The *Erwinia chrysanthemi* EC16 *hrp/hrc* Gene Cluster Encodes an Active Hrp Type III Secretion System That Is Flanked by Virulence Genes Functionally Unrelated to the Hrp System

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Erwinia chrysanthemi is a host-promiscuous plant pathogen that possesses a type III secretion system (TTSS) similar to that of the host-specific pathogens *E. amylovora* and *Pseudomonas syringae*. The regions flanking the TTSS-encoding *hrp/hrc* gene clusters in the latter pathogens encode various TTSS-secreted proteins. DNA sequencing of the complete *E. chrysanthemi* *hrp/hrc* gene cluster and approximately 12 kb of the flanking regions (beyond the previously characterized *hecA* adhesin gene in the left flank) revealed that the *E. chrysanthemi* TTSS genes were syntenic and similar (>50% amino-acid identity) with their *E. amylovora* orthologs. However, the *hrp/hrc* cluster was interrupted by a cluster of four genes, only one of which, a homolog of lytic transglycosylases, is implicated in TTSS functions. Furthermore, the regions flanking the *hrp/hrc* cluster lacked genes that were likely to encode TTSS substrates. Instead, some of the genes in these regions predict ABC transporters and methyl-accepting chemotaxis proteins that could have alternative roles in virulence. Mutations affecting all of the genes in the regions flanking or interrupting the *hrp/hrc* cluster were constructed in *E. chrysanthemi* CUCPB5047, a mutant whose reduced pectolytic capacity can enhance the phenotype of minor virulence factors. Mutants were screened in witloof chicory leaves and then in potato tubers and *Nicotiana clevelandii* seedlings. Mu dIII1734 insertion in one gene, designated *virA*, resulted in strongly reduced virulence in all three tests. *virA* is immediately downstream of *hecA*, has an unusually low G+C content of 38%, and predicts an unknown protein of 111 amino acids. The *E. chrysanthemi* TTSS was shown to be active by its ability to translocate AvrPto-Cya (a *P. syringae* TTSS effector fused to an adenylate cyclase reporter that is active in the presence of eukaryote calmodulin) into *N. benthamiana* leaf cells. However, VirA₍₁₋₆₁₎-

Cya was not translocated into plant cells, and *virA* expression was not affected by mutations in *E. chrysanthemi* Hrp regulator genes *hrpL* and *hrpS*. Thus, the 44-kb region of the *E. chrysanthemi* EC16 genome that is centered on the *hrp/hrc* cluster encodes a potpourri of virulence factors, but none of these appear to be a TTSS effector.

Type III secretion systems (TTSSs) are used by many bacterial pathogens of plants and animals to deliver virulence effector proteins into host cells (Cornelis and Van Gijsegem 2000; Galán and Collmer 1999). In plant pathogens, the TTSS is encoded by *hrp/hrc* genes, which are so named because mutants typically are unable to elicit the defense-associated hypersensitive response (HR) in nonhosts or in resistant host plants or to be pathogenic in host plants. The *hrc* genes represent a subset of *hrp* genes that are conserved in the TTSSs of both plant and animal pathogens. The Hrp TTSS has been studied most extensively in host-specific biotrophic pathogens, where several effectors, known as avirulence (Avr) proteins, have been shown to be delivered into plant cells by the Hrp system and to determine whether the outcome of the interaction is the HR or pathogenesis (Dangl and Jones 2001; Keen 1990). In contrast, the function of the TTSS and Hrp effectors in host-promiscuous necrotrophic pathogens, such as *Erwinia chrysanthemi*, has received less attention and is poorly understood.

The *hrp/hrc* gene clusters have been characterized extensively or fully sequenced in *Pseudomonas syringae* pv. *syringae* (Huang et al. 1995; Lidell and Hutcheson 1994), *P. syringae* pv. *tomato* (Fouts et al. 2003; Preston et al. 1995), *Ralstonia solanacearum* (Van Gijsegem et al. 1995), *Xanthomonas axonopodis* pv. *citri* and *X. campestris* pv. *campestris* (Da Silva et al. 2002), *E. amylovora* (Bogdanove et al. 1996; Kim et al. 1997), *Pantoea stewartii* subsp. *stewartii* (Coplin et al. 1992), *E. herbicola* pv. *gypsophilae* (Mor et al. 2001), and *E. herbicola* pv. *betae* (Nizan et al. 1997). The Hrp systems characterized so far fall into two groups that differ by the presence or absence of certain *hrp* genes, the organization of *hrp/hrc* genes within operons, and regulatory components (Alfano and Collmer 1997). Group I Hrp systems are present in *P. syringae* pathovars and *Erwinia* and *Pantoea* spp. and are activated by an alternative sigma factor, HrpL. Group II Hrp systems are present in *R. solanacearum* and *Xanthomonas* spp. and are activated by AraC-type regulators. Regardless of the group, *hrp* genes usually are repressed in complex media and induced in media that mimic apoplastic fluids, and they are flanked by genes encoding Hrp effectors and accessory type III secretion or translocation

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factors (Alfano and Collmer 1997; Kim and Alfano 2002). The latter observation reflects the general importance in bacterial pathogens of pathogenicity islands, which are clusters of genes with related functions in virulence that often show indications of horizontal acquisition (Hacker and Kaper 2000).

The presence of *hrp/hrc* genes in the soft rot pathogens *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* is puzzling because these pathogens attack their hosts primarily with an arsenal of type II-secreted pectolytic enzymes that cause tissue maceration and cell killing (Barras et al. 1994; Robert-Baudouy et al. 2000). The role of the Hrp system in *E. carotovora* is uncertain. In *E. chrysanthemi* EC16, the Hrp system appears to contribute to an early stage of pathogenesis, as demonstrated by *E. chrysanthemi* EC16 *hrp* mutants that were quantitatively reduced in the initiation of infection in witloof chicory leaves (Bauer et al. 1994; Yang et al. 2002). Similarly, *E. chrysanthemi* 3937, *hrpG*, and *hrcC* mutants are impaired in lesion formation and growth in African violet leaves (Yang et al. 2002). In both strains, *hrp* mutations had stronger virulence phenotypes in mutants that produce only the secondary pectate lyase (Pel) isozymes because of deletions affecting the major Pels. The *E. chrysanthemi* EC16 *hrp/hrc* cluster has been cloned and shown to enable *Escherichia coli* to secrete *P. syringae* Hrp effectors (Deng et al. 2003; Ham et al. 1998). However, *Erwinia chrysanthemi* Hrp effectors have not been found, and the only native protein that is a candidate for TTSS secretion by *E. chrysanthemi* is the HrpN harpin (Bauer et al. 1995).

In an attempt to identify candidate Hrp effector genes in *E. chrysanthemi*, we have been exploring the regions flanking the *hrp/hrc* genes, because these regions have been found to be rich in effectors in other plant pathogens (Alfano et al. 2000; Kim and Alfano 2002). For example, among pathogens with group I Hrp systems, the *E. amylovora* *hrp/hrc*-flanking regions contain genes encoding the harpins HrpN and HrpW and genes encoding the Hrp effector protein DspE and its likely chaperone, DspF (Kim and Alfano 2002). Similarly, *Pantoea stewartii* subsp. *stewartii* has a *dspE* homolog linked to the *hrp/hrc* gene cluster (Frederick et al. 2001). In *Pseudomonas syringae*, homologs of *hrpW*, *dspE*, and other genes encoding other Hrp effectors are located in the conserved effector locus, which flanks *hrpR* in the divergent strains *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728A. In these strains, an exchangeable effector locus flanks the other side of the *hrp/hrc* cluster, beyond *hrpK* (Alfano et al. 2000). The presence of genes encoding Hrp effectors in the regions flanking the *hrp/hrc* gene cluster is also true for pathogens having group II Hrp systems. These genes include *popA*, *popB*, and *popC* in *R. solanacearum* (Salanoubat et al. 2002); *xopA*, *xopD*, and *hrpF* in *X. campestris* pv. *vesicatoria* (Noel et al. 2002); and *hpaI* in *X. oryzae* pv. *oryzae* (Zhu et al. 2000).

The region flanking the *E. chrysanthemi* EC16 *hrp/hrc* gene cluster beyond *hrpN* contains an adhesin-encoding gene (*hecA*) and its cognate transporter-encoding gene (*hecB*) (Kim et al. 1998b), whose contribution to virulence is associated with adhesion to the plant epidermis and bacterial autoagglutination (Rojas et al. 2002). Genomic sequencing of the regions flanking the *hrp/hrc* cluster in *E. carotovora* subsp. *atroseptica* also revealed *hecA* and *hecB* homologs as well as sequences homologous to rhizobacterial genes involved in opine catabolism. More importantly, homologs of *E. amylovora* *dspE* and *dspF* were found adjacent to *hecB* (Bell et al. 2002), which would suggest that soft-rot *Erwinia* spp. also produce Hrp effectors.

Although the regions flanking the *hrp/hrc* cluster in several plant-pathogenic bacteria encode candidate Hrp effectors, not all of the genes in these regions encode proteins that travel the Hrp pathway. Additional criteria that have been used to iden-

tify candidate Hrp effector genes in genomic sequences include linkage with promoters that are responsive to the HrpL alternative sigma factor and the presence of N-terminal amino-acid patterns and compositional biases that are characteristic of Hrp effector proteins (Fouts et al. 2002; Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). The application of such sequence-based search methods and functional assays to the genome of *P. syringae* pv. *tomato* DC3000 has revealed the presence of 38 effector genes and several more candidates in the Hrp pathogenicity island and in other locations of the DC3000 genome (Buell et al. 2003; Collmer et al. 2002). Thus, Hrp promoter sequences, TTSS substrate-associated patterns, and homology with a growing list of effectors found in sequenced pathogen genomes provide new resources for identifying candidate effector genes in sequenced regions of the *E. chrysanthemi* genome.

We report here i) the complete sequence of the *E. chrysanthemi* EC16 *hrp/hrc* gene cluster; ii) DNA sequence analysis 7.9 kb beyond *hrcU* (right flank of the *hrp/hrc* cluster) and 4.4 kb beyond *hecA* (left flank of the *hrp/hrc* cluster) and evaluation of these regions for features that often are associated with Hrp effector genes; iii) construction of mutations affecting each open reading frame (ORF) (other than the *hrp/hrc* genes) in the Hrp region of the *E. chrysanthemi* genome; iv) demonstration of a significant contribution of ORF11 (*virA*) to the virulence of *E. chrysanthemi* in witloof chicory leaves, potato tubers, and *Nicotiana clevelandii* seedlings; and v) evidence that the *E. chrysanthemi* TTSS is active in planta but does not activate *virA* gene expression or translocate the VirA protein into plant cells.

RESULTS

The *hrp/hrc* cluster in *E. chrysanthemi* is collinear with the *E. amylovora* *hrp/hrc* cluster but also contains an intervening region in which three out of four ORFs appear functionally unrelated to the Hrp system.

We completed the sequence of the *E. chrysanthemi* EC16 *hrp/hrc* cluster, which confirmed a gene organization similar to the previously characterized *hrp/hrc* clusters from *E. amylovora* (Bogdanove et al. 1996; Kim et al. 1997), *Pantoea stewartii* subsp. *stewartii* (Frederick et al. 2001), and *E. herbicola* pv. *gypsophillae* (Mor et al. 2001), which have group I Hrp systems (Fig. 1). The *E. chrysanthemi* EC16 *hrp/hrc* operons are preceded by Hrp box promoter sequences, and the predicted proteins exhibited more than 50% identity to their *E. amylovora* orthologs. Previous sequence analysis of the *E. chrysanthemi* *hrp/hrc* genes revealed several ORFs in an intervening region within the *hrp/hrc* gene cluster. These are ORF2, ORF3, ORF4, and *plcA* (Fig. 1). None of these ORFs are preceded by recognizable Hrp promoters, and the N-terminal amino acids of these proteins have at least two deviations from the patterns associated with substrates of group I Hrp pathways (Petnicki-Ocwieja et al. 2002) (Fig. 1). For example, ORF2 begins with four hydrophobic amino acids and is not rich in Ser or other polar amino acids (Fig. 1B). In contrast, the HrpN harpin is preceded by a Hrp promoter and has all of the patterns associated with Hrp-exported proteins (Fig. 1B). ORFs 2 and 3 do not show any significant similarity to other proteins in the databases, but ORF4 has similarity to lytic transglycosylases.

The regions flanking the *E. chrysanthemi* *hrp/hrc* cluster contain ORFs with no apparent functional relationship with the TTSS.

We next sequenced 4.4 kb of *E. chrysanthemi* DNA in the left flank beyond *hecA* and 7.9 kb in the right flank beyond

hrcU and searched for ORFs that were preceded by Hrp promoter sequences, or showed similarity to known Hrp-related proteins, or possessed N-terminal amino-acid patterns associated with proteins that are secreted by the Hrp system (Fig. 1B). In contrast to other plant pathogens whose Hrp regions had been similarly sequenced, *E. chrysanthemi* had no ORFs that appeared to be Hrp TTSS substrates (Table 1). With the exception of ORF1, which resides between *hecB* and *hrpN*, no ORFs were preceded by recognizable Hrp promoter sequences (Fig. 1A). The N-terminal amino acids of ORF1 and all other proteins encoded in the regions flanking the *hrp/hrc* cluster have at least two deviations from the patterns associated with substrates of group I Hrp pathways (Petnicki-Ocwieja et al. 2002). For example, ORF1 lacks a hydrophobic amino acid in positions 3 or 4 and is not rich in Ser and other polar amino acids (Fig. 1B).

However, some of these ORFs have homologies that suggest a potential role in virulence or in interactions with the biotic environment (Table 1). ORF1 in the left flanking region is similar to one of the products of a polyketide synthesis gene cluster that is responsible for the production of the antibiotic curamycin (Kim et al. 1998b). ORFs 5 to 10 in the right flanking region encode putative membrane proteins that have sequence similarity to ABC transporters and a chemotaxis protein. The closest homologs to these proteins are in other enterobacterial pathogens. ORFs located in the left flanking region also include *hecA* and *hecB*, whose role in virulence has been reported (Rojas et al. 2002). ORFs 12 and 13 show sequence similarity to a transcriptional regulator in *Pseudomonas aeruginosa* and a primase in *Aquifex aeolicus*, respectively. Other ORFs in this region do not show any significant similarity to other proteins in the databases.

Table 1. Open reading frames (ORF) found in regions inserted within or flanking the *Erwinia chrysanthemi hrp/hrc* cluster and their similarity with proteins in other organisms

ORF ID	Predicted protein size (aa)	Homolog	Accession no.	BLASTP E value
ORF1	133	Hypothetical protein, <i>Mesorhizobium loti</i>	NP_103170	4e ⁻²²
ORF2	146	None	...	...
ORF3	195	None	...	...
ORF4	436	Lytic murein transglycosylase, <i>Xanthomonas axonopodis</i> pv. <i>citri</i>	NP_644735	1e ⁻⁸⁸
ORF5	411	ABC transporter, <i>Salmonella typhimurium</i>	NP_459371	e ⁻¹³⁶
ORF6	655	Methyl accepting chemotactic protein, <i>Yersinia pestis</i>	NP_407489	6e ⁻⁷⁶
ORF7	278	Putative permease of ABC transporter, <i>Y. pestis</i>	NP_668714	5e ⁻⁶⁵
ORF8	424	Putative permease of ABC transporter, <i>Y. pestis</i>	NP_668713	2e ⁻¹⁴³
ORF9	377	Putative transport system periplasmic protein, <i>Y. pestis</i>	NP_406351	e ⁻¹³⁷
ORF10	161 (partial)	AttK, <i>Agrobacterium tumefaciens</i> , partial sequence	AAL13073	8e ⁻⁵⁴
ORF11	111	None	...	...
ORF12	120	Transcriptional regulator, <i>Pseudomonas aeruginosa</i>	NP_248738	1e ⁻¹¹
ORF13	509	DNA primase, <i>Aquifex aeolicus</i>	NP_214030	1e ⁻²¹
ORF14	248	None	...	...
ORF15	211	None	...	...

Fig. 1. *Erwinia chrysanthemi hrp/hrc* cluster and flanking regions. **A**, The *hrp/hrc* gene organization is depicted with line arrows representing putative transcription units and small block arrows indicating predicted HrpL-regulated promoters (note that the *hrpJ* operon is 10 kb). Gray boxes represent open reading frames (ORFs) within the *hrp/hrc* gene cluster. Numbers below boxes indicate ORF designations. Plasmids pCPP3101 and pCPP3083 were used to sequence the flanking regions beyond *hecA* and *hrcU*, respectively, and the ORFs found are shown with their respective direction of transcription in expanded views of the region. Triangles on ORFs 11 to 15 indicate Mu dIII734 phage insertions that were introduced into the chromosome of *E. chrysanthemi*. Plasmid pCPP2511 was used to create the $\Delta plcA$ -ORF4 deletion mutant CUCPB5097, and plasmid pCPP3231 was used to create the deletion mutant CUCPB5137 encompassing ORFs 5 to 9. **B**, Sequence of the N-proximal 40 amino acids in HrpN and in ORFs located in the regions that interrupt or flank the *hrp/hrc* gene cluster.

***virA* (ORF11) is the only gene in the regions that interrupt or flank the *E. chrysanthemi* *hrpL*/*hrpC* gene cluster that has a major virulence phenotype.**

To determine whether any genes, other than *hecA* or *hecB*, that are located in regions that interrupt or flank the *hrpL*/*hrpC* gene cluster play a role in virulence, we constructed strains containing either deletions of several genes or insertions in individual genes in *E. chrysanthemi* CUCPB5047 (Fig. 1A). We introduced these mutations into CUCPB5047 because it is a $\Delta(pelA/pelE)\Delta(pelB/pelC)::28bp \Delta(pelX) \Delta4bp \Delta(pchX)::\Omega^{Cm}$ derivative of AC4150 (Alfano et al. 1995). The reduced maceration capacity of CUCPB5047 favors the detection of subtle virulence phenotypes, as does the use of witloof chicory as a test host plant (Beaulieu et al. 1993). Mutants initially were screened for reduced virulence in chicory leaves using pairwise comparisons of each mutant and its CUCPB5047 parent (Fig. 2). Only CUCPB5356 (ORF11::Mu dIII1734) showed a significant reduction in virulence and, consequently, ORF11 is being designated *virA*. Statistical analysis (*t* test) of lesions in witloof chicory leaves showed no significant difference in virulence between other mutant strains and CUCPB5047 (*P* values ≥ 0.479).

CUCPB5356 and CUCPB5047 showed the same growth in King's B medium (data not shown). pCPP3134, which carries PCR-subcloned *virA* in pBluescript SK⁻, was electroporated into CUCPB5356. CUCPB5047, CUCPB5356, and CUCPB5356(pCPP3134) were tested for virulence in chicory leaves and potato tuber slices. Representative results reveal that *virA* mutation also reduces the virulence of CUCPB5047 in potato tuber slices and that complementation with pCPP3134 restores virulence in both chicory and potato (Fig. 3A). Quantitative assays with chicory confirmed that pCPP3134 restores the virulence of CUCPB5356 to nearly the same level as the parental strain CUCPB5047 (Fig. 3C). Axenically grown *Nicotiana* seedlings are a third host that often is used in virulence assays involving soft-rot *Erwinia* spp. (Pirhonen et al. 1991). Because of the weak virulence in *N. clevelandii* seedlings of CUCPB5047, we marker exchanged the *virA*::Mu dIII1734 mutation into wild-type strain AC4150 to assess the virulence cost of *virA* mutation in *N. clevelandii* seedlings (Fig. 3B). The resulting mutant, CUCPB5339, was strongly reduced in virulence unless carrying the complementing plasmid pCPP3134.

Fig. 2. Relative virulence in witloof chicory leaves of *Erwinia chrysanthemi* CUCPB5047 and mutant derivatives deficient in open reading frames that are between *hrpF* and *hrpE* or flanking *hecA* and *hrpU*. Witloof chicory leaves were inoculated in 20 replicates with bacterial strains at 1×10^8 CFU/ml and incubated in a moist chamber for 48 h. Length and width of the lesions were measured, and mean lesion area was plotted. Error bars indicate standard error. Each mutant was independently compared with CUCPB5047.

***E. chrysanthemi* can translocate AvrPto-Cya into *N. benthamiana* cells in an Hrp-dependent manner, but *virA* is not activated by HrpL or HrpS, and VirA₍₁₋₆₁₎-Cya is not translocated.**

Although *virA* is not preceded by a recognizable Hrp promoter, and the N-terminal region of the predicted VirA protein does not have amino-acid patterns associated with TTSS substrates, the possibility remained that VirA is a Hrp effector protein. In *P. syringae*, some genes in the Hrp regulon are not

Fig. 3. Virulence phenotypes in witloof chicory leaves, potato tuber slices, and *Nicotiana clevelandii* seedlings of *Erwinia chrysanthemi* *virA* (open reading frame 11) mutants. **A**, Representative macerated lesions produced in chicory leaves and potato by CUCPB5047, CUCPB5356, and CUCPB5356(pCPP3134). The photograph was taken 48 h after inoculation. **B**, Replicate axenically grown *N. clevelandii* seedlings drop inoculated with AC4150, CUCPB5047, CUCPB5339, and CUCPB5339(pCPP3134). The photograph was taken 48 h after inoculation. **C**, Quantitative analysis of the virulence in chicory leaves of CUCPB5047, CUCPB5356, and CUCPB5356(pCPP3134). Strains were grown in King's B medium until optical density at 600 nm reached 0.7.

preceded by Hrp promoters (Fouts et al. 2002), and at least one protein that is translocated into plant cells by the Hrp TTSS lacks diagnostic features associated with TTSS substrates (Schechter et al. 2004). Therefore, we experimentally questioned whether *virA* expression is dependent upon HrpL or HrpS and whether VirA can be translocated into plant cells by the *E. chrysanthemi* Hrp TTSS.

We previously had shown that the *E. chrysanthemi* EC16 *hrpN* gene is expressed in King's medium B, but it is not expressed in *hrpL* or *hrpS* mutants (Ham et al. 2004). To determine if *virA* is part of the Hrp regulon, we used reverse-transcriptase polymerase chain reaction (RT-PCR) and primers for internal fragments of *hrpN* and *virA* to investigate *virA* expression levels in *E. chrysanthemi* strains that were either wild type (AC4150), or deficient in *hrpL* (CUCPB5118) or *hrpS* (CUCPB5360). The 169-bp RT-PCR product from *virA* transcripts was detected in the wild-type strain as well as the *hrpL* and *hrpS* mutant strains (Fig. 4). In the same reaction, however, the 326-bp RT-PCR product from *hrpN* transcripts was detected only in the wild-type strain but not in the *hrpL* or *hrpS* mutant strains, which is consistent with the previous report that *hrpN* expression is dependent on *hrpL* and *hrpS*.

The *E. chrysanthemi* EC16 *hrpL*/*hrpS* gene cluster, heterologously expressed in *Escherichia coli*, previously had been shown to direct TTSS-dependent secretion of the *P. syringae* AvrPto effector (Ham et al. 1998). Here, we addressed the further question of whether the native *Erwinia chrysanthemi* Hrp system was capable of translocating AvrPto into plant cells. We used *E. chrysanthemi* CUCPB5006 because it carries deletions affecting the production of the four major pectate lyase isozymes that are responsible for much of the macerating activity of the parental strain (He and Collmer 1990), and the associated cell killing likely would interfere with the translocation assays. Translocation was assayed using an AvrPto-Cya fusion and *N. benthamiana* plants, as described (Schechter et al. 2004). The assay is based on the ability of Cya (*Bordetella pertussis* adenylate cyclase) to produce cAMP in a calmodulin-dependent manner in eukaryotic cells (Casper-Lindley et al. 2002; Sory and Cornelis 1994). *N. benthamiana* leaf tissue inoculated with CUCPB5006 expressing AvrPto-Cya produced significant levels of cAMP (Fig. 5). In contrast, no cAMP was produced in leaf tissue inoculated with CUCPB5038, an *hrcT* mutant derivative of CUCPB5006, when expressing AvrPto-Cya.

TTSS targeting signals are present in the N-terminal region of TTSS substrates (Lloyd et al. 2001; Ramamurthi and Schneewind 2002), and Cya reporter fusions involving the first approximately 50 amino acids of phytopathogen TTSS substrates have been shown sufficient to direct translocation into plant cells (Casper-Lindley et al. 2002; Schechter et al. in

press). We were unable to construct a fusion of the full-length VirA to Cya because of apparent toxicity to *Escherichia coli*. Consequently, we constructed and tested VirA₍₁₋₆₁₎-Cya. The construct directed the production of cAMP in *E. coli* lysates supplemented with calmodulin (data not shown), but it failed to produce cAMP in *N. benthamiana* tissue inoculated with CUCPB5006 (Fig. 5). Thus, VirA does not appear to be a substrate for the *Erwinia chrysanthemi* Hrp TTSS.

DISCUSSION

To better understand the function of the Hrp system in *E. chrysanthemi*, we searched for genes that encode candidate Hrp effectors or have any virulence phenotype in a 44-kb region of the strain EC16 genome that is centered on the *hrpL*/*hrpS* cluster. Our findings highlight several unusual features of the *E. chrysanthemi* Hrp system: no candidate effector genes are evident in the entire region and, other than the previously characterized *hecA* and *hecB* genes (Rojas et al. 2002), we have found *virA* (ORF11) to be the only gene flanking the *hrpL*/*hrpS* cluster that has a major virulence phenotype. These observations and other features of the Hrp region warrant further discussion.

There is considerable evidence that the Hrp systems of various plant-pathogenic bacteria can deliver effector proteins into plant cells (Cornelis and Van Gijsegem 2000). The *E. chrysanthemi* Hrp system functioning heterologously in *Escherichia coli* can secrete the *P. syringae* AvrB and AvrPto effectors and also elicit an AvrB-dependent HR in *N. clevelandii* (Ham et al. 1998). Given the evidence that AvrB acts within plant cells in HR elicitation (Gopalan et al. 1996; Leister et al. 1996; Wu et al. 2003), it appears that the *Erwinia chrysanthemi* Hrp system is like other Hrp systems in being capable of injecting virulence proteins into plant cells. Here we have shown that *E. chrysanthemi* itself is capable of translocating the model effector AvrPto into plant cells in a TTSS-dependent manner. This experimental confirmation that the native *E. chrysanthemi* Hrp system is active in planta leaves more puzzling our failure to find any TTSS effector genes in the region of the *hrpL*/*hrpS* cluster, and we are left with the possibility that effector genes may be located elsewhere in the genome.

Fig. 4. Expression of *virA* is independent of HrpL and HrpS. AC4150, CUCPB5118 (*hrpL*:: Ω Cm^r), and CUCPB5360 (*hrpS*:: Ω Cm^r) strains were grown in King's B medium until optical density at 600 nm reached 0.7. Total RNA was isolated and analyzed by reverse-transcriptase polymerase chain reaction using primer pairs that directed products of 169 and 326 bp from *virA* and *hrpN* transcripts, respectively.

Fig. 5. Time course of cAMP accumulation in *Nicotiana benthamiana* leaves following inoculation with *Erwinia chrysanthemi* CUCPB5006 expressing AvrPto-Cya and VirA₍₁₋₆₁₎-Cya. Bacteria (optical density at 600 nm = 0.3) were infiltrated with a blunt syringe into *N. benthamiana* leaf tissue and, at the indicated times, samples of the inoculated tissues were assayed for cAMP accumulation. cAMP was quantified in triplicate for each sample, and the standard deviations are indicated by error bars. The experiment was repeated twice with similar results. The strains tested were CUCPB5006 (Δ pelABCE) carrying pCPP3221 (AvrPto-Cya) (squares), CUCPB5038 (*hrcT*::Tn10-mini-Km derivative of CUCPB5006) carrying pCPP3221 (AvrPto-Cya) (triangles), and CUCPB5006 carrying pCPP3381 (VirA₍₁₋₆₁₎-Cya) (crosses).

We have shown that the *E. chrysanthemi* *hrp/hrc* cluster is similar to other group I clusters in its gene order and the presence of the HrpL alternative sigma factor and Hrp box promoter sequences. However, we also found that it is unusual among *hrp/hrc* clusters in several aspects. The first unusual feature is the presence of three unique ORFs (*plcA*, ORF2, and ORF3) inside the *hrp/hrc* gene cluster (Kim et al. 1998b) that do not appear to be functionally related to the Hrp system. *plcA* encodes a phospholipase C that has been reported to carry an N-terminal signal peptide and to be secreted in culture by *E. chrysanthemi* and *Escherichia coli* DH5 α transformants (Keen et al. 1992). A fourth ORF in this region encodes a putative lytic transglycosylase (lysozyme-like protein). Such proteins commonly are associated with *hrp/hrc* clusters, contain a peptidoglycan binding domain, and have been suggested to be required for remodeling of the periplasmic peptidoglycan during the assembly of the type III secretion apparatus (Alfano et al. 2000; Noel et al. 2002; Zhu et al. 2000). Similar to the observations with the putative lysozyme-like HpaH in *X. campestris* pv. *vesicatoria* (Noel et al. 2002), mutations in *Erwinia chrysanthemi* ORF4 have no effect on virulence.

Comparison of the regions flanking the *hrp/hrc* clusters of *E. chrysanthemi* and *E. carotovora* subsp. *atroseptica* is interesting because of the notable absence of genes encoding Hrp-secreted proteins in *E. chrysanthemi*. That is, in the left flank-

ing region, both species have *hecA* and *hecB* homologs, but only *E. carotovora* subsp. *atroseptica* has *hrpW* and *dspE* (Bell et al. 2002). In the right flanking region, neither species has apparent effector genes, but both have genes associated with ABC transporters and opine catabolism.

In other plant-pathogenic enterobacteria, virulence-associated genes not related with Hrp secretion also have been found in the *hrp/hrc* flanking regions. One is *rlsA*, which encodes a regulator of levan production in *E. amylovora* (Zhang and Geider 1999). In *E. chrysanthemi*, only *virA* (ORF11) was found to make a significant contribution to *E. chrysanthemi* virulence. The predicted VirA protein sequence yields no clue to function, and its mechanism of action remains to be elucidated. Our observations that *virA* expression is independent of HrpL and HrpS and that a VirA₍₁₋₆₁₎-Cya hybrid is not translocated into plant cells by *E. chrysanthemi* argues against VirA functioning in association with the Hrp system. In this regard, it is worth noting that *virA* mutants are more impaired in virulence than the corresponding *hrcT* mutants in each of the virulence assays that we used (Bauer et al. 1994; Rojas et al. 2002). This provides further evidence for an action of VirA that is independent of HrcT and other components of the *E. chrysanthemi* TTSS.

Mutations in the other genes located in the *hrp/hrc* flanking regions showed no phenotype in virulence assays. However, microarray analysis in *E. chrysanthemi* strain 3937 revealed that

Table 2. Bacterial strains and plasmids used in this work

Designation	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i>		
MC4100	FaraD139 Δ (<i>lacIPOZYA</i> ⁻ <i>argF</i>)U169 <i>rpsL thi</i>	Casadaban and Cohen 1979
POII1734	MC1040 (Mu cts) with Mu dIII1734 <i>lac</i> ⁺	Castilho et al. 1984
<i>Erwinia chrysanthemi</i>		
AC4150	Spontaneous Nal ^R derivative of wild-type strain EC16	Chatterjee et al. 1983
CUCPB5006	Δ (<i>pelA/pelE</i>) Δ (<i>pelB/pelC</i>):28bp derivative of AC4150	He and Collmer 1990
CUCPB5038	<i>hrcT</i> ::Tn10-mini-Km derivative of CUCPB5006	Bauer et al. 1994
CUCPB5047	Δ (<i>pelA/pelE</i>) Δ (<i>pelB/pelC</i>):28bp Δ (<i>pelX</i>) Δ 4bp <i>pehX</i> : Ω Cm	Alfano et al. 1995
CUCPB5093	Δ <i>plcA</i> -ORF4::nptII-sacB-sacR derivative of CUCPB5047	This work
CUCPB5097	Δ <i>plcA</i> -ORF4::28-bp derivative of CUCPB5093	This work
CUCPB5118	<i>hrpL</i> :: Ω Cm ^r derivative of AC4150	This work
CUCPB5354	Δ ORF5-ORF9 replaced by Ω ^{Sp} cassette derivative of CUCPB5047	This work
CUCPB5356	<i>virA</i> ::Mu dIII1734 derivative of CUCPB5047	This work
CUCPB5357	ORF12::Mu dIII1734 derivative of CUCPB5047	This work
CUCPB5358	ORF14::Mu dIII1734 derivative of CUCPB5047	This work
CUCPB5359	ORF15::Mu dIII1734 derivative of CUCPB5047	This work
CUCPB5370	ORF13::Mu dIII1734 derivative of CUCPB5047	This work
CUCPB5382	ORF1:: Ω ^{Sp} derivative of CUCPB5047	This work
CUCPB5339	<i>virA</i> ::Mu dIII1734 derivative of AC4150	This work
Cosmids and plasmids		
pCPP2156	pCPP19 carrying <i>Erwinia chrysanthemi</i> <i>hrp/hrc</i> genes and 10 kb of the right flanking region, Sp ^r	Ham et al. 1998
pCPP5329	Cosmid clone containing genes at the left flanking region of the <i>E. chrysanthemi</i> <i>hrp/hrc</i> genes	This work
pCPP2354	3.1-kb <i>SalI/BamHI</i> fragment of pCPP1221 in pWSK29 that harbors <i>hrpF</i> , <i>hrpG</i> , and <i>hrcC</i>	Kim et al. 1998a
pCPP2359	7.5-kb <i>BamHI/BglIII</i> clone in pUC18 that contains part of <i>hrpC</i> operon, <i>plcA</i> , ORF2, ORF3, ORF4	This work
pCPP2360	4.3-kb <i>BamHI/BglIII</i> clone in pUC18 that contains <i>hrpA</i> operon	This work
pCPP2504	6.1-kb <i>BglIII</i> clone in pBluescriptSK+ that contains <i>hrpA</i> to <i>hrpY</i>	This work
pCPP2505	10.5-kb <i>BglIII</i> clone in pBluescriptSK+ that contains <i>hrpL</i> to <i>hrcR</i> .	This work
pCPP2511	Δ <i>plcA</i> -ORF4 construct	This work
pCPP5112	Δ <i>plcA</i> -ORF4 construct	This work
pCPP2513	1.8-kb <i>SacII</i> clone in pUC18 that contains <i>hrcJ</i> , <i>hrpD</i> , and <i>hrpE</i>	This work
pCPP3083	<i>BglIII</i> subclone containing <i>hrcR</i> , <i>hrcS</i> , <i>hrcT</i> , <i>hrcU</i> , and 10-kb right flanking region in pBluescript SK-	This work
pCPP3040	4.8-kb <i>EcoRV</i> fragment from pCPP3083 in pBluescript SK-	This work
pCPP3099	4-kb <i>KpnI</i> fragment from pCPP3083 in pBluescript SK-	This work
pCPP3094	30-kb <i>NorI</i> fragment containing genes at the left flanking region in pBluescriptSK- beyond <i>hecA</i>	This work
pCPP3101	7-kb <i>PstI</i> fragment from pCPP3094 in pBluescriptSK-	This work
pCPP3231	Δ ORF5-ORF9 construct in pUCP24	This work
pCPP3234	pVLT35 containing Gateway Reading Frame B cassette	Schechter et al. 2004
pCPP3287	ORF1:: Ω ^{Sp} in pBluescriptSK-	This work
pCPP3314	ORF11 in pBluescriptSK-	This work
pCPP3381	Codons 1-61 of <i>virA</i> fused with <i>cya</i> in pCPP3234	This work

^a ORF = open reading frame, Km = kanamycin, Cm = chloramphenicol, Sp = spectinomycin.

several ABC transporters are upregulated during infection of African violet (Okinaka et al. 2002), and screening with reporter gene fusions revealed methyl-accepting chemotactic proteins and transcriptional regulators to be induced in witloof chicory disks (Aguilar et al. 2002). These observations suggest that the ORFs found in the flanking regions of the *hrp/hrc* cluster might contribute to virulence by allowing bacterial survival in planta either by conferring resistance to antibiotic peptides, as shown before with the Sap operon, or by aiding nutrient uptake (Lopez-Solanilla et al. 1998). Although the mutants we constructed had no phenotype in our virulence assays, it is possible that they have a function in virulence that is masked by redundancy.

Whether the *hrp/hrc* genes in *E. chrysanthemi* are part of a pathogenicity island is an open question. The average G+C contents for the ORFs interrupting the *hrp/hrc* gene cluster and for the ORFs located in the right flanking region are 55.83 and 56.48%, respectively, which is similar to the G+C content for the rest of the genome (55 to 57%). The average G+C content for the *hrp/hrc* genes and the left flanking region is slightly higher (58%). Strikingly, the G+C content for *virA* is 38%. Although transposon and IS elements were observed in partial sequences of the region beyond ORF15, no tRNA sequences were observed (data not shown). In summary, the regions flanking the *hrp/hrc* gene cluster of *E. chrysanthemi* EC16 differ from that of host-specific pathogens in lacking any apparent TTSS substrate genes and, instead, encoding the functionally disparate virulence genes *hecA* and *virA*. This is consistent with the apparent role of the Hrp TTSS as just one of many virulence systems contributing to the ability of *E. chrysanthemi* to attack a broad range of plants.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA manipulation techniques.

Bacterial strains and plasmids are listed in Table 2. *Escherichia coli* strains were grown at 37°C in LM medium supplemented with ampicillin (Ap) at 100 µg/ml or spectinomycin (Sp) at 25 µg/ml. *Erwinia chrysanthemi* strains were grown at 30°C in King's B medium supplemented with kanamycin (Km) at 50 µg/ml or chloramphenicol (Cm) at 20 µg/ml. Plasmids and cosmids were isolated from *Escherichia coli* DH5α following standard procedures (King et al. 1954; Sambrook et al. 1989). DNA fragments for cloning were purified using Prep-a-Gene (Bio-Rad, Richmond, CA, U.S.A.) and ligated to pBluescriptSK[−] (Stratagene, LaJolla, CA, U.S.A.), pBluescriptSK⁺ (Stratagene), pUC18 (Fermentas, Hanover, MD), or pUCP24 (Schweizer 1991).

Sequencing of *hrp/hrc* genes.

Sequences of the *hrpN* and *hrpC* operons have been reported previously (Bauer et al. 1995; Kim et al. 1998b). Plasmid pCPP2359 was used to sequence from *plcA* to the 3' end of ORF4, pCPP2504 was used to sequence the rest of ORF4, the entire *hrpA* operon and *hrpY*, and pCPP2505 was used to sequence the region from *hrpX* to *hrcR*. Sequencing from these plasmids was initiated using universal and reverse primers followed by primer walking. DNA sequencing was performed with an ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.) at the Cornell University BioResource Center. Sequences were assembled using SEQMAN 5.03 (DNASTar, Madison, WI, U.S.A.), and ORFs were identified using MapDraw (DNASTar). Assembled sequences were used to search the GenBank nonredundant database using the BLASTX program (Altschul et al. 1990). The accession number for the sequence is AY169276.

Sequencing of regions flanking *hrp/hrc* genes.

Plasmid pCPP3083 containing *hrcR* to *hrcU* and the right flanking region of the *hrp/hrc* genes was subcloned into plasmids pCPP3040 and pCPP3099. pCPP3040 was sequenced using universal and reverse primers followed by primer walking. pCPP3094, derived from cosmid pCPP5329, was subcloned into pCPP3101. pCPP3099 and pCPP3101 were electroporated into *Escherichia coli* POII1734, which contains phage Mu dIII1734 (Castilho et al. 1984). Phage insertions in the plasmids were transduced into *E. coli* MC4100 following standard procedures (Miller 1992). Plasmids were isolated from transductants, and different insertions were chosen to sequence outwards from the phage using primers 5'-CGCTTTCGGGTTTTCGTG-3' and 5'-ATCCCGAATAATCAATGTCCTC-3'; gaps in contigs were filled with custom-made primers and sequences were assembled and analyzed as described before. The accession numbers for the sequences are AY169277 (left side beyond *hecA*) and AY169278 (right side beyond *hrcU*).

Deletion of the intergenic region between the *hrpA* and *hrpC* operons.

pCPP2360, containing the *hrpA* operon, was digested with *Sac*II, and a 1.8-kb *Sac*II fragment was blunt-ended and ligated to *Sma*I-digested pUC18 to create pCPP2513. pCPP2354, containing *hrpF*, *hrpG*, and *hrpC*, was digested with *Kpn*I and *Sac*I and the 3.1-kb fragment was isolated and ligated to *Kpn*I/*Sac*I-digested pCPP2513 to generate pCPP2511. The latter plasmid was digested with *Kpn*I, and the protruding 3' ends were blunted and ligated with the *nptII-sacB-sacR* cassette from pUM24Cm, which contains the pUM24-derived cassette in pPR328 (Quigley and Reeves 1987; Ried and Collmer 1987). This produced pCPP2512, which was used to mutate *Erwinia chrysanthemi* CUCPB5047 by marker exchange to produce CUCPB5093. The *nptII-sacB-sacR* cassette then was removed from CUCPB5093 by marker eviction employing pCPP2511 (Ried and Collmer 1987), which produced CUCPB5097 carrying a 28-bp scar marking the *plcA*-ORF4 deletion.

Construction of ORF1 mutation.

Primers 5'-GTTTCTAGATATTAACGACAGCC-3', containing an *Xba*I site, and 5'-CGACTAGTGTCCGGCCTGACAA-3', containing a *Spe*I site, were designed to amplify 39 bp from the 5' end of ORF1 and an approximately 2-kb region upstream. Primers 5'-CCACTCGAGAACTGCCCTATTTTGTGCG-3', containing an *Xho*I site, and 5'-AGGGTACCCGCTGGTCGCCGCAA-3', containing a *Kpn*I site, amplified a region that includes bases 59 to 399 in ORF1 and approximately 2 kb downstream. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.), digested with the enzyme pair combinations, gel-purified, and cloned into pBluescriptSK[−], generating plasmids pBSSK::ORF1A and pBSSK::ORF1B, respectively. pBSSK::ORF1A was digested with *Bam*HI and ligated to a purified Ω^{Sp} cassette. This construct, pBSSK::ORF1A::Ω^{Sp}, was digested with *Xho*I and *Xba*I, and the 4-kb fragment was purified and ligated to pBSSK::ORF1B to produce plasmid pCPP3287.

Plasmid construction.

Plasmid pCPP3314, which carries ORF11, was constructed by PCR amplification. Primers 5'-GTCGAATTCGGGATGATGCCAA-3' and 5'-CGGCTCGAGATTGAGCGGAGAT-3', containing *Eco*RI and *Xho*I sites, respectively, were used to amplify a 500-bp fragment containing ORF11 and 168 bp 5' of the 3' end of ORF12. The PCR product was purified with the QIAquick PCR purification kit (Qiagen), digested, gel-puri-

fied, and ligated to pBluescriptSK– digested with *EcoRI* and *XhoI*, generating plasmid pCPP3314. pCPP3381, which expresses the first 61 amino acids of VirA fused to Cya (VirA₁₋₆₁-Cya), was created using Gateway cloning technology (Invitrogen, Carlsbad, CA, U.S.A.). First, the first 61 codons of *virA* were amplified by PCR using primers 5'-CAC-CATGCAAGAGCCAGTAAGATTAATTG-3' and 5'-TAATA TTAGACCAGCCTTATGACCAG-3', and this fragment was cloned into the pENTR/SD/D-TOPO entry vector according to the manufacturer's instructions. pCPP3381 then was created by a site-specific recombination reaction (or LR reaction) between the *virA61* entry vector and pCPP3234 (Schechter et al., in press).

Deletion of genes located in the right flanking region.

A 1.9-kb *KpnI-EcoRI* fragment spanning from *hrcR* to part of ORF5 was isolated from pCPP3083 and cloned into pUCP24. A 2-kb region from ORF7 to ORF9 was cloned into pBluescriptSK– by PCR amplification using primers 5'-CC ACTAGTCCGTCTCATCAGGCACATCTT-3' and 5'-GTTCT AGACGCCGACGGTGGTAGG-3', containing *SpeI* and *XbaI* sites, respectively. pBSSK::ORF7-ORF9 was digested with *BamHI* and ligated to a purified Ω^{Sp} cassette. This construct, pBSSK::ORF7-ORF9:: Ω^{Sp} , was digested with *XbaI* and *SmaI*, and the 4-kb fragment was isolated and ligated to pUCP24::hrcR-ORF5 to create pCPP3231.

Mutagenesis of *E. chrysanthemi*.

Plasmids containing phage insertions in ORFs 10, 11, 12, 13, and 14 and the plasmids pCPP3231, pCPP2511, and pCPP3287 were electroporated into $\Delta(\text{pelA/pelE})\Delta(\text{pelB/pelC})$::28bp $\Delta(\text{pelX})$ Δ 4bp *pehX*:: Ω^{Cm} mutant CUCPB5047. Transformants obtained after electroporation were used for marker exchange following standard procedures (Roeder and Collmer 1985). CUCPB5118 was constructed by introducing the *hrpL*:: Ω^{Cm} mutation into AC4150 as previously described (Ham et al. 2004). Mutant constructs were confirmed by DNA gel blot analysis.

RT-PCR analysis.

E. chrysanthemi AC4150, CUCPB5118, and CUCPB5360 were grown in King's B medium until an optical density at 600 nm (OD₆₀₀) reached 0.7. Total RNA from 100 μ l of culture was extracted and eluted in 30 μ l of diethylpyrocarbonate-treated H₂O with the RNeasy Mini Kit (Qiagen). Then, 2 μ l of each RNA sample was further treated with Dnase I, amplification grade (Invitrogen), to eliminate remaining DNA in 10 μ l of total reaction volume. After 20 min of incubation, Dnase I was inactivated with 1 μ l of 25 mM EDTA and heat treatment (70°C, 10 min). Of a total 11 μ l of reaction mixture, 1 μ l was used as the template for RT-PCR. Two primer pairs (5'-TCGC TGGGTTCAGCGTGGAT-3' and 5'-CATCTGGCTGGCGT TCAGCAT-3' for *hrpN* and 5'-AGGATCTCATATCGGTCTAT T-3' and 5'-TCATCTGGTCATAAGGCTGGT-3' for *virA*) were added to the RT-PCR reaction mixture in order to detect *hrpN* and *virA* transcripts simultaneously. RT-PCR was performed with Superscript One-Step RT-PCR kit (Invitrogen) following the manufacturer's protocol. The program for RT-PCR was 50°C for 30 min; 94°C for 2 min; 40 cycles of 94°C for 15 s, 50°C for 30 s, and 68°C for 40 s; and 72°C for 5 min.

Plant assays.

E. chrysanthemi strains were grown in King's B plates at 30°C overnight and resuspended in 5 ml of 10 mM MgCl₂ to an OD₆₀₀ of 0.3. Witloof chicory leaves and potato tubers were wound inoculated with 50 μ l of a bacterial suspension containing 1×10^8 CFU/ml and incubated in a moist chamber at 30°C

for 48 h, following previously described procedures (Bauer et al. 1994; López-Solanilla et al. 2001). Because of quantitative variation in the susceptibility of different batches of chicory leaves to *E. chrysanthemi*, assays for the virulence phenotype of any mutant were done in direct comparison with the CUCPB5047 parental strain using the batch available.

Maceration was scored 48 h after inoculation. Axenically grown *N. clevelandii* seedlings were inoculated by pipetting 50 μ l of bacterial suspension to the apical meristem and incubating at 25°C (Rojas et al. 2002). Symptoms were photographed 48 h after inoculation. Assays for Cya-dependent cAMP production in *N. benthamiana* leaves were performed as described previously, except that bacteria were suspended in 5 mM MES, pH 6.5 (Schechter et al. in press). Plants were bagged and incubated at 27°C during the course of the experiment.

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