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Export of the HR Eliciting Protein, $Harpin_{ES}$, of the Maize Pathogen *Erwinia stewartii* is Species-Specific but is Independent of the Growth Temperature

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Abstract: The extra-cellular export of the HR-eliciting protein, $Harpin_{Es}$ of the maize pathogen *Erwinia stewartii* was studied to find out if the protein needs any species-specific signal for its export and to determine if the export of the protein to the medium is affected in any way by the growth temperature. Based upon the experimental evidence, it was proved that the protein (i.e., $Harpin_{Es}$) does require its own export system (species-specific) to get out of the bacterial cell and can not be exported by the export system of even the very closely related bacterium, *Erwinia amylovora*. It was also found that the export of $Harpin_{Es}$ is, unlike the case of $Harpin_{Ea}$ (HR-eliciting protein of *Erwinia amylovora*), independent of the growth temperature.

Key words: *Erwinia (Pantoea) stewartii*, *Erwinia amylovora*, *hrpN* gene, Harpins, TTSS

INTRODUCTION

Pathogens, including bacterial pathogens, possess several classes of genes that are essential for causing disease (pathogenicity genes) or for increasing the amount of disease (virulence genes) on one or a few hosts. A class of general pathogenicity genes called *hrp* (hypersensitive response and pathogenicity) genes is usually found in Gram-negative plant pathogenic bacteria (Agrios, 2005). These genes enable the bacteria carrying them to incite either a Hypersensitive Response or HR (the death of host cells around the point of infection by pathogen) on resistant hosts or non-hosts, or a pathogenic response on susceptible hosts. Harp (*hrp*) mutants are unable to cause either an HR on resistant hosts or non-hosts, or a pathogenicity on susceptible hosts.

One gene of the *hrp* clusters of the phytopathogenic bacteria encode a protein product (Harpin) which causes HR in resistant hosts or non-hosts. DNA sequence analysis of Harpin producing genes of various plant pathogenic bacteria reveal that their protein products (i.e., Harpins) are hydrophilic in nature, i.e., they lack the N-terminal stretch of 20-25 hydrophobic amino acids and the 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 pathway. So, such proteins (i.e., Harpins) can not be exported out of the bacterial cell via the Sec-dependent (Type II) pathway. Hence, if such proteins are to be exported, there should be some special pathway that would not require the N-terminal stretch of hydrophobic amino acids and the N-terminal signal peptide. This special secretion pathway is called the Type Three Secretion System (TTSS) which is a pilus-like protein channel (made up of 15-20 proteins associated with the bacterial cell membrane) delivering proteins directly from the pathogen's cytoplasm to the host cell membrane (Rantakari *et al*., 2001; Li *et al*., 2002; Szurek *et al*., 2002; Greenberg and Vinatzer, 2003). This pathway is formed of proteins called Hrp proteins which are also encoded by *hrp* genes of the *hrp* clusters.

Erwinia stewartii, a gram-negative, rod-shaped, non-motile plant pathogenic bacterium that causes a vascular wilt and leaf blight of corn, has been found to have (McCammon *et al*., 1985) 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 and the left half was cloned in an another cosmid designated pES411. The entire cluster is 28 kb in size and contains seven complementation groups (Coplin *et al*., 1986). 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 (clone containing *E. amylovora hrp* cluster) and pES1044 was able to restore the Hrp^+ phenotype to several *Erwinia amylovora hrp* mutants (Beer *et al*., 1990). The secretion of $Harpin_{Ea}$ to the medium have been reported to dependent on the growth temperature in *Erwinia amylovora* i.e., *E. coli* DH5α(pCCP430) produces $Harpin_{Ea}$ (HR eliciting protein of *Erwinia amylovora*) at

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both 37 and 25°C but exports it to the medium only if cells are grown at 25°C (Wei and Beer, 1993). Since the two bacteria are vary closely related, we were interested to know if similar situation was present in *Erwinia stewartii*. Also, we wanted to know if the TTSS of the two bacteria could export each other's Harpins which would indicate that the export signal was not species-specific.

In this study, we report studies conducted to find out whether or not *Erwinia stewartii* and *Erwinia amylovora*, the two very closely related bacteria, could secrete each other's Harpins and to determine if the secretion of $Harpin_{Es}$, like the secretion of $Harpin_{Ea}$, was dependent on the growth temperature.

MATERIALS AND METHODS

Bacterial strains, plasmids and media: These experiments were done at the department of plant pathology, college of agriculture, The Ohio State University, Columbus Ohio, USA, during 2001-03. 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 (Coplin *et al*., 1981), respectively. Culture media, growth of bacteria and mating conditions for *E. stewartii* have been described previously (Coplin, 1978; Coplin *et al*., 1986). 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, was prepared as described previously (Ahmad *et al*., 2001). Plasmids were mobilized from *E. coli* HB 101 into *E. stewartii* by pRK 2013::Tn7 (Coplin *et al*., 1986) 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 (Sambrook *et al*., 1989). 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.

Mutagenesis of the *hrpN* gene: *E. coli* S17-1 λ*pir* (pDM2530) was mutagenized with λTn*5* (Dolph *et al*., 1988). Two plasmids were identified that had insertions in the 1.8-kb *Hind*III fragment (containing the *hrpN* gene). 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.

Western blots of the culture supernatants: To obtain Culture Supernatants (CS) from DC283 (pRF205) cells and cells of other strains, the bacteria were grown overnight and CSs were extracted as described previously (Ahmad *et al*., 2001). For Western blotting, CSs were electrophoresed and electroblotted on an ImmunoSelect (GIBCO BRL) nitrocellulose membrane (Sambrook *et al*., 1989). The filter was probed with anti-$Harpin_{Es}$ serum and immunodetection of the bands was performed with rabbit alkaline phosphatase-conjugated secondary antibody (Protoblot® II AP system kit) according to the manufacturer's (Promega) protocol.

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	15
DM 760	DC 283 *hrpN* 189:: Tn*5*	22
MA 1	DC 283 *hrpN* 123:: Tn*5*	Present study
MA 2	DC 283 *hrpN* 254:: Tn*5*	Present study
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-kb *Hind*III fragment cloned in pBS (SK), Ap^r	Present study
pMA2	Same as above but cloned in pT7-7	Present study
pDM 2530	3-kb *Hind*III/*BamH*I *hrpN*$^+$ fragment of pES411 cloned in pGP704	Majerczak[b]
pDM 2530::Tn5	Contains *Tn5* insertion in *hrpN*	Majerczak[b]
pCPP 1012	A part of *hrp* gene cluster (containing $hrpN_{Ea}$) of *Erwinia amylovora* Ea321, Sp^r	Beer[c]
pRF205	1.8-kb *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; [b]Department of Plant Pathology, The Ohio State University, Columbus; [c]Department of Plant Pathology, Cornell University, Ithaca, New York; [d]Department of Biological Chemistry, Harvard Medical School, Boston

Western blots of the cell free elicitor preparations: To obtain the Cell Free Elicitor Preparations (CFEPs) from the *hrpN* mutants of *Erwinia stewartii* complemented with *Erwinia amylovora hrpN*-containing clone pCPP1012 i.e., DM760(pCPP1012),MA1(pCPP1012)andMA2(pCPP1012) and from the *hrpN* mutants of *Erwinia stewartii* having the *hrp* gene enhancer clone pRF205 i.e., DM760(pRF205), MA1(pRF205), MA2(pRF205), the cells were grown overnight and centrifuged to get the pellet as described elsewhere (Ahmad *et al*., 2001). The pellet was resuspended in 10 mL of 50 mM potassium phosphate buffer (pH 6.5) and transferred to sterile metal tube. The cells were disrupted by sonication using the procedure of Wei *et al*. (1992). The CFEPs were concentrated with 10% TCA and used for Western blots as described before (Ahmad *et al*., 2001).

Bioassays for HR in tobacco: Tobacco plants (*Nicotiana tabacum* L. var.Wisconsin) were grown and used for hypersensitive response bioassays for *Erwinia stewartii hrpN* mutants carrying the *Erwinia amylovora hrpN*-containing clone pCPP1012 and *Erwinia stewartii hrpN* mutants carrying the *hrp* gene enhancer clone pRF205 as described before (Ahmad *et al*., 2001).

RESULTS AND DISCUSSION

A Tn5 insertion mutation was produced near the center of the open reading frame of the *hrpN* gene and marker-exchanged into the chromosome of the wild-type strain DC283 to produce the mutant strain DM760 (Frederick *et al*., 2001). This mutant was HR$^-$ on tobacco but fully pathogenic on corn (Stover, unpublished). In order to make the studies more reliable and conclusive, additional *hrpN*::Tn5 mutants were needed. 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 (Fig. 1). The 1.8 kb *Hind*III fragment (lower bands in lane 1 and 9) and 2.4 kb *Bam*HI fragment (lower band in lane 5) have been replaced by two Tn5 junction fragments of the predicted sizes in the *hrpN* mutants confirming the insertion of Tn5 sequence into the open reading frame of the *hrpN* gene.

The immunoblot analysis of Cell-free Elicitor Preparations (CFEPs) from *Erwinia stewartii hrpN* mutants complemented with *Erwinia amylovora hrpN*-containing clone pCPP1012 and reacted with Harpin$_{Ea}$ antiserum is shown in Fig. 2. The results indicate that the mutants i.e., DM760(pCPP1012), MA1(pCPP1012) and MA2(pCPP1012), did produce the *Erwinia amylovora*

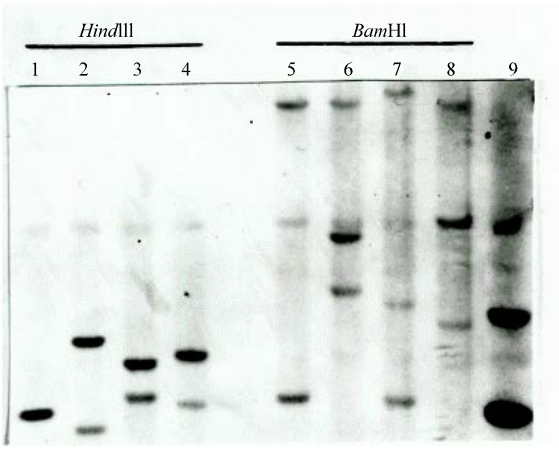

Fig. 1: Southern blot of genomic DNA from wild-type strain DC283 and *hrpN* mutants to verify marker-exchange of Tn5 insertions into the chromosome. Genomic DNA was digested with *Hind*III or *Bam*HI, electrophoresed through an 0.8% agarose gel, stained with ethidium bromide, photographed and then used for Southern transfer. The blot was probed with the 1.8 kb *Hind*III insert from pDM2507 containing the *hrpN* region. 1.8 kb *Hind*III fragment (lower bands in lane 1 and 9) and 2.4 kb *Bam*HI fragment (lower band in lane 5) have been replaced by two Tn*5* junction fragments of the predicted sizes in the *hrpN* mutants. Lane 1 and 5 = DC283 DNA, Lane 2 and 6 = MA1 DNA, Lane 3 and 7 = MA2 DNA, Lane 4 and 8 = DM760 DNA and Lane 9 = HindIII-digested pDM2507 DNA bands (upper band = 3 kb vector DNA and lower band = 1.8 kb insert DNA fragment)

Harpin intracellularly as is evident from the bands of 44 kd size (the size of Harpin$_{Ea}$) reacting with anti-Harpin$_{Ea}$ serum in the lanes of CFEPs from all the mutants. However, all these mutants (each having the *Erwinia amylovora hrpN*-containing clone pCPP1012) were found to be not able to export the foreign Harpin i.e., Harpin$_{Ea}$ out of the cell as neither of them could cause an HR on tobacco (Table 2). It should be noted that the *Erwinia amylovora hrpN*-containing clone pCPP1012 does provide *hrpN* gene for Harpin$_{Ea}$ production but no genes for TTSS.

We demonstrated (Ahmad *et al*., 2005a, b) that *Erwinia stewartii* produces a Harpin (protein product of the *hrpN* gene) which is homologous to Harpin$_{Ea}$ (protein product of the *hrpN* gene of *Erwinia amylovora*) through its entire length and to the C-terminal half of Harpin$_{Ech}$ (protein product of the *hrpN* gene of *Erwinia chrysanthemi*). Comparison of these three proteins

Table 2: Tobacco HR assay of cell-free elicitor preparations (CFEPs) from *Erwinia stewartii hrpN* mutants containing *Erwinia amylovora* $hrpN_{Ea}^{+}$ clone pCPP1012

		Hr[b] by CFEPs[a] (%)			
Strain	HR by cells (%)	Expt.I	Expt.II	Expt.III	Mean±SD
MA1(pCPP1012)	0	100[c]	100	100	100
MA1(pRF205)	0	0	0	0	0
MA2(pCPP1012)	0	85	90	70	81±10
MA2(pRF205)	0	0	0	0	0
DM760(pCPP1012)	0	90	88	95	91±3
DM760(pRF205)	0	0	0	0	0
$Harpin_{Ea}$	100	100	70	95	91±16

[a]CFEPs were extracted from bacteria grown overnight at 28°C in IM (pH 5.5) according to Wei *et al.* (1992) and then infiltrated into tobacco leaves; [b]% HR was determined by estimating the percentage of the infiltrated area that was necrotic at 24 h; [c]Each value is the mean of three replicates±Standard Deviation from the mean

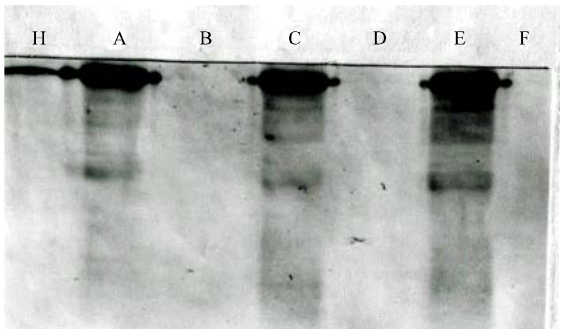

Fig. 2: Immunoblot of cell-free elicitor preparations from *Erwinia stewartii hrpN* mutants complemented with *Erwinia amylovora hrpN*-containing clone pCPP1012 reacted with $Harpin_{Ea}$ antiserum. The low molecular weight cross-reacting bands are due to Harpin degradation. The experiment was repeated once with similar results. H = $Harpin_{Ea}$, A = DM760(pCPP1012), B = DM760(pRF205), C = MA1(pCPP1012), D = MA1(pRF205), E = MA2(pCPP1012), F = MA2(pRF205)

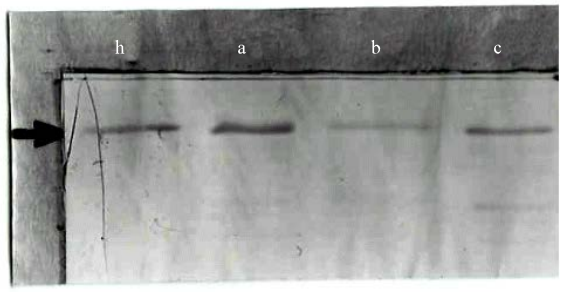

Fig. 3: Western blot analysis of $Harpin_{Es}$ production and secretion by DC283(pRF205) grown at three different temperatures. Harpin is indicated by the arrow. Cells were pelleted and discarded. The concentrated samples were used for SDS PAGE and Western blots. h = purified Harpin, a = CS from cells grown at 25°C, b = CS from cells grown at 28°C and c = CS from cells grown at 32°C

suggests that the Harpins of *Erwinia stewartii* and *Erwinia amylovora* are more closely related to each other than they are to harpin from *Erwinia chrysanthemi*. Nevertheless, all three Harpins share many physical and chemical properties with Harpins from other plant pathogenic bacteria.

Although Harpins have extensive sequence homology, it appears that the secretion signal is species-specific. *Erwinia 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.* (1995) reported that *Erwinia chrysanthemi* and *Erwinia amylovora* could not secrete each other's Harpins. A similar problem have been noticed with heterologous secretion of pectic lyases and cellulases via the Out pathway in *Erwinia chrysanthemi* and *Erwinia carotovora* (He *et al.*, 1991; Pye *et al.*, 1991).

Western blot analysis of $Harpin_{Es}$ production and secretion by DC283(pRF205) grown at three different temperatures is shown in Fig. 3. The results show that *Erwinia stewartii* produces and exports $Harpin_{Es}$ at all the three temperatures (i.e., 25, 28 and 32°C) tested. This is evident from the bands of 43 kd size (the size of $Harpin_{Es}$) reacting with anti-$Harpin_{Es}$ serum in the lanes of Culture Supernatants (CSs) from DC283(pRF205) cells grown at 25, 28 and 32°C. Neither of these temperatures adversely affects or enhances the production or secretion of $Harpin_{Es}$. The detection of $Harpin_{Es}$ in culture supernatants from DC283(pRF205) cells grown at three different temperatures suggests that $Harpin_{Es}$ secretion is not restricted to any of the temperatures tested. This is in contrast to the findings of Wei and Beer (1993) that *E. coli* DH5α(pCCP430) produces $Harpin_{Ea}$ (HR eliciting protein of *Erwinia amylovora*) at both 37 and 25°C but exports it to the medium only if cells are grown at 25°C. It should be kept in mind that *E. coli* DH5α(pCCP430) carries the full length clone i.e., the 39 kb *hrp* cluster of

Erwinia amylovora and so the TTSS of *E. coli* is actually made up of Hrp proteins of *Erwinia amylovora* origin. In *Erwinia amylovora*, *hrp* gene expression is favored by low temperature (Wei *et al*., 1992). Nevertheless, our results are qualitative, so it is possible that we missed a more subtle effect of temperature on $Harpin_{Es}$ secretion.

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