone-precipitated $harpin_{Es}$ produced in *E. coli* was electrophoressed several times on SDS-PAGE (12%) gels to check its purity. The protein was found to be almost 90% homogeneous and was used to raise antibodies in rabbits. The resulting serum reacted with the 43 kDa $harpin_{Es}$ band in Westerns, but not with the pre-immune serum. Likewise, the antiserum did not react with CFEPs from *wts* mutant (Fig. 1). In addition, antibodies to $harpin_{Ea}$ cross-reacted with $harpin_{Es}$ and vice versa.

$Harpin_{Es}$-deficient mutants of *E. stewartii* are fully pathogenic on corn: The pathogenicity of mutants MA1 *hrpN*123 :: Tn*5*, MA2 *hrpN*254 :: Tn*5* and DM760 *hrpN* 189 :: Tn*5* on corn was compared to that of wild-type strain DC283 with respect to ED_{50} (Table 2), disease

Table 2: ED_{50} of wild-type strain DC 283 and *hrpN* mutants on corn seedlings

	ED_{50}[a]		
Strain	Experiment 1[b]	Experiment 2[b]	Average
DC 283	17	20	18±2
DM 760	26	27	26±1
MA1	26	40	33±10
MA2	20	27	24±5

[a]Number of bacteria/plant needed to cause disease symptoms in 50% of the inoculated plants

[b]Plants were inoculated (pseudo-stem inoculation method) with different concentrations of the appropriate strains as described in the text. Thirty plants/strain/concentration were used. ED_{50} values were determined graphically by plotting dose (bacterial cell numbers) vs the probit of the proportion of the inoculated plants showing disease symptoms eleven days after inoculation

Table 3: Disease severity rating of wild-type strain DC 283 and *hrpN* mutants DM 760, MA1, MA2 and DM 3020Δ *wts* on 8-day-old corn seedlings using three different inoculation methods

Strain	Pseudo-stem[a]	Tooth-pick[b]	Whorl[c]
DC 283	4.3[d]±0.8A[e]	4.98±0.2A	2.77±0.3A
DM 760	4.6±1.1A	4.97±0.2A	2.64±0.7A
MA 1	4.2±1.2A	4.88±0.3A	2.67±0.6A
MA 2	4.4±1.1A	4.98±0.3A	2.53±0.7A
DM 3020	1.1±0.4B	0.00B	0.00B

[a]Five µL of inoculum (1250 cells/plant) were pipeted onto the cut ends of the decapitated 8-day-old corn seedlings (grown as described in the text) and plants were rated 10 days after inoculation using a 1-5 scale: 1 = no symptoms, 2 = scattered lesions, 3 = slight wilting, 4 = severe wilting and 5 = dead

[b]The seedlings were inoculated with sterile tooth-picks dipped into fresh bacterial cultures and inserted into the plants 1 cm above the soil line. The plants were rated as above 10 days after inoculation

[c]Two hundred microliter of inoculum (10^7 cells mL^{-1}) in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.2% Tween 40 were placed in the whorls of corn seedlings. The plants were rated 10 days after inoculation using 0-3 scale: 0 = no symptoms, 1 = a few lesions but no ooze, 2 = many lesions and some ooze and 3 = coalescing lesions and ooze

[d]Each value is the average of 25 plants rated±standard deviation from the mean. The experiment was repeated once with similar results

[e]Means followed by the same letter are not significantly different at p=0.05 level according to the paired t test

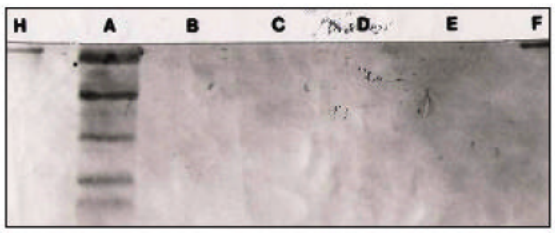

Fig. 1: Western blot of CFEPs from wild-type strain DC283 and several *hrpN* mutants reacting with antiserum to $harpin_{Es}$. All strains contained pRF205 to increase harpin production. The low molecular weight cross-reacting bands in lane A are due to harpin degradation. H=$harpin_{Es}$, A=DC283 (pRF205), B = MA1(pRF205), C=MA2 (pRF205), D=DM760 (pRF205), E=DM3020 (pRF205), F=harpin (from *Erwinia amylovora*)

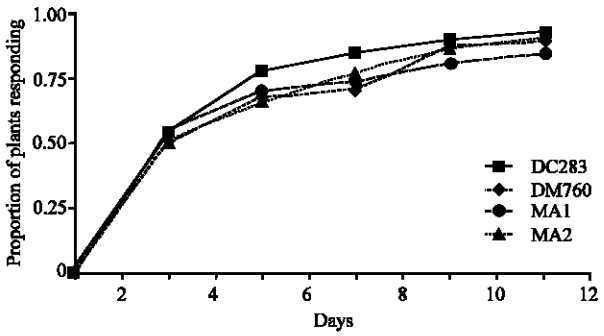

Fig. 2: Response time of wild-type strain DC283 and *hrpN* mutants on corn seedlings. Plants were inoculated with 1250 cells/plant as described in the text. Thirty plants/strain were used. At intervals of 1, 3, 5, 7, 9 and 11 days after inoculation, any plants showing disease symptoms were counted. The experiment was repeated twice with similar results. At any of the above intervals, the proportion of DC283-inoculated plants showing disease symptoms was not significantly different from the proportion of DM760-, MA1-, or MA2-inoculated plants showing disease symptoms using a non-parametric t-test with n_1+n_2-2 degrees of freedom and p = 0.05

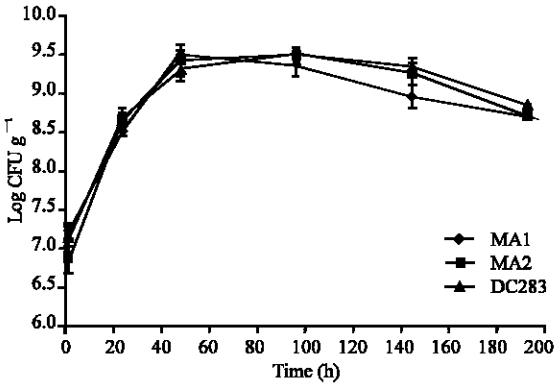

Fig. 3: Growth of wild-type strain DC283 and *hrpN* mutants in corn seedlings. Plants were inoculated with DC283 *hrpN*+, MA1 *hrpN*123::Tn5 and MA2 *hrpN*254::Tn5 as described in the text. At intervals entire plants were sampled (two plants/sample and three samples/strain/interval). The experiment was repeated once with similar results. Bacterial populations are given as the log CFU g^{-1} (fresh weight). Error bars indicate standard deviations.

severity (Table 3), response time (Fig. 2) and growth rate (Fig. 3). ED_{50} ranged from 17 to 26 cells/plant and were not significantly different. The differences between the response times for mutants and the wild-type were not statistically significant. The mutants grew in corn seedlings as good as the wild-type. The results showed that mutations in *hrpN* did not affect the pathogenicity of *E. stewartii*; the mutants were neither quantitatively nor qualitatively different from the wild-type controls.

E. 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, Δ *pelABCE* out of *E. chrysanthemi* elicits rapid necrosis in tobacco leaves. Tn 10 mini-*kan* mutagenesis of $hrpN_{Ech}$ indicated that this gene is required for the HR elicitation in tobacco and contributes in a minor way to pathogenicity on witloof chicory[5,19]. A similar HR-elicitor from *P. syringae* pv. *syringae*[4], $harpin_{pss}$, encoded by *hrpZ*, is required to cause wilting on peas, but it is not needed for pathogenicity on beans[33]. Another HR-elicitor protein, *PopA*, is produced by *P. solonacearum*. The *popA* gene is adjacent to the *hrp* cluster and is co-regulated by HrpB. However, *PopA* is not needed for pathogenicity and is not the sole HR-elicitor made by this bacterium[6], *popA* mutants are fully pathogenic on tomatoes and susceptible petunia lines. Moreover, the *popA* mutant still produces a high molecular weight product that elicits an HR on tobacco and petunia St 40[6]. This suggests that additional proteins, acting as disease determinants, must be secreted via the Hrp pathway.

$Harpin_{Ea}$ is needed for both the elicitation of HR on tobacco and other non hosts and for pathogenicity on apples and pears. Insertion of Tn*5tacI* into the *hrpN* gene rendered *E. amylovora* HR on tobacco and non-pathogenic on pears[15]. However, its role in pathogenesis is not clear yet. He *et al.*[4] suggested that harpin likely functions in pathogenesis to release nutrients from plant cells into the apoplast. Nevertheless, purified $harpin_{Ea}$, in contrast to live bacteria, does not cause necrosis in susceptible apple leaves or immature pear fruit. Nor does it elicit a K^+ efflux/ H^+ influx reaction in apple cell suspension cultures. In an extra-cellular complementation test, $harpin_{Ea}$ added to inoculum could neither restore pathogenicity to $hrpN_{Ea}$ mutants nor their ability to grow in host plants, although it was found to be relatively stable inside host tissue[34]. This suggests that harpin must be introduced directly into apple cells by the *hrp* secretion system or it probably requires additional co-acting proteins in order to affect host cells. One such co-acting protein is the product of *dspE* of *Erwinia amylovora*[13,35] which has been reported to be critical for the pathogenicity of the bacterium on its host.

The extensive similarity between the *hrp* gene cluster of *E. amylovora* and virulence loci of *Yersinia* sp. suggested the transfer via a type III secretion system of

elicitor proteins from a plant pathogen into a plant cell[20]. Type III secretion system has been found in a number of enteric bacteria pathogenic to mammals[11,26]. It is a needle-like protruding structure, resembling bacterial flagella in its structure and function[36], with a channel along which proteins travel. Using this pathway, the effecter proteins of the bacterial pathogens can be delivered either directly into the plant cells or into the extracellular spaces[4,32,37]. Direct transfer of elicitor proteins of plant pathogenic bacteria to the plant cytosol may explain why it has been difficult to isolate *avr* gene products from extra-cellular media and why the predicted products of many of the plant resistance genes cloned to date appear to be cytoplasmic[3] and Avr proteins have no extra-cellular activity on plant cells.

We have been un-able to show even minor effects of a *hrpN* mutation on the ED_{50}, response time or symptoms severity of *E. stewartii* in corn. This finding suggests that additional pathogenicity proteins, possibly acting as cell leakage factors, must be produced and secreted by the *wts/hrp* system. This is supported by our observation that *wtsE* mutants are HR^+ but non-pathogenic on corn (Frederick and Coplin, unpublished). These mutants still produce and secrete $harpin_{Es}$ but cannot cause water-soaking or wilting on corn seedlings. Therefore, genes in the *wtsE* complementation group may encode extra-cellular disease determinants that are secreted in the same way as $harpin_{Es}$.

An important question is why does *E. stewartii* retain the ability to make harpin, if it has no obvious role in pathogenicity. One possibility is that *hrpN* gene of *E. stewartii* may have only weak selective value that is difficult to assay individually.

Another possibility would be that *E. stewartii* has not co-evolved with corn and we are not testing it on its original host. The bacterium reportedly can cause latent infections in a number of hosts[2] suggesting that it is really an endophyte or minor pathogen of North American grasses and it only later became problem on modern cultivars of sweet corn and maize after they were introduced. For this reason it is possible that *hrpN* is needed to colonize a host other than corn. The notion that harpin is, in some way, advantageous to the bacterium is also supported by the fact that it is produced by the related *Erwinia* spp. and *Erwinia* (*Pantoea*) *stewartii* subsp. *indologenes* strains isolated from grasses. CFEPs and culture supprnatants (data not shown) from these strains reacted with $harpin_{Es}$ antibodies. To explore this possibility, the *hrpN* mutant(s) would be tested against the wild-type, using a large number of North American grasses, to see if the wild-type colonizes any of these grasses any better than the mutant(s).

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