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Molecular Characterization of the *hrpN* Gene of *Erwinia* (*Pantoea*) *stewartii*, a Bacterium that Causes Vascular Wilt and Leaf Blight of Corn

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Abstract: The collinear hybridization of the portions of *wts* cluster of *E. stewartii* with the *hrp* cluster of *E. amylovora* suggested that the two clusters were related and that the *wts* and *hrp* genes had a common function in pathogenicity. So, we used $hrpN_{Ea}$ as a probe and located a 1.8 kbp *Hind* III fragment within the *wts* cluster of *E. stewartii*. This gene was designated as $hrpN_{Es}$. Sequencing of this gene revealed a 1,146 bp open reading frame with a typical ribosome-binding site located 6 bases upstream of an ATG translational initiation codon. A promoter sequence, *hrp* box, was found 74 bp upstream of the start of the *hrpN* open reading frame. The *hrpN* ORF encodes a 382 amino acid polypeptide and has a predicted molecular mass of 43 kDa and a PI of 4.28. The polypeptide is rich in glycine, has only three tyrosines and lacks cysteine. The protein is highly hydrophilic and does not show any transmembrane domains. To confirm that the ORF of $hrpN_{Es}$ produces $harpin_{Es}$, the gene was over-expressed in *E. coli* using T 7 system. The estimated molecular mass of this protein, as determined by SDS-PAGE, was 44 kDa which agrees with the sequence data.

Key words: *Erwinia stewartii*, *Pantoea stewartii*, *hrpN* gene

INTRODUCTION

Random transposon mutagenesis of necrogenic and wilt-inducing bacteria has yielded general pathogenicity mutants, called *hrp* (hypersensitive response and pathogenicity) mutants, that are unable to incite either a Hypersensitive Response (HR) on resistant hosts or non-hosts, or a pathogenic response on susceptible hosts. *hrp* genes were initially identified as major determinants of pathogenicity in *Pseudomonas syringae* pv. *phaseolicola* in the mid 1980's[1]. Since then, they have been found in a wide range of Gram-negative plant pathogenic bacteria including *P. syringae* pv. *syringae, glycinea,* tabaci, tomato and *pisi*; many *Xanthomonas campestris* pathovars; *Pseudomonas solanacearum*; *Erwinia amylovora* and *Erwinia chrysanthemi*. These genes are clustered on the chromosome (or on the mega plasmid in *P. solanasearum*) and consist of many complementation groups, which show inter-species DNA sequence homology to each other[2,3]. In some cases, inter-generic sequence homology has also been found[4].

Among the more interesting *hrp* clusters are those of *P. syringae* pv. *syringae* and *E. amylovora*. The *P. syringae* pv. *syringae* cluster is 36 kbp in size and has been cloned on a single cosmid, pHIR 11. This clone restores Hrp^+ phenotype to all *hrp* mutants of *P. syringae* pv. syringae and it also enables non pathogens such as *P. fluorescens* and *Escherichia coli* to cause an HR on tobacco[5]. Mutagenesis of pHIR 11 with Tn*phoA* revealed eleven complementation groups, two of which appear to encode either transmembrane or exported proteins[6]. The *hrp* cluster of *E. amylovora* is the largest of all the *hrp* clusters known so far. It spans over 39 kbp and contains at least nine complementation groups[7]. A full length clone, pCPP 430, enables *E. coli* and other bacteria to produce an HR in tobacco[8].

Although substantial progress in terms of molecular analysis of *hrp* genes was made, their function remained unknown for a long time. One of the problems in predicting the biological function of the product of *hrp* genes has been their hydrophilic nature, i.e. the lack of an N-terminal stretch of 20-25 hydrophobic amino acids and the absence of an N-terminal leader/signal peptide. These characteristics are needed for a protein to be located in the outer membrane or exported via a Sec-dependent (i.e. protein employ the Sec machinery to cross the inner membrane) pathway. The products of most of the *hrp* genes were therefore considered to be cytoplasmic enzymes that had only an indirect role in plant bacteria interactions. Interestingly, nucleotide sequence comparisons of *hrp* genes with genes from mammalian

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pathogenic bacteria (e.g., *Yersinia enterocolitica* and *Y. pestis*) that encode proteins involved in the production of pathogenecity determinants revealed a possible common secretion function. Such an export system could secrete elicitors that interact with plant cells to induce disease or the HR[9].

The above findings prompted Beer *et al.*[8] to look for an unstable, extracellular elicitor encoded by the *hrp* cluster of *E. amylovora*. PMSF (phenyl methyl sulfonyl fluoride, SIGMA)-treated Cell-Free Elicitor Preparations (CFEP) from *E. amylovora* strain 321 and *E. coli* DH5a (pCPP 430) revealed a 44 kDa cell-envelop-associated polypeptide, which was named harpin. This protein causes tobacco leaf lamina to collapse and it increases the pH of bathing solutions of suspension-cultured tobacco cells. The N-terminal sequence of harpin was determined and used to design a synthetic oligonucleotide probe. Using this probe, the harpin gene (*hrpN*) was mapped to the middle of the *hrp* cluster of *E. amylovora*. A mutant carrying a non-polar Tn*5tac* mutation in *hrpN* was non-pathogenic on pear, did not cause an HR in tobacco and did not produce harpin. The non-pathogenic phenotype of the *hrpN* mutant suggests that the harpin is a primary determinant of pathogenicity in *E. amylovora*. That harpin has an essential role in both susceptible and resistant interactions could be based on differential proteolysis of harpin or differential expression of *hrp* genes in host and non-host plants[8].

The genetics of pathogenicity of *E. stewartii*, a Gram-negative, rod-shaped, non-motile plant pathogenic bacterium that causes a vascular wilt and leaf blight of corn, has been studied in detail. In an attempt to identify and clone pathogenicity genes from *E. stewartii*, McCammon *et al.*[10] discoverd a large cluster of general pathogenicity genes, the *wts* genes which enable the bacterium to cause water-soaked lesions on young corn leaves. The right half of this *wts* cluster was cloned in a cosmid designated pES1044 that contains *wtsA, wtsB, wtsC* and *wtsI*. The remainder of the *wts* cluster was cloned in a cosmid designated pES411. The entire cluster is 28 kbp in size and contains seven complementation groups, *wts E, F, D, A, C, I* and *B*[11-13].The *wts* cluster of *E. stewartii* is similar to *hrp* clusters of other bacteria, especially *E. amylovora*. Cosmid pES 1044 and pES411 share considerable homology with pCPP430 and pES1044 was able to restore the Hrp^+ phenotype to several *E. amylovora hrp* mutants[14]. The conservation of function was reflected by collinear hybridization of portions of pES1044 and pES411 with pCPP430[2]. This suggested that *wts* and *hrp* genes had a common function in pathogenicity. We therefore, wanted to know if the *wts* genes cluster of *E. stewartii* had a $hrpN_{Ea}$ homolog.

This study was an attempt to find, clone, mutagenize, sequence and characterize a $hrpN_{Ea}$ homolog from within the *wts* cluster of *E. stewartii*.

MATERIALS AND METHODS

Bacterial strains, plasmids and media: The various experiments in this paper were started earlier 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 [15]; respectively. Culture media, growth of bacteria and mating conditions for *E. stewartii* have been described previously[11,16]. The following antibiotics were used in selective media in the amounts indicated (μgmL^{-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% 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::Tn7[11] or introduced by electroporation using a BIO-RAD Gene Pulsar™ (Model 1652076) according to manufacturer's protocol.

General DNA manipulations: Plasmid DNA isolations, agarose gel electrophoresis, restriction analysis, transformation, ligation, Southern hybridization and random primer labeling were performed according to standard protocols[17]. Non-radioactive Southern blots were done with the Photogene Nucleic Acid Detection System (version 2.0) as described by the manufacturer (GIBCO BRL) except that Western Blue™ Stabilized Substrate for Alkaline Phosphatase (Promega Corporation, Madison, WI) was used for the detection of the hybridizing bands.

To obtain the nucleotide sequence of the 1.8 kbp *Hind* III fragment containing *hrpN*, eight custom primers were designed from the previously sequenced DNA (Fig. 1).

To sequence the *hrpN* promoter region, a primer was designed from the DNA sequence just upstream of the start of the ORF. The single-stranded DNA sequence obtained was compared to *hrp* boxes of other bacteria[18]. All primers were synthesized by DNAgency, Malvern, PA (Table 2). Double-stranded DNA sequence templates were prepared as described by Applied Biosystems, Inc.

Table 1: Bacterial strains and plasmids

Bacterial strains/plasmids	Relevant characteristics[a]	Reference/source
Bacterial strains:		
Erwinia (*Pantoea*) *stewartii*		
DC 283	SS 104 Nal^r	[15]
DM 760	DC 283 *hrpN* 189:: Tn*5*	[22]
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 hsdR⁻ hsdM⁺*RP4-2-Tc::Mu Sm^r Tp^r	Beer[c]
Plasmids:		
pMA1	1.8-kbp *Hind*III fragment cloned in pBS (SK), Ap^r	This study
pMA2	Same as above but cloned in pT7-7	This study
pDM 2513	3 kbp *Hind*III/*BamH*I *hrpN*⁺ fragment of pES411 cloned in pBluescript KS^+ AP^r	Majerczak[b]
pDM 2530	Same as above but cloned in pGP704	Majerczak[b]
pDM 2530::Tn5	Contains *Tn*5 insertion in *hrpN*	Majerczak[b]
pDM 2510	1.1 kbp *Hind*III/*BamH*I fragment of pMA1 cloned in pBluescript SK^-, Ap^r	
pDM 2501	0.7 kbp *Hind*III/*BamH*I fragment of pMA1 cloned in pBluescript SK, Ap^r	Majerczak[b]
pCPP 430	Sp^r, *hrp* gene cluster of *Erwinia amylovora* Ea321	Beer[c]
pES 411	*wts*⁺ clone (*wtsA,C,I,D, F,N,* and *E*) in pVK100 from *E. stewartii* chromosome	[12]
pRF205	1.8 kbp *Hind*III fragment (having *wtsA*) from pES1044 in pVK100	Frederick[b]
pT7-7	Cloning vector, Ap^r	Tabor[d]
pBluescript $KS^+$and SK^-	Ap^r	Stratagene

[a] 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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[d] Department of Biological Chemistry, Harvard Medical School, Boston

Table 2: Primers used in the automated sequencing of the *hrpN* region

Primer	%GC	Sequence		
A	55	5'	CTCGTCATCAGGTCTTCG	3'
B	72	5'	CCACTTCCCTGGCCAGCG	3'
C	66	5'	GTGCCGAGCCTGAACCAC	3'
D	66	5'	GTGGTTCAGGCTCGGCAC	3'
E	72	5'	CGCTGGCCAGGGAAGTGG	3'
F	55	5'	CGAAGACCTGATGACGAG	3'
G	61	5'	CTGCCTGCCTTATGGTGC	3'
H	61	5'	GCACCATAAGGCAGGCAG	3'
P	66	5'	GATGTGCCCAGCGGACTC	3'

(ABI, Foster City, CA). The PCR-based dye-terminator labeling reactions and the preparation of the labeled samples for sequencing were done as recommended by ABI. Automated sequencing was done on an ABI Model 373A sequencer by the OSU Biological Instrumentation Center. Comparison of $HrpN_{Es}$, $HrpN_{Ea}$ and $HrpN_{Ech}$ was done with the Clustal V multiple sequence alignment program[19].

Transposon mutagenesis and marker-exchange: *E. coli* S17-1 λ*pir* (pDM2530) was mutagenized with λTn*5*[20]. Two plasmids were identified that had insertions in the 1.8 kbp *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.

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 of a 3 mL disposable plastic transfer pipet pressed against the lower leaf surface[13]. 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

Molecular cloning and sequencing of the *E. stewartii hrpN* gene: A 1.3 kbp *Hind*III fragment from cosmid pCPP430, containing *hrpN* of *Erwinia* 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 kbp *Hind*III fragment located in the region of the *wts* cluster that was expected to be collinear with the $hrpN_{Ea}$. This fragment was subcloned into pBluescript SK and the resulting clone was designated as pMA1. However, the insert in pMA1 is oriented opposite to vector P*lac* promoter and all attempts to re-clone this in the other orientation failed. Therefore, this clone could not be used for the complementation of *hrpN* mutants.

The nucleotide sequence of the entire 1.8 kbp *Hind*III fragment of pMA1 was determined. A 1,146 bp open reading frame was identified by homology with *E. amylovora* and *E. chrysanthemi hrpN* genes and designated as $hrpN_{Es}$. A typical ribosome-binding site, consisting of GAGGAA was located 6 bases upstream of an ATG translational initiation codon. A promoter sequence, *hrp* box, was found 74-bp upstream of the start of the *hrpN* ORF (Fig. 2). The $hrpN_{Es}$ open reading frame

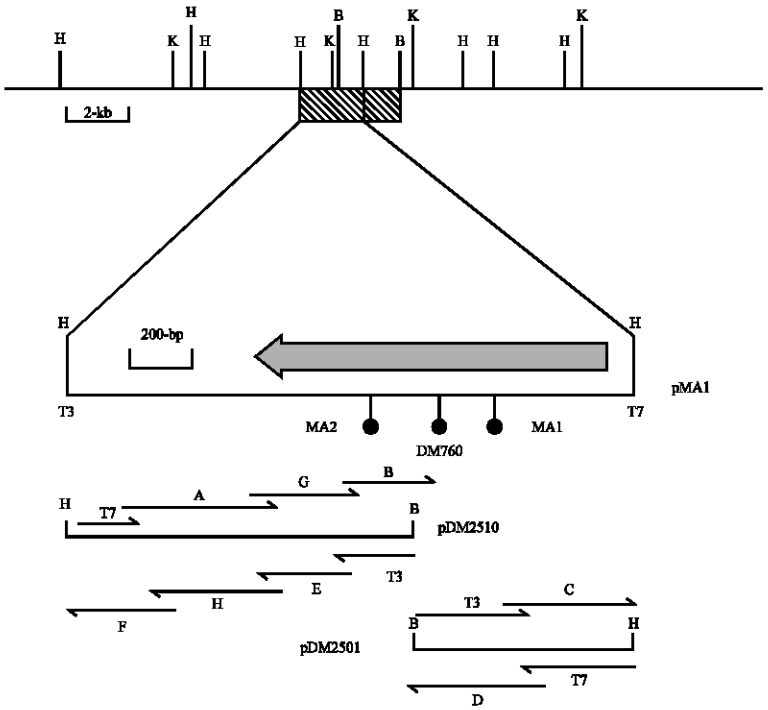

Fig. 1: Physical and genetic map of part of the *E. stewartii* wts region showing the location of the *hrpN* gene. The DNA sequencing strategy for *hrpN* is shown by the arrows below the expanded map of the 1.8 kbp *Hind*III fragment. The lettered arrows represent sequence obtained from the primers listed in Table 2. The shaded arrow above the expanded map indicates the *hrpN* ORF and the direction of transcription. The shaded box indicates the 3 kbp pDM2513 clone used for Tn*5* mutagenesis. Filled circles show the location of Tn*5* insertions *hrpN*123, *hrpN*189 and *hrpN*254 in mutants MA1, DM760 and MA2, respectively. B = *Bam*HI; K = *Kpn*I; H = *Hind*III

1 CTGTTATCTCTGTTTGGACAAGTGAGAACAAACTTTCTTGCTGGTATCAGTTAAGGGAAG

61 AGGGCGCTGATGACAAGCATCAGATTGCACAACTTTTTCAGCTAGCCGGCATCGAAACTG

21 AAGTCTGGATCTGAGTAAGTTTCATCTTTTATGATCTTCCACCTACCTCTGCTAACCCAC

181 TACGCTTTGTATTTACAGCTTTTTTCCACATCGCTGTGCCGCTTTTATTTGCAAATTTCG

241 TTCTTCTGACCCAAACGAAAGTCACGCCCTGTAAACGTCAAATTCCCCCATCACGTGTGA

301 TTATTTTCATTTCAGACAGGAACCAGCACCGCGATAGCGGCACTTAATAAAAGCTTTGCC

361 CGGACACTCACATAGCGCACCGTATGCCAAGTAATCATCACTTATTCAAAGAGGAAGAGG

421 ACATGAGTATGAATACG

Fig. 2: Nucleotide sequence of the promoter region (*hrp* box) of the *hrpN* gene of *Erwinia stewartii*. The putative promoter sequence and the start codon of the *hrpN* ORF are underlined

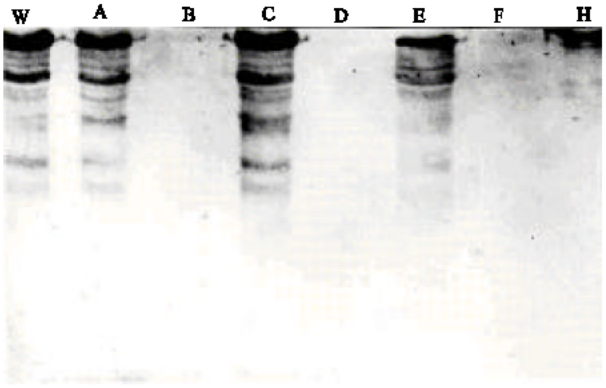

Fig. 3: Immunoblot of CFEPs from DC283(pES411) and several *hrpN* mutants complemented with pES411 reacting with anti-harpin$_{Es}$ serum. pES411*hrpN*189::Tn5 has a transposon insertion in the ORF of *hrpN* gene at amino acid position 189. The low molecular weight cross-reacting bands are due to degradation products of harpin. W = DC283(pES411), A = MA1(pES411), B = MA1(pES 411 *hrpN*189::Tn5), C = MA2 (pES 411), D = MA2(pES 411 *hrpN*189::Tn5), E = DM760 (pES411), F=DM760(pES411 *hrpN*189::Tn5), H = harpin$_{Es}$

encodes a 382 amino acid polypeptide and has a predicted molecular mass of 43 kDa and a pI of 4.28[21]. The polypeptide is rich in glycine (20%), has only three tyrosines and lacks cysteine. Unlike harpin$_{Pss}$, harpin$_{Es}$ does not have C-terminal direct repeats and as expected, has no N-terminal signal sequence for Sec-dependent secretion. The protein is highly hydrophilic and does not show any transmembrane domains. Comparison of the amino acid sequences of predicted harpin$_{Es}$ and harpin$_{Ea}$ proteins revealed significant homology throughout their entire lengths except for a stretch of 10 amino acids in the N-terminus and two regions of 9 and 11 amino acids in the C-terminus. The overall identity and similarity of harpin$_{Es}$ to harpin$_{Ea}$ is 58 and 78%, respectively. Harpin$_{Es}$ is also homologous to harpin$_{Ech}$. The two proteins are 41% identical and 66% similar to each other with higher homology occurring in their C-terminal halves.

Expression of *hrpN* in *E. coli*: To confirm the production of harpin$_{Es}$ by its predicted open reading frame and determine its molecular size, the 1.8 kbp *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). The estimated molecular mass of this protein, as determined by SDS-PAGE, was 44 kDa, which agrees with sequence data.

Mutagenesis of *hrpN*: 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[22]. This mutant was HR$^-$ on tobacco but fully pathogenic on corn (E. Stover, unpublished). Although this result suggested that *hrpN* might not be required for pathogenesis, additional *hrpN*:: Tn*5* mutants were needed to rule out the possibility that the pathogenicity of DM760 was due to the production of a truncated harpin$_{Es}$ molecule that was still biologically active. Two new Tn5 insertions into *hrpN* were isolated and marker-exchanged into the genome of the wild-type strain DC283 to produce mutant strains MA1 and MA2. Marker-exchange of the transposon mutations and the excision of pDM2530 were confirmed by Southern blotting. Direct sequencing placed the respective mutations in DM760, MA1 and MA2 at amino acids 189, 123 and 254 from the N-terminus of the *HrpN*$_{Es}$ peptide. To confirm that the mutants did not produce truncated harpin$_{Es}$ proteins, the CFEPs from the mutants and wild-type strains containing pRF205 were assayed for harpin in Western blots using harpin$_{Es}$ antibodies. The CFEPs from the mutants did not contain any cross-reacting proteins, whereas a protein ladder, typical of harpin, was observed in the parent strain (Fig. 3). In tobacco leaves, the mutants failed to elicit any necrosis at 2.5×10^8 cells mL^{-1}, even when *wts* gene expression was increased by the introduction of pRF205. In contrast, DC283 (pRF205) caused a strong HR within 18 h.

To complement *hrpN* mutants, wild-type cosmid pES411 and a derivative containing *hrpN* :: Tn*5* were introduced into them by triparental matings. The HR phenotype of the *hrpN* :: Tn*5* mutants, with or without pES411, was tested in tobacco. Panels of leaves were infiltrated with 2.5×10^8 cells mL^{-1} of DC283(pES411) and those of the mutants, MA1(pES411), MA1(pES411 *hrpN* 189:: Tn*5*), MA2(pES411), MA2(pES411 *hrpN*189:: Tn*5*), DM760(pES411) and DM760(pES411 *hrpN* 189:: Tn*5*). Only DC283(pES411), MA1(pES411), MA2(pES411) and DM760(pES411) produced a necrotic response. Likewise, when the CFEPs from the above strains were tested, only the CFEP from DC283(pES411) and those from the mutants with pES411 gave an HR or reacted with anti-hapin$_{Es}$ serum in Western blots (Fig. 3). These results indicate that the *hrpN* gene is required for harpin production and the elicitation of the HR by *E. stewartii*.

We demonstrated that *E. stewartii* produces a harpin which is homologous to harpin$_{Ea}$ through its entire length and to the C-terminal half of harpin$_{Ech}$. Comparison of these three proteins suggests that *E. stewartii* and *E. amylovora* harpins are more closely related to each other than they are to harpin from *E. chrysanthemi*.

Nevertheless, all three harpins share many physical and chemical properties with harpins from other plant pathogenic bacteria.

wtsB, *wtsD*, *wtsF* and *wtsE* have been shown to be regulated by *wtsA*[22]. The observation that the harpin content of CFEPs is greatly increased by the presence of a *wtsA* plasmid suggests that *hrpN* is part of the same regulon as the other *wts* genes. *hrpN* appears to constitute a separate transcription unit. This is supported by the following observations: I) mutations in the upstream complementation group *wtsF* do not have a polar effect on *hrpN*, ii) mutations in *hrpN* do not have a polar effect on the downstream operon *wtsE*, iii) Tn*5* insertions mapping immediately upstream and downstream of the *hrpN* are still HR^+ and pathogenic [22] and iv) the *hrpN* ORF is preceded by a *hrp* consensus promoter (*hrp* box) (Fig. 2) and is followed by a fairly strong terminator and another *hrp* box, 5' to *wtsE*.

Although hapins have extensive sequence homology, it appears that the secretion signal is species-specific. *E. stewartii* synthesized $harpin_{Ea}$, but could not secrete the heterologous protein, suggesting that the property of $harpin_{Es}$ which targets it for secretion is different from that of $harpin_{Ea}$. In similar experiments, Bauer *et al.*[23] reported that *E. chrysanthemi* and *E. amylovora* could not secrete each other's harpin. A similar problem have been noticed with heterologous secretion of pectic lyases and cellulases via the Out pathway in *E. chrysanthemi* and *E. carotovora*[24,25].

We demonstrated that $hrpN_{Es}$ is needed for the production of $harpin_{Es}$ and for causing HR on tobacco by *E. stewartii*. However, $hrpN_{Es}$ seems to be of no value for the bacterium in causing pathogenicity on corn. To verify this notion, the three *hrpN* mutants i.e. DM760, MA1 and MA2 will be tested against the wild-type DC 283, in a separate study, using different pathogenicity parameters.

REFERENCES

1. Lindgren, P.B., R.C. Peet and N.J. Panopoulos, 1986. Gene cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity of bean plants and HR on non-hosts. J. Bacteriol., 168: 512-522.
2. Laby, R.J. and S.V. Beer, 1992. Hybridization and functional complementation of the *hrp* gene cluster from *Erwinia amylovora* strain Ea 321 with DNA of other bacteria. Mol. Plant-Microbe Interact., 5: 412-419.
3. Willis, D.K., J.J. Rich and E.M. Hrabak, 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact., 4: 132-138.
4. Gough, C.L., S. Genin, C. Zischeck and C.A. Boucher, 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol. Plant-Microbe Interact., 5: 384-389.
5. Huang., H.C., R. Schurink, T.P. Denny, M.M. Atkinsin, C.J. Baker., I. Yucel, S.W. Hutcheson and A. Collmer, 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *P. fluorescens* to elicit the hypersensitive response in tobacco plants. J. Bacteriol., 170: 4748-4756.
6. Huang., H.C., S.W. Hutcheson and A. Collmer, 1990. Tn*phoA* tagging of *Pseudomonas syringae* pv. *syringae hrp* genes encoding potentially exported proteins. Phytopathology, 80: 984.
7. Bauer, D.W. and S.V. Beer, 1991. Further characterization of an *hrp* gene cluster of *Erwinia amylovora*. Mol. Plant-Microbe Interact., 4: 493-499.
8. Wei, Z.M., R.J. Laby, C.H. Zumoff, D.W. Bauer, S.Y. He, A. Collmer and S. V. Beer, 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science., 257: 85-88.
9. Fenselau, S., I. Balbo and U. Bonas, 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant-Microbe Interact., 5: 390-396.
10. McCammon, S., D.L. Coplin and R. Rowan, 1985. Isolation of avirulent mutants of *Erwinia stewartii* using bacteriophage Mu pf7701. J. Gen. Microbiol., 131: 2993-3000.
11. Coplin, D.L., R.D. Frederick and E.S. Haas, 1986. Molecular cloning of virulence genes from *Erwinia stewartii*. J. Bacteriol., 168: 619-623.
12. Coplin, D.L., R.D. Frederick and D.R. Majerczak, 1992. New pathogenicity loci in *Erwinia stewartii* identified by random *Tn5* mutagenesis and molecular cloning. Mol. Plant-Microbe Interact., 5: 266-268.
13. Bauer, D.W., A.J. Bogdanove, S.V. Beer and A. Collmer, 1994. *Erwinia chrysanthemi hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. Mol. Plant-Microbe Interact., 7: 573-581.
14. Beer, S.V., R.J. Laby, B.J. Sneath and D.L. Coplin, 1990. Complementation of *hrp* mutants of *Erwinia amylovora* with DNA of *Erwinia stewartii*. Phytopathology, 80: 985.

15. Coplin, D.L., R.G. Rowan, D.A. Chisholm and and R.E. Whitmoyer, 1981. Characterization of plasmids in *Erwinia stewartii*. Applied Environ. Microbiol., 42: 599-604.
16. Coplin, D.L., 1978. Properties of F and P group plasmids in *Erwinia stewartii*. Phytopathology, 68: 1637-1643.
17. Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning. A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. Wei, Z.M. and S.V. Beer, 1995. *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF sub-family of factors. J. Bacteriol., 177: 6201-6210.
19. Higgins, D.G., A.J. Bleasby and R. Fuchs, 1992. Clustal V: Improved software for multiple sequence alignment. Computer Applications in the Biosciences (CABISO), 8: 189-191.
20. Dolph, P.J., D.R. Majerczak and D.L. Coplin, 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii*. J. Bacteriol., 170: 865-871.
21. Chou, P.Y. and G.D. Fasman, 1978. Prediction of secondary structure of proteins from their amino acid sequences. Adv. Enzymol., 47: 45-147.
22. Frederick, R.D., M. Ahmad, D.R. Majerczak, A.S. Arroyo-Rodriguez, S. Manulis and D.L. Coplin, 2001. Genetic organization of *Pantoea stewartii* subsp. *stewartii hrp* gene cluster and sequence analysis of the *hrpA*, *hrpC, hrpN* and *wtsE* operons. Mol. Plant-Microbe Interact, 14: 1213-1222.
23. Bauer, D.W., Z.M. Wei, S.V. Beer and A. Collmer, 1995. *Erwinia chrysanthemi harpin*$_{Ech}$: An elicitor of the hypersensitive response that contributes to soft rot pathogenesis. Mol. Plant-Microbe Interact., 8: 484-491.
24. He, S.Y., M. Lindeberg, A.K. Chatterjee and A. Collmer, 1991. Cloned *Erwinia chrysanthemi* Out genes enable *E. coli* to selectively secrete a diverse family of heterologous proteins to its milieu. Proc. Natl. Acad. Sci. USA., 88: 1079-1083.
25. Pye, B., G.P.C. Salmond, M. Chippsux and F. Barras, 1991. Secretion of cellulases in *Erwinia chrysanthemi* and *Erwinia carotovora* is species-specific. FEMS Microbiol. Lett., 79: 315-322.