Research Note

The *hrpC* and *hrpN* Operons of *Erwinia chrysanthemi* EC16 Are Flanked by *plcA* and Homologs of Hemolysin/Adhesin Genes and Accompanying Activator/Transporter Genes

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The *hrpC* operon of *Erwinia chrysanthemi* EC16 encodes five genes conserved in *Erwinia amylovora* and *Pseudomonas syringae*. Mutagenesis indicated that *hrcC* is required for elicitation of the hypersensitive reaction in tobacco leaves. The unexpected presence of *plcA* and homologs of hemolysin/activator genes in the regions flanking the *hrcC* and *hrpN* operons is reported.

Additional keywords: *Bordetella*, Hrp pathway, phospholipase C, type III secretion.

Clusters of *hrp* genes required both for elicitation of the hypersensitive reaction (HR) on nonhost plants and pathogenicity on host plants are conserved in many phytopathogenic bacteria (reviewed in Alfano and Collmer 1997). The majority of *hrp* genes encode components of a protein secretion system called the Hrp (type III) pathway. Some of the *hrp* secretion genes are widely conserved even among animal-pathogenic bacteria such as *Yersinia*, *Salmonella*, and *Shigella* spp.; these have been designated *hrc* (Bogdanove et al. 1996a). The existence of *hrp/hrc* genes in soft-rotting bacteria like *E. carotovora* and *E. chrysanthemi*, which do not normally induce the HR, was suggested based on Southern hybridization (Laby and Beer 1992). Furthermore, mutants of *E. chrysanthemi* EC16 deficient in pectate lyases were demonstrated to cause a typical HR (Bauer et al. 1994). To date, two *hrp/hrc* genes, *hrpN* and *hrcT*, have been identified from *E. chrysanthemi* (Bauer et al. 1994, 1995).

Among the proteins of the Hrp pathway, HrcC is of particular interest because it is a member of the broadly conserved PulD/pIV superfamily of proteins that function in outer-membrane translocation of both the type II and type III secretion pathways (Russel 1994). We characterized the genomic region of *E. chrysanthemi* EC16 encompassing the

hrpN gene and the *hrpC* operon that contains the *hrcC* gene, and compared it with the corresponding region of *E. amylovora*. We report here (i) similarities in the *hrp* systems of two bacterial plant pathogens that employ different attack strategies, (ii) evidence that *hrcC* is required by *E. chrysanthemi* to elicit the HR, (iii) the discovery of *hrp*-linked potential virulence genes seemingly unrelated to the Hrp system, and (iv) evidence that this gene arrangement is conserved among diverse strains of *E. chrysanthemi*.

Sequence analysis of Hrp mutants indicated that *hrpN* and *hrcU* mark the ends of the *hrp* gene cluster of *E. amylovora*, and that *hrcT* is situated upstream of *hrcU* (Wei et al. 1992; Wei and Beer 1993; Bogdanove et al. 1996b). Based on the presence of both *hrpN* and *hrcT* together in individual cosmid clones, pCPP2156, pCPP2157, and pCPP2158 (data not shown), the *hrp/hrc* genes of *E. chrysanthemi* EC16 also seemed clustered and, therefore, *hrcC* was likely to be contained in these cosmids. Southern hybridization was used to confirm the presence of *hrcC* in the three cosmids. The cosmids were digested with *Bam*HI or *Sal*I, and then incubated with the ³²P-labeled, 2.1-kb *Bam*HI-*Hpa*I fragment from pCPP1036 that contains *hrcC* of *E. amylovora* Ea321 (Kim et al. 1997). Restriction fragments of 8 to 11 kb in size from each cosmid hybridized to the probe (data not shown). One of them, the 8.2-kb *Sal*I fragment of pCPP2157, was the insert of pCPP2142 that had been constructed to isolate *hrpN_{Ech}* (Bauer et al. 1995).

The nucleotide sequence upstream of *hrpN_{Ech}* in pCPP2142 was determined with an ABI 373A automated DNA sequencer by the DNA Sequencing Facility of the Cornell University Biotechnology Program, using synthesized primers. An apparent five-cistronic operon, which is preceded by an HrpL-dependent promoter-like sequence, GGAACC-N₁₅-CCACAC AA, was identified and named *hrpC* (Fig. 1A). Open reading frames (ORFs) in *hrpC*, designated *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV*, were remarkably similar and collinear to corresponding ORFs in the *hrpC* operons of *E. amylovora* and *P. syringae* (Kim et al. 1997; Huang et al. 1992, 1995; W.-L. Deng, G. Preston, H.-S. Huang, and A. Collmer, *in preparation*) (Table 1). Among the five encoded proteins in the *hrpC* operon of the three bacteria, HrcC showed the highest amino

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The nucleotide sequence reported in this paper has been deposited in GenBank under accession number L39897, which contains the previously published *hrpN_{Ech}* sequence (D. W. Bauer, Z.-M. Wei, S. V. Beer, and A. Collmer, Mol. Plant-Microbe Interact. 8:484-491, 1995).

acid identities. HrpT has an N-terminal lipoprotein signal peptide, and it could be a periplasmic chaperone for HrcC, as in type II secretion systems (Hardie et al. 1996). As in type II secretion systems (Hardie et al. 1996). As in *E. amylovora* and *E. chrysanthemi*, the 5' region of *hrpN* of *E. carotovora* subsp. *carotovora* (Mukherjee et al. 1997) contains *hrpV_{Ecc}* (J. F. Kim, personal observation), indicating conservation of the location of the *hrpC* operon relative to *hrpN* among the three *Erwinia* spp.

To determine the role of *hrcC_{Ech}* in HR elicitation, a non-polar *hrcC* mutant was generated by marker exchange-eviction mutagenesis (Ried and Collmer 1987) of CUCPB5006, a PelABCE⁻ derivative of *E. chrysanthemi* EC16 that causes a typical HR in tobacco leaves (Bauer et al. 1994). The 3.9-kb *Bam*HI-*Eco*RI fragment of pCPP2142, which contains the entire *hrpC* operon, was cloned into pBC SK (-) and pBluescript II KS (-) (Stratagene, La Jolla, CA), producing pCPP1221 and pCPP2355, respectively (Fig. 1A). pCPP1221 was digested with *Nar*I to eliminate most of *hrcC*,

the protruding ends were filled in with dGTP/dCTP (Perkin-Elmer, Branchburg, NJ) and the Klenow fragment (Boehringer Mannheim, Indianapolis, IN) to make the *hrcC* deletion in frame, and the deleted region was replaced with the *nptI-sacB*-

Table 1. Comparison of proteins in the *hrpC* operon of *Erwinia chrysanthemi* EC16 with the corresponding proteins of *Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae*^a

Bacterium	Protein amino acids similar/identical (%/%) with those of <i>E. chrysanthemi</i>				
	HrpF [75] ^b	HrpG [144]	HrcC [691]	HrpT [61]	HrpV [136]
<i>E. amylovora</i>	76/62	57/39	82/69	65/48	55/39
<i>P. syringae</i> pv. <i>syringae</i>	47/26	39/21	69/48	58/37	48/21

^a BESTFIT program (GCG software package, version 7.3), with default parameters, was used to compare the proteins.

^b Number of amino acid residues of the *E. chrysanthemi* proteins.

Fig. 1. The *hrpC* and *hrpN* region of *Erwinia chrysanthemi* EC16 and the requirement of *hrcC* for hypersensitive reaction (HR) elicitation. **A**, Genetic organization of the *hrpC* and *hrpN* region of *E. chrysanthemi* EC16 (Keen et al. 1992; Bauer et al. 1995; this study) and subclones used for sequencing and marker-exchange mutagenesis. Corresponding region in *E. amylovora* (Wei et al. 1992; Kim et al. 1997; Kim 1997) is shown for comparison. Directions of transcription indicated by arrow boxes. Filled triangles are putative HrpL-dependent promoters; vertical black bars are KdgR-box-like sequences. The hairpin indicates a putative transcription terminator site. Open reading frames indicated by asterisks contain potential signal peptides in their deduced protein sequences. Restriction enzymes: H, *Hind*III; B, *Bam*HI; Hp, *Hpa*I; P, *Pst*I; S, *Sal*I; N, *Nar*I. **B**, Tobacco leaf showing effect of non-polar *hrcC* mutation. Panels: 1, CUCPB5006 (contains intact *hrpC*); 2, CUCPB5089 (*hrcC* mutant); 3, CUCPB5089(pCPP2354; contains *hrpF*G*hrcC*). Leaf was photographed 24 h after infiltration.

Table 2. Comparison of HecB and HecA with homologs^a

Bacteria containing homologs of HecB and HecA, and similar/identical amino acid percentages of the homologous proteins							
<i>Erwinia chrysanthemi</i> proteins	<i>Bordetella pertussis</i>	<i>Serratia marcescens</i>	<i>Edwardsiella tarda</i>	<i>Proteus mirabilis</i>	<i>Haemophilus ducreyi</i>	<i>Haemophilus influenzae</i>	<i>Haemophilus influenzae</i>
HecB	FhaC (49/26)	ShlB (47/30)	EthB (49/28)	HpmB (48/26)	HhdB (50/24)	HMW2B (45/22)	HxuB (46/20)
HecA ^b	FhaB (63/44)	ShlA (60/39)	EthA (56/38)	HpmA (51/37)	HhdA (52/33)	HMW2A (51/26)	HxuA (48/23)
^a Determined by BESTFIT program (GCG software package, version 7.3).							
^b Only the N-terminal 258 amino acids of HecA were compared.							
HecB-Ech	- - - - -	- [M] P V S S R K T [G] L [I] A C [L] S [L] I G Y T - - A [S] A F [S] A S [L] N P A [E] - [R] S [E]	35				
FhaC-Bpe	M T D A T N R F R P G L V G R A L V R A [G] L L - - F A V A A C A - - Q A Q L L P G A R D L N - R I [D]	45					
ShlB-Sma	- - - - -	- [M] I K K I T A L T L L V S T A L S A E T L P D [S] H M M Q D [M] S M G [E] S [R] R A	38				
HecB-Ech	I [Q] Q R [Q] S [E] V - - - [I] E [Q] S [R] Q [Q] R E A L [Q] Q L N D I A [Q] A P L K A D N A P Q [G] P C F T L R D I R	82					
FhaC-Bpe	D R [Q] R K E [Q] L Q R D I E R A L T R P P V E L N P Q S [E] A A A P A R K P D A T S [G] H T V T V H A V D	95					
ShlB-Sma	L [Q] D S T R [E] V N Q L [I] E [Q] R R Y [Q] Q L K Q [Q] R L L A E P A A P A L P Q S A - - - Q [C] L P I A G V Y	85					
HecB-Ech	[F] N H S [T] L L R P S [D] Q A T L V A [G] Y L N R C N N L E Q I N Q L V H [D] V S N W [Y] I Q R G Y I T S R A	132					
FhaC-Bpe	[L] D F G [V] E G R L F D P A P L V Q D Y L N R P L D N E Q L F L L V K A L S A A L Y D R G Y A T S I V	145					
ShlB-Sma	[L] Q G V [T] L L S [P] A D L S A L - S [G] L P E Q C I S S N D I N R L T R E L T R L Y V V Q K G Y I T A R V	134					
HecB-Ech	F [L] S E Q D L [S] G G V L Q L E I L E G R L E K I T I N N Q T E R M T R - - - - - T A F P A H E G E	176					
FhaC-Bpe	T F V P P G V V D G V L K L K V E W G R I K G W L I D G K P L E G T R D R M M V F S A M P G W Q D K	195					
ShlB-Sma	Q I V R P N - [S] Q G E L G L S V T E G F I E K I - - - - - E G G D R W V N S R L L F P G L E G K	176					
HecB-Ech	[I] L N L R D I E Q G M E Q M N R M P S [Q] Q V T I E I Q P G S [Q] P G Y S V V N L T R E A H L P F S G G	226					
FhaC-Bpe	V L N [V] F D I D Q [A] I Y N I N N - G G K T G N I T I V P A D E Y G Y S Y L D L Q L Q R R A L P R V S	244					
ShlB-Sma	P L K L T E L D Q G L D Q A N R L Q S N T T K L D I L P G R Q V G G S V I R L R N Q H A K P W L I T	226					
HecB-Ech	[L] T F D N S G Q K S T G E E - - Q L N G S L A L D N V F G L A D Q W F I S A G H S S R F A T S H D A	274					
FhaC-Bpe	[L] G M D N S G P G T P E N G R Y K Y N A S V T A N D L L G L N D T L G L Y I G N R Y Y R D A G H D A	294					
ShlB-Sma	A [G] T D N Y G Q K S T - - G R W L A R A T A T L D S P F G L S D - F V S L N A N S T L E N P A H R Y	273					
HecB-Ech	[E] - S L Q A G F S M P Y G Y W N L G Y N Y [S] Q S R Y R N T F I N R D F P W H S T G D S D T H R F S L	323					
FhaC-Bpe	[E] R N Y D L M Y S [V] P L G R T R L D L Q T G Y S T Y R N L L K T R Y G Q Y Q S A G N S R S F G L K A	344					
ShlB-Sma	N R A Y T L L Y S L P Y G A F T F S G F A S F S S Y E N H Q Q L P H N V V K L H G Q T Q Q Y G L R S	323					
HecB-Ech	S R V V F R D G T M K T A I A G T F S Q R T G N N Y L N G S L L P S S S R K L S S V S L G V N H S Q	373					
FhaC-Bpe	T R L L Y R D T R S Q F S V Y G G L K L R Q N K N Y L A G T R L D V S S K H Y S D V T V G M Q Y S T	394					
ShlB-Sma	D Y V F Y R D H D Q I D S L S G Q L T Y K R I D N Y F E S V R L E V S S P T L T L A E L S A S H L Q	373					
HecB-Ech	K [L] W G G L A T F N P - T Y N R G V R W L G S E T D T - - D K [S] A D E P R A E F N K W T L S A S Y -	419					
FhaC-Bpe	Q - R G A N A Y F G D L S F T R G V G V N N G K Y A A Y D E R G P Q G N V S R F N G S L A W T R Y -	442					
ShlB-Sma	I [L] P N G V - F S A N L S V E Q G M P W L G A G - - - R H P S S V H L D S Q F T K G K L F A N L S	418					
HecB-Ech	- - Y H P V T D S [I] T Y L G S [L] Y G Q Y S A R A [L] Y G S E Q L T L G G E S S I R G F R E Q Y T - S G	466					
FhaC-Bpe	- - M A L A G Q P I Q W A S Q L G F Q Y S R Q Q L L N S Y Q I T V G D E Y T V R G Y N L R T S Q S G	490					
ShlB-Sma	Q R L R L G D A T Y Q L N N L F Y G Q Y S R D P L P G V E W L S L T D R S A V R G F S - R S T Q S G	467					
HecB-Ech	N R G A Y W R N E L N W Q A W Q L P V L G N V T F M A A V D G G H L Y N H K Q D N S T A A S L W G G	516					
FhaC-Bpe	D S G V Y L S N T L T V P V - Q F S L L G K Q A S V A P F V G A D V G A L K S N H P D A R T I R M A	539					
ShlB-Sma	D N G W Y L Q N T L S R - - - - S F N L G A T T L T P R L G A D V G R I L P R Q D N S G W R S S A	512					
HecB-Ech	A [V] G M T V A S R W L S Q Q [V] T V G W P I S Y P A W L Q P D T M V V G - - - - Y R V G L S F	558					
FhaC-Bpe	[G] L A A G V - - R F D L P Y A R M S F T Y S K P V G A Q P G G A P R A P V W L Y I N A G L S F	584					
ShlB-Sma	G I S T G A T L R Y Q R A L V D L E V S R G W I L S N H A T P E D P V Q V L A R F S Y T F - -	557					

Fig. 2. Alignment of HecB and its homologs by PILEUP program (GCG software package, version 7.3) with default parameters. Ech, *Erwinia chrysanthemi*; Bpe, *Bordetella pertussis*; Sma, *Serratia marcescens*.

sacR cartridge, which confers Km resistance and sucrose sensitivity to create the *hrcC_{Ech}::nptI-sacB-sacR* plasmid pCPP2365. This plasmid was electroporated into *E. chrysanthemi* CUCPB5006 with a Gene Pulser (BioRad, Richmond, CA) and subsequently marker exchanged into the CUCPB5006 chromosome to produce CUCPB5088 (Km^r, Suc^r). A similar $\Delta hrcC$ construct, pCPP2366, was made by digestion with *NarI*, end filling, and self-ligation of pCPP2355 (Fig. 1A). pCPP2366 was then electroporated into CUCPB5088 and introduced into the chromosome through double cross-over. One of the Km^r, Ap^r, and Suc^r colonies, which has a 1,905-bp in-frame deletion of *hrcC*, was designated CUCPB5089. To assay in tobacco, CUCPB5089 was grown on King's B plates (King et al. 1954) for 24 h, and the colonies were suspended to approximately 5×10^8 CFU/ml in 5 mM morpholino-ethanesulfonic acid, pH 6.5. Tobacco leaves (cv. Xanthi) infiltrated with CUCPB5089 did not exhibit the HR, demonstrating that *hrcC* is required for HR elicitation (Fig. 1B). Transformation of the mutant with pCPP2354, which carries *hrpF*, *hrpG*, and *hrcC*, restored the HR, indicating the nonpolar nature of the mutation.

Fig. 3. Polymerase chain reaction amplification of DNA between (A) *plcA* and *hrpF*, and (B) *hrpN* and *hecB*. Chromosomal DNA used from: *Erwinia chrysanthemi* strains EC16 (lane 1), ATCC27385 (lane 2), NCPPB1861 (lane 3), NCPPB427 (lane 4), NCPPB2543 (lane 5), 113-2 (lane 6), CFBP1448 (lane 7), CUCPB1237 (lane 8), 0-596 (lane 9), PDDCC2349 (lane 10), 3937 (lane 11), 4227 (lane 12); *E. carotovora* subsp. *atroseptica* ATCC33260 (lane 13); *E. carotovora* subsp. *carotovora* strains ATCC15713 (lane 14), DB17 (lane 15); *E. stewartii* DC283 (lane 16); *E. amylovora* Ea321 (lane 17); and *E. herbicola* Eh252 (lane 18). M indicates 1-kb DNA ladder (Life Technologies, Gaithersburg, MD).

Sequence analysis upstream of *hrpC_{Ech}* revealed *plcA*, the gene encoding phospholipase C (Keen et al. 1992) (Fig. 1A). In *E. amylovora* and *P. syringae*, the comparable location is occupied by another *hrp* secretory operon (Kim et al. 1997; Huang et al. 1995). Differences were also evident in the region downstream of *hrpN_{Ech}*. The deduced translation product of the ORF that is transcribed convergent to *hrpN*, designated ORF1, showed similarity to proteins that are involved in biosynthesis of aromatic polyketides such as CurC of *Streptomyces curacoi* (50% similar, 26% identical) (Bergh and Uhlen 1992).

BLAST searches (Altschul et al. 1990) of the two ORFs next to ORF1 (Fig. 1A) indicated homology to Ca²⁺-independent hemolysins and the outer-membrane hemolysin activator/transporter proteins of several Proteobacteria (Hirono et al. 1997 and references therein) (Table 2). These ORFs were named *hecA* (hemolysin homolog) and *hecB* (hemolysin activator/transporter homolog), after ORFs encoding hemolysin-like proteins in *E. chrysanthemi*. Other homologs of HecA and HecB included adhesins and accessory processing proteins such as FhaB/FhaC of *Bordetella pertussis* and HMWA/HMWb of nontypeable *Haemophilus influenzae* (Willems et al. 1994; Barenkamp and St. Geme 1994). Both HecA and HecB contained potential N-terminal signal peptides. HecB was predicted to contain many amphiphilic β -sheets that could form a β -barrel, and the sequence ended with phenylalanine, which is typical of outer-membrane proteins (Fig. 2). The N-terminal, 258-amino-acid portion of HecA was most similar to FhaB of *B. pertussis*, which is processed into filamentous hemagglutinin (Makhov et al. 1994). In animal-pathogenic bacteria, phospholipases, hemolysins, and adhesins have been implicated as important pathogenicity/virulence determinants. However, Keen and colleagues (1992) reported that a *plcA* mutant exhibited the same degree of virulence as the wild-type strain on potato tubers and in chrysanthemum stems. The role, if any, of the *plcA*, *hecB*, and *hecA* products in the virulence of *E. chrysanthemi* is under investigation.

In the AT-rich intergenic region between ORF1 and *hecB* (33.7% G+C), we found a sequence, AATAAAAGGTA ATTTATTT, that resembles the KdgR-box consensus sequence (Hugouvieux-Cotte-Pattat et al. 1996). A similar sequence, AAATAATTCGCGTTTTATT, was found in the 107-bp intergenic space between *hecB* and *hecA*, which also has high AT content (36.4% G+C). This implies that some or all of ORF1, *hecB*, and *hecA* could be part of the KdgR regulon, which includes operons involved in pectinolysis and type II protein secretion, although this could be coincidental and requires experimental testing. Interestingly, the upstream regulatory regions of *pel* genes that possess KdgR-binding sites also are AT-rich (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1992).

To determine whether the unusual gene arrangement of the *hrpC* and *hrpN* region found in strain EC16 is conserved in other strains of *E. chrysanthemi*, a hot-start polymerase chain reaction procedure (94°C for 2 min, followed by 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min for a total of 30 cycles, ending with 7 min of elongation at 72°C) was performed with *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD) and AmpliWax (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ). Primers derived from the region between *plcA* and *hrpF* of EC16, 5-CTTCATTACTGAGCAA

ATGGG-3 and 5-GTTGAAGTCATTGATGATGGC-3, amplified products of approximately 0.9 kb in size from genomic DNA of nine strains of *E. chrysanthemi* (Fig. 3A). In addition, strains 0-596 and 3937 produced single amplified products of slightly different sizes. Another set of primers designed from *hrpN* and *hecB*, 5-GATGGGTATGATCAAAAGCG-3 and 5-ATGTCGTTTAACTGCTGTAACG-3, produced distinctive bands of approximately 1.2 kb from four strains belonging to subgroups II and III of *E. chrysanthemi* (Dickey 1979), including EC16 itself (Fig. 3B). Other strains produced several weak bands of various sizes. Although not definitive, these results suggest gene organizations around the *hrpCN* region in other strains of *E. chrysanthemi* that are similar to that determined for EC16.

It is well established that diverse plant- and animal-pathogenic bacteria utilize the type III pathway to secrete proteins involved in pathogenicity and virulence (Galán and Bliska 1996). Our work on the *hrpC* operon and an earlier study with *hrcT* (Bauer et al. 1994) confirm that the soft-rotting bacterium *E. chrysanthemi* employs a Hrp (type III) pathway. As *hrp* clusters generally are conserved and collinear among even distantly related bacteria such as *E. amylovora* and *P. syringae* (Bogdanove et al. 1996b; Kim et al. 1997), it is unexpected that *hrp* operons of *E. chrysanthemi* are interrupted by genes that may function in virulence but are not known to be associated with the Hrp system. The *hrpCN*-linked location of *plcA* and *hecBA* supports the idea that the *hrp* region of *E. chrysanthemi* may constitute a "pathogenicity island" (Groisman and Ochman 1996) with many virulence proteins of diverse functions, controlled by diverse regulatory proteins, and secreted by diverse pathways.

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