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Alternative Sigma Factor HrpL of *Pectobacterium carotovorum* 35 is Important for the Development of Soft-rot Symptoms

Hyo-Song Nam†, Ju Yeon Park[1†], Beom Ryong Kang[2], Sung-Hee Lee[3], Jae Soon Cha[4] and Young Cheol Kim[1]*

BioControl Center, Jeonnam Bioindustry Foundation, GokSeung 516-942, Korea
[1]Institute of Environmentally-Friendly Agriculture, Chonnam National University, Gwangju 500-757, Korea
[2]Jeonnam Agricultural Extension Service Center, Naju 520-715, Korea
[3]Chungbuk Agricultural Research and Extension Services, Ochang 363-833, Korea
[4]Department of Agricultural Medicine, Choongbuk National University, Chungju 361-763, Korea
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A bacterial artificial chromosome library of *Pectobacterium carotovorum* 35 was constructed to characterize the genome and to sequence its *hrp* region. The *hrp* cluster of *P. carotovorum* 35 consisted of 26 open reading frames in five operons. A promoter-based green fluorescent protein technology was used to identify the genes regulated by the alternative sigma factor, HrpL, in *P. carotovorum* 35. The majority of the selected clones contained the *hrpJ* operon promoter sequence, which harbors a *hrp* box, but no putative *hrp* boxes were detected within the promoter sequences of two other *hrpL*-regulated genes encoding for pectate lyase and large repetitive protein. Although the promoters of five other *hrp* operons also contained *hrp* boxes, their expression was not HrpL-dependent in the promoter-based selection in *E. coli*. However, transcriptional analysis showed that expression from all operons harboring *hrp* boxes, except for the *hrpN* operon, was reduced significantly in the *hrpL* mutant. The severity of soft-rot symptoms when the *hrpL* mutant was applied to the surface of tobacco leaves, mimicking natural infection, was greatly attenuated. These results indicate that the *hrpL* gene of *P. carotovorum* 35 may be involved in the development of soft-rot symptoms.

Keywords **: BAC end sequencing, Bacterial genomics, Hypersensitive and pathogenicity genes, Promoter-based expression technology, Soft-rot disease**

Introduction

Pectobacterium carotovorum strains, which cause soft-rot disease, are plant pathogens with relatively broad host ranges (Perombelon and Salmond, 1995). The major virulence factors in soft-rot caused by *P. carotovorum* are extracellular enzymes, which macerate plant tissues. These enzymes include pectin-degrading enzymes, cellulases, and proteases (Andro et al., 1984; Collmer and Keen, 1986). Owing to the economic importance of soft-rot diseases, *P. atroseptica* SCRI1043 has been sequenced (Bell et al., 2004), and draft genome sequences for *P. carotovorum* and *P. brasiliensis* have been published (Glasner et al., 2008). Currently, we are working with the *P. carotovorum* 35 strain, which is responsible for massive losses of Chinese cabbage crops in Korea (Shin, 2004). In order to explore the genome of this strain, we generated a large-insert bacterial artificial chromosome (BAC) library. Such libraries have been extensively exploited in map-based gene cloning studies of eukaryotes (Zhang and Wing, 1997) and also in functional analyses of complex genomes (Antonarakis, 2001).

The hypersensitive response and pathogenicity (*hrp*) gene cluster in plant pathogens plays a function in both pathogenicity and the hypersensitive response associated with plant resistance. The genes within the *hrp* cluster encode for a type III-secretion machinery and bacterial effectors. The regulation of *hrp* gene expression is partially dependent on an alternate sigma factor, HrpL, which is encoded by the *hrpL* gene within the *hrp* cluster (Frederick et al., 2001; Wei and Beer, 1995; Wei et al., 2000). HrpL activates transcription by binding to a common "*hrp* box", 5-

*Corresponding author
Phone) +82-62-530-2071, Fax) +82-62-530-2079
Email) yckimyc@chonnam.ac.kr

†These authors are equally contributed to this work.

GGAACCNA-N_{15-16}-CCACNNA-3, located in the promoters of the responding genes (Fouts et al., 2002; Nissan et al., 2005; Xiao and Hutcheson, 1994). A promoter analysis of the HrpL-regulated genes in *Pantoea agglomerans* pv. *gypsophilae* demonstrated that the first five nucleotides of -35 (GGAAC) and the 3 nucleotide of the -10 (ACNNA) are crucial for HrpL recognition (Nissan et al., 2005). Genetic manipulation increasing the promoter strength of the HrpL-responding genes enhanced gall formation on *P. agglomerans* susceptible hosts (Nissan et al., 2005).

Recently, five *hrpL* up-regulated genes, *hrpA*, *hrpK*, *dspE*, *yijC* and *yecF* of *Dickeya dadantii* (*Erwinia chrysanthemi*), were identified by a green fluorescence (GFP)-based *E. coli* promoter-probe system (Shi and Cooksey, 2009). The functional roles of a few *hrp* genes of plant pathogenic *Pectobacterium*-related bacteria have also been identified. Mutants of the *hrpL*-regulated genes in *D. dadantii* evidenced reduced pathogenicity (Shi and Cooksey, 2009). Mutation of the HrpL-regulated *hrcC* gene in *P. carotovorum* did not impair pathogenicity, even though early growth on *Arabidopsis* was reduced (Rantakari et al., 2001). High expression levels of *hrpL* and *hrpN* genes in *P. carotovorum* were shown to negatively impact disease symptoms on *Arabidopsis* plants (Lehtimaki et al., 2003). These results indicate that HrpL may be a regulator of pathogenesis in *P. carotovorum*, even though no direct evidence supporting this notion has yet been published.

In this study, we identified a complete *hrp* cluster in *P. carotovorum* 35 from our BAC library, by comparison with the *hrp* cluster identified in *P. carotovorum* SCCI (Lehtimaki et al., 2003). In an effort to identify the genes regulated by HrpL in *P. carotovora* 35, we employed the GFP-based strategy used for *D. dadantti* (Shi and Cooksey, 2009), which identifies the promoters required for pathogenesis (Shi and Cooksey, 2009; Yang et al., 2004). We selected genes that were expressed only in the presence of HrpL. We evaluated expression from *hrp* operons harboring the Hrp box in the wild-type and *hrpL*-deficient mutants by quantitative reverse transcription-polymerase chain reaction analysis, and also assessed the role of HrpL in the virulence of *P. carotovorum* 35. Our findings indicated that the alternative sigma factor, HrpL, regulates the *hrp* box-harboring operons or genes in *P. carotovorum* 35, and may also be involved in the development of soft-rot symptoms.

Materials and Methods

Bacterial strains and growth conditions. *P. carotovorum* strain 35, isolated from the roots of the Chinese cabbage (Shin, 2004), was stored in 15% glycerol at −80°C. Luria-Bertani (LB) medium containing : Bacto-Tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g, 1 l of distilled water and an *hrp*-inducing minimal medium containing : KH_2PO_4 2 g, K_2HPO_4 7.7 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, glucose 4 g, 1 l of distilled water were used for growth (Huynh et al., 1989). For the liquid cultures, *P. carotovorum* 35 cells were grown with agitation at 100 rpm at 27°C. *Escherichia coli* cells containing the BAC clones were grown in LB or LB supplemented with 12.5 μg/ml of chloramphenicol. The media were amended with the appropriate antibiotics: tetracycline (12.5 μg/ml), ampicillin (50 μg/ml), or kanamycin (100 μg/ml).

DNA manipulation. DNA manipulations for cloning and subcloning were conducted as described previously Ausubel et al. (1989) and Sambrook et al. (1989). Plasmids were isolated using a mini-plasmid purification system (Bioneer Inc., Daejeon, Korea). Transformation was conducted using an Electroporator (Cell-porator, Gibco BRL., USA). Nucleotide sequence analyses were conducted with an ABI1301 DNA sequencer (Applied Biosystems, Foster City, CA) at the Korea Basic Science Institute (KBSI), Gwangju Branch.

BAC library construction. High molecular weight chromosomal DNA of *P. carotovorum* 35 was prepared in low melting agarose gel, as previously described (Bell et al., 2002). The plugs containing high molecular weight *P. carotovorum* 35 chromosomal DNA were chopped into a slurry with a razor blade, washed, and partially digested with *Hin*dIII. The digested DNA samples were fractionated with a Bio-Rad CHEF Mapper and large fragments were cloned into the BAC cloning vector, pIndigoBAC-5 (Ma et al., 2000). Recombinant BAC clones were manually picked and stored in duplicate in 384-well plates at −80°C.

BAC end sequencing and cloning of *hrp* cluster. The BAC clones were grown on LB containing chloramphenicol (12.5 μg/ml) and the DNA was isolated by the alkaline lysis miniprep method (Birnboim and Doly, 1979). The nucleotide sequences of the 504 BAC clones were determined with an ABI1301 DNA sequencer (Applied Biosystems, Foster City, CA) with the forward sequencing primer (5'-GGA TGT GCT GCA AGG CGA TTA AGT TGG-3') and the reverse sequencing primer (5'-CTC GTA TGT TGT GTG GAA TTG TGA GC-3') at Solgent Inc (Daejeon, Korea). The nucleotide sequences of the BAC clones were analyzed using the NCBI Blast program (http://www.ncbi.nlm.nih.gov).

On the basis of the BAC end sequencing results, the

Table 1. Primers used in this study

Primer		Sequence
hrpN	F	5'-CCA TTC ATC CAG CCT GAA AT-3'
	R	GAC ATT ACC CCG GTA TGC TG
hrpN~hrcC	F	ACA GCT GTT CTG CGA TGT TG
	R	CGC TTT CAG TGG TTC GTG TA
hrcC~hrpE	F	GTC AGA GAC CGA GGC ATA GC
	R	CGA TGC GAT GAA GCT GAT TA
hrpF~hrpB	F	GTC AGA GAG CGA GGC ATA GC
	R	CGA TGC GAT GAA GCT GATTA
hrpB~hrpA	F	TTG TAC ACT TTT CGC CAC CA
	R	TGT CAA CAT GGC GCT GTA GT
hrpS~hrpY	F	TGG CCG ATC TGC TAT TTA CC
	R	TGG TGC CAG AGG TTA CAT CA
hrpY~hrpL	F	CTA CTC CTG AAG CCC AGA CG
	R	ACC CGT CGT TTT TCA CTG AC
hrpL	F	TTC CGT CAG TTT CTC GAT
	R	AAA TCG CAT CCT TGA TAC GG
hrpJ	F	TTG CTG ACT CTC CGT TGT TG
	R	GCG TGG TGG AAA TGG AGA T
hrpJ~hrcQ	F	AAC GGC GAT CCA ACT GAT AC
	R	CAC CAG ACG CAG GTT GTC TA
hrcQ~hrcN	F	GTT GGC AGC TAC AGG AGA GC
	R	GTA TCG GCA TCA GCA GGT TC
hrcN	F	CTG CGT CTG GTG AGA ATT GA
	R	GTA TCG GCA TCA GCA GGT TC
hrpO~hrcS	F	CAA GCA GGT TGA AAT GCT GA
	R	TGA TAG CCA ACT GCA TCA GC
hrcR~hrcT	F	GAT GCA GTT GCA GGATCA GA
	R	ATC GGC GTC TTC TAT GTT GG
hrcT~hrcU	F	CGG CCT GTT CTA CAG CTA CC
	R	GTC CCC GTA GCC AGA CAA TA

BAC clone E16 was ascertained to harbor the *hrp* gene cluster of *P. carotovorum* 35. DNA was isolated from the E16 clone by the alkaline lysis method and digested with various restriction enzymes, after which the fragments were subcloned into the designated plasmids. The *hrp* gene sequences were determined from the products of polymerase chain reactions (PCR), using primers based on the gene sequences in the *hrp* cluster of *P. carotovorum* SCC1 (GenBank accession number AY293288) (Table 1). The PCR products were purified and cloned into pGEM T-Easy vector (Promega, Madison, WI).

Replacement of *hrpL* promoter. The promoter of the *hrpL* gene from *P. carotovorum* 35 was replaced with *nptII* and the construct was housed in *E. coli* DH5α with pUC18. A reverse primer (5'-TCG GCA ACG CTC AAA TGC ATG-3') and forward primers harboring the nucleotide sequences of the partial N-terminal sequence of *P. carotovorum* 35 *hrpL* (underlined nucleotide sequences) were utilized in the construction of the *nptII-hrpL* sequence (5'-GCA CTA GAG CCC GGA ATT GCC AGC TGG GGC GCC CTC TGG TAA GGT TGG GAA GCC CTG CAA ATG GAA ATG TCT ACC CTG AAA CAC ATC G-3'). PCR analysis with these primers yielded a 1.0-kb product, which was purified by the methods described in the Nucleogen manual (NucleoGen Inc., Korea), cloned into pGEM T-Easy vector (Promega Inc., Madison, WI) and transferred into pUC18 prior sequencing in order to confirm the replacement of the promoter sequence.

A promoter probe-based GFP screening strategy. To construct the GFP promoter based library of *P. carotovorum* 35, the genomic DNA of *P. carotovorum* 35 was isolated by the CTAB-NaCl method (Ausubel et al., 1987). The genomic DNA of *P. carotovorum* 35 was partially digested with *Sau*3A1 and the approximately 2-kb genomic DNA fragments from agarose gels were isolated and purified with a Zymoclean gel DNA recovery kit (Zymo Research Inc., Orange, CA). The purified genomic DNA fragments of *P. carotovorum* 35 were cloned into the *Bam*HI site of the promoter-probe vector, pPROBE-NT (Miller et al., 2000). A library of more than 20,000 clones was constructed, and each of the library plasmids was isolated by the CTAB method (Del Sal et al., 1989). The *hrpL* plasmid harboring the *nptII*-promoter-*hrpL* fusion was electroporated into *E. coli* DH5α, followed by individual purified plasmids with potential promoter-*gfp* fusions. Following electroporation, more than 20,000 ampicillin-resistant (selecting for the *nptII-hrpL* plasmid) and kanamycin-resistant colonies were isolated. The relative intensity of green fluorescence of each colony was measured with a Fluorometer (FLx800™ Multi-Detection Microplate Reader, Bio-Tek Inc., USA). Plasmids from clones with high GFP expression levels were isolated and re-introduced into *E. coli* DH5α, which did not harbor the *nptII-hrpL* gene. Plasmids that evidenced low expression levels in the absence of HrpL were selected for further study. The plasmids of the selected clones were then purified, and each of the clones was subjected to DNA sequencing.

The promoter sequences of the *hrpN, hrpG, hrpA, hrpJ,* and *hrcN* operons from *P. carotovorum* 35, which harbored the putative *hrp* boxes, were amplified with the specific primer sequences (Table 1). The PCR products were subsequently cloned into pGEM T-Easy vector and DNA sequencing was performed to confirm the promoter sequences of each gene. Each of the PCR products was digested with *Eco*RI and cloned by ligation into the promoter-probe vector, pPROBE-NT, then electroporated into *E. coli* DH5α harboring the plasmid containing the *nptII-hrpL* fusion. The relative green fluorescence of each colony was assessed with

the Fluorometer (FLx800™ Multi-Detection Microplate Reader, Bio-Tek Inc., USA).

Quantitative reverse transcription polymerase chain reaction analysis. *P. carotovorum* 35 cells were grown in *hrp*-inducing minimal and LB media. The cells were harvested at $OD_{600\ nm}$=0.1 for early-log phase cells, and at 2.0 for stationary-log phase cells. To confirm the expression of the *hrp* box containing genes, *P. carotovorum* 35 wild-type and *hrpL* mutant cells were grown to stationary phase in *hrp*-inducing minimal medium. Reverse transcription polymerase chain reaction (RT-PCR) analysis of RNA isolated from the *P. carotovorum* 35 cells was conducted using Trizol™ (GIBCO BRL, Rockville, MD, USA) or an RNeasy Mini Kit with RNA protect Bacterial Reagent and RNase free DNase digestion (Qiagen, Valencia, CA) in accordance with the guidelines provided in the user's manual in order to evaluate the expression of the *hrp* genes.

Quantitative RT-PCR was conducted using the QuantiTect SYBR Green reverse transcription-PCR kit (Qiagen Cat. No. 204243, Valencia, CA). The specific primers were as follows: forward (*hrpL*: 5'-ATC TGT CGA TTG CGA ACA GG-3', *hrpJ*: 5'-AAC GGC GAT CCA ACT GAT AC-3') and reverse (*hrpL*: 5'-CTC CCG CAA ATT TAT CCT GA-3', *hrpJ*: 5'-ACA GAT TCA TGG CTG CTC CT-3'). Specific primers for the 16S rRNA gene, forward (5'-TGG CTC AGA ACG AAC CCT GGC GGC-3') and reverse (5'-CCC ACT GCC TCC CGT AAG GA-3'), were used as internal standards. A 25 μl mixture was incubated for 30 min at 50°C for reverse transcription, followed by quantitative PCR. A Rotor-Gene 2000 Real Time Cycler machine (Corbett Research Inc., Sydney, Australia) was operated for 35 of following cycles: denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and a 30 s extension step at 72°C. Software manufactured by Corbett Research Inc., Australia, was used to determine the expression of the target genes. The comparative C_T method ($2^{-\Delta\Delta CT}$ method) was employed to determine the expression level of the analyzed genes (Livak and Schmittgen, 2001). The expression of the target genes was normalized using the 16S rRNA gene fragment as a housekeeping gene. Fold units were calculated by dividing the normalized expression values of the target genes in the *hrpL* mutant by the normalized expression values of those in the wild-type strain. The results are expressed as the mean and standard deviation of three replicates.

Production of extracellular enzymes. Growth conditions, preparation of culture supernatants, and assay conditions for cellulase, pectate lyase, polygalacturonase, and protease were described previously (Chatterjee et al., 1985; Murata et al., 1991). After 16 h to 18 h at 28°C, pectate lyase and polygalacturonase assay plates were developed with 4 N HCl, and the cellulase assay plates were developed with Congo red and NaCl solutions solutions (Barras et al., 1987; Chatterjee et al., 1985). Halos around the wells arising as the result of protease activity became visible in the protease assay plates within 24 to 36 h without any further treatments.

Construction of *hrpL* mutant. The *hrpL* gene was disrupted by the insertion of an *Eco*RI fragment containing a kanamycin resistance gene from plasmid pRL648 (Elhai and Wolk, 1988) into the unique *Mfe*I site within the *hrpL* ORF. The chromosomal *hrpL* gene in *P. carotovorum* 35 was exchanged for the disrupted version using the exchange vector pRK415, as previously described. *P. carotovorum* 35 transconjugants were cultured twice in *hrp*-inducing minimal medium (Hyun et al., 1989). The putative mutants were selected for their kanamycin resistance and tetracycline sensitivity. The primer (forward: 5'-TTC CGT CAG TTT CTC GAT-3', reverse: 5'-AAA TCG CAT CCT TGA TAC GG-3') was designed on the basis of the *hrpL* from *P. carotovorum* 35, and the identification of the *hrpL* mutant was confirmed by PCR.

Virulence test. Four weeks after seeding in microtiter plates, tobacco plants were challenged with wild type *P. carotovorum* 35 or the *hrpL* mutant by pipetting 20 μl of inoculum onto each leaf. The bacteria were grown for 24 h in LB broth, harvested by centrifugation, washed in sterile water, and resuspended in sterile distilled water at $OD_{600\ nm}$=1.0 (about 1×10^8 cfu/ml). The wild-type and mutant cells were cultivated and centrifuged, washed in sterile water, and resuspended in sterile distilled water at $OD_{600\ nm}$=1.0. Distilled water was used as a control. For the studies conducted with the potato plant, the bacterial suspensions were pipetted onto potato slices, and incubated for 24 h at 30°C. Virulence was scored according to the intensity of the water-soaked rotted lesions surrounding the inoculation site.

Results

BAC-end sequencing of the *P. carotovorum* 35 BAC library. The sequence analysis of a BAC library of 504 clones harboring partial fragments of genomic DNA from *P. carotovorum* 35 evidenced an average insert size of approximately 150 kb, and enabled the construction of a physical map of *P. carotovorum* 35 for comparison with the genome of *P. atroseptica*

SCRI1043. Detailed information concerning the BAC end sequences of *P. carotovorum* 35 is available at the homepage of the Plant Pathology Laboratory of Chonnam National University (http://ppmpmi.chonnam.ac.kr). The results of BAC end sequencing analysis demonstrated that the BAC library of *P. carotovorum* 35 harbored most of bacterial chromosome which covers approximately 4.6 Mb chromosome (data not shown).

Cloning of the *hrp* cluster from BAC library. Sequencing of the subclones from the BAC clone E16 revealed a 24 kb *hrp* cluster with 26 open reading frames, which were grouped into five operons (Fig. 1) in the same order as the *hrp* gene cluster of *P. carotovorum* 35 (Lehtimaki et al., 2003). The deduced amino acid sequences of the *hrp* genes of *P. carotovorum* 35 evidenced a 92–99% identity with their *P. carotovorum* SCCI homologues (Table 2). The putative genes and their encoded functions are summarized in Table 2. Using *hrpL* and *hrpJ* as reference loci, we predict that the *hrp* genes are likely to be transcribed in two directions, with the cluster of *hrpN*, *hrpV*, *hrpT*, *hrcC*, *hrpG*, *hrpF*, *hrpE*, *hrpD*, *hrcJ*, *hrpB*, *hrpA*, *hrpS*, *hrpY*, and *hrpX* being transcribed in the same direction as *hrpL*, and opposite the transcriptional direction of *hrpJ*, *hrcV, hrpQ, hrcN, hrpO, hrpP, hrcS, hrcT,* and *hrcU*

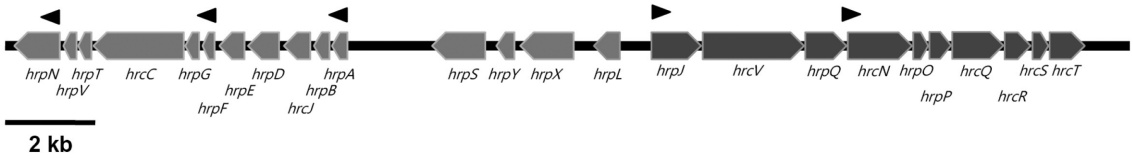

Fig. 1. The *hrp* gene cluster of *Pectobacterium carotovorum* subsp. *carotovorum* 35. The arrowheads indicate the predicted direction for the transcription of each gene. The *hrp*-boxes are marked with black arrowheads. The nucleotide sequences of the *P. carotovorum* 35 *hrp* genes are deposited in GenBank under Accession No. EU420066.

Table 2. Putative functions and identities of the *Pectobacterium carotovorum* 35 *hrp* genes

Name of gene	Hypothetical MW(kDa)/pI	Putative function of the gene	Identity with *Erwinia carotovora* SCCI (%)
hrpN	38/6.74	HR-inducer harpin	93
hrpV	12/5.83	A negative regulator of transcription of the *hrp* regulon	92
hrpT	6.9/8.63	A putative lipoprotein	94
hrcC	75/6.51	A secretion associated outer membrane	98
hrpG	14/4.82	An OmpR subclass of two-component response regulator	98
hrpF	8/4.46	A putative type III translocon protein	96
hrpE	22/6.10	A *hrp* pilin	94
hrpD	23/6.95	A bitopic membrane protein	94
hrcJ	23/5.28	A putative outer membrane lipoprotein	95
hrpB	15/8.03	A positive regulator of pathogenicity gene	96
hrpA	6/5.96	A putative type III translocon protein	98
hrpS	40/7.73	A putative NtrC like regulatory protein	95
hrpY	23/8.65	A putative two-component response regulator	96
hrpX	54/8.47	A putative two-component sensor kinase	96
hrpL	20/6.22	An alternative sigma factor for hrp genes	99
hrpJ	98/4.97	A putative hydrophilic-secretion protein	97
hrcV	176/4.85	Type III secretion protein	96
hrpQ	76/5.02	Type III secretion protein	97
hrcN	112/4.93	Type III secretion cytoplasmic ATPase	96
hrpO	35/5.17	Secretion component, pathogenicity factor	96
hrpP	44/5.13	Type III secretion protein	96
hrcQ	97/4.96	Type III secretion protein	95
hrcR	54/5.11	Type III secretion protein	96
hrcS	21/5.29	Type III secretion protein	97
hrcT	68/5.06	Type III secretion protein	95

Table 3. Hrp boxes found in the promoter regions of various *hrp* genes in *Erwinia carotovora* SCC1 and *Pectobacterium carotovorum* 35

Organism and operon	Promoter sequences and consensus sequences	Reference
E. carotovora SCC1		
hrpA	5'- CTTTTAGTTGGAACTCACTGACCCGCTCTCCCACTTAATGAATGAA-3'	Rantakari et al. (2001)
hrpC	CGTACGATGGGAACTGAGCAGGCAAGAAAATCACTTAATGGGGGAG	Rantakari et al. (2001)
hrpN	CTGCCACTGGGAACTGCACGCTTAGGTTAACCACTCACTATTGAGT	Rantakari et al. (2001)
hrpJ	TCACGTCGGGGAACCCATCCTTTTCTGCGTCCACACAGCAATACAT	Lehtima et al. (2003)
hrcN	AAAAAAGCTGGAACGGATGCTCGCTCAACTCCACCAACGTTACAGA	Lehtima et al. (2003)
P. carotovorum 35		
hrpA	CTTTCCGTTGGAACTCACTGACCCGCTCTCTCACTTAATGAATGA	This study
hrpG	TGCTAATGGGAACTGCACGCCGGGGTTAACCACTCACTATTGAGT	This study
hrpN	CATAAGACGGGAACTGAGCACGCAAGGAAATCACTTAATGGGGGA	This study
hrpJ	TCACGTCGGGGAACCCATCCTTTTCTGCATCCACATAGCAATACAT	This study
hrcN	AAAAAAGCTGGAACGGATGGCTCGCTCAACTCCACCAACGTTATAG	This study
Consensus	GGAACc --------- 16bp space --------- cCACtcA	Fouts et al. (2002)

Table 4. Relative green fluorescence of HrpL-regulated genes from *Pectobacterium carotovorum* 35 as screened by the promoter-based GFP technology[a]

Clone No.	Gene or function in *E. carotovora* ssp. *atroseptica*	GFP with *hrpL* (RLU)	GFP without *hrpL* (RLU)[b]
J11B and 100 clones	*hrpJ*, typeIII secretion protein	1,500–2,000	100–200
J11C	*pel*, pectate lyase	1,671±234	234±54
J75B	large repetitive protein	829±86	87±23

[a] The relative green fluorescence of each construct grown in LB broth was measured using a Fluorometer (FL×800™ Multi-Detection Microplate Reader, Bio-Tek Inc., USA). Three independent experiments were repeated and the relative expression values are expressed as the means±SE of three experiments.
[b] The control was *Escherichia coli* DH5α containing vector only without co-expression of the *hrpL* gene.

(Fig. 1). Putative *hrp* boxes were detected upstream of *hrpJ, hrpA*, *hrpG*, and *hrpN* (Fig. 1). The consensus sequence of the *hrp* boxes, (GGAACc --16 bp space--cCACtcA), was identical between the SCCI and 35 strains (Table 3).

Screening of *hrpL*-regulated genes in *P. carotovorum* 35. In order to screen for HrpL-regulated genes, we employed an *E. coli* construct that constitutively generated HrpL via a plasmid harboring a construct of the open reading frame ligated to the promoter of the kanamycin resistance gene (*nptII*). The *nptII* promoter fusion resulted in a 13,000-fold stronger relative expression than that generated by the native promoter (data not shown). An initial screening revealed approximately 102 constructs that evidenced HrpL-dependent GFP expression (Table 4). The sequence analysis of these fusions showed that the majority of the selected clones contained the promoter for the *hrpJ* operon. Four other operons from the *hrp* cluster, *hrpN, hrcN, hrpG*, and *hrpA,* all of which harbored *hrp* boxes, were not detected by the promoter-probe technology (Table 3). However, two other HrpL-dependent clones contained promoter sequences of a *pel* gene encoding for pectate lyase, and a gene encoding for large repetitive protein.

Expression of *hrp* box-containing genes in the presence of *hrpL* by promoter-probe technology. Via functional screening using the *E. coli* promoter-probe strategy, we determined that the *hrpJ* operon from *P. carotovorum* 35 was HrpL-dependent, whereas no enhanced expressions from the promoter sequences of the *hrpN, hrcN, hrpG*, and *hrpA* operons were noted in these constructs (data not shown). However, the *hrpJ* promoter construct generated a stronger GFP signal (Table 4).

Expression and transcriptional regulation of *hrpL* and *hrpJ* from *P. carotovorum* 35. Expression from *hrpL* and *hrpJ* was higher in the early log-phase *P. carotovorum* 35 cells ($OD_{600\ nm}$=0.1) grown on the minimal medium than those grown in rich medium (Fig. 2). In both media, the transcript levels of *hrpJ* and *hrpL* increased from the mid-log phase to the stationary phase (Fig. 2). The highest expression levels were detected in the stationary phase, bolstering the proposal of Chatterjee et al. (2002) that *hrp* genes are

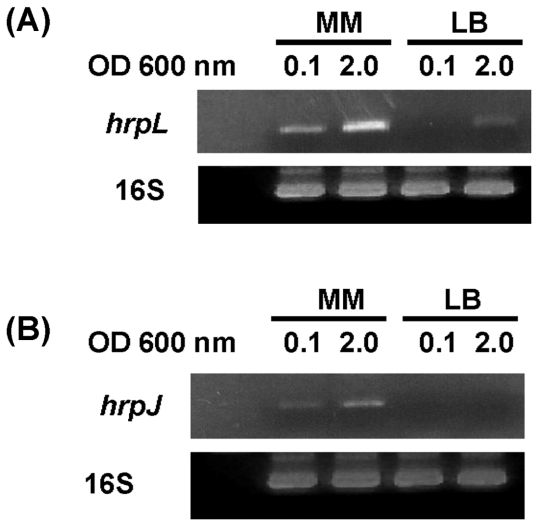

Fig. 2. Transcript accumulation of *hrpJ* and *hrpL* in *Pectobacterium carotovorum* 35 at different growth phases and in two growth media. RNA was isolated from early log-phase ($OD_{600\,nm}$=0.1) and stationary-phase ($OD_{600\,nm}$=2.0) cells grown in *hrp*-inducing minimal medium and LB broth. RT-PCR analysis was conducted using specific primers for *hrpL* (**A**) and *hrpJ* (**B**) or for the 16S rRNA gene transcripts as positive control. The PCR reactions were halted at the end of the 30th cycle, and the PCR products were loaded onto 2% agarose gels for detection. The data shown are from one of three studies that generated similar results.

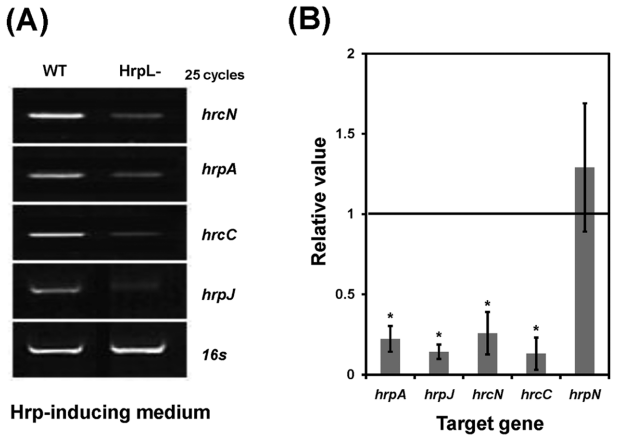

Fig. 3. Expression of *hrpJ, hrpN, hrcC, hrcN,* and *hrpA* in *Pectobacterium carotovorum* 35 and *hrpL* mutants, as detected by qRT-PCR analysis. Total RNAs were isolated from *P. carotovorum* 35 and *hrpL* mutant cells grown to late-log phase ($OD_{600\,nm}$=1.8) on the Hrp-inducing medium containing 0.1% glucose. Quantitative RT-PCR analysis was conducted using specific primers for *hrp* genes or for the 16S rRNA gene transcripts as positive controls. The PCR reactions were halted at the end of the 25th cycle, and the PCR products were loaded onto 2% agarose gels for detection. The data shown are one of three studies that generated similar results. The relative expression values are from three independent experiments, and the mean expression values are provided. *indicates a significant difference in the relative expression of the genes as shown by ANOVA analysis ($P<0.05$).

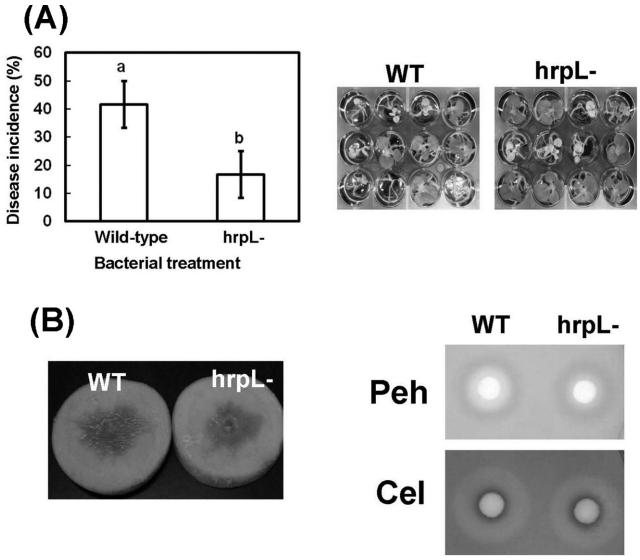

Fig. 4. Virulence test of the wild-type and mutant strains on tobacco plants and potato slices. (**A**) Three-week-old plants were inoculated with bacterial suspensions of the wild-type or *hrpL* mutant containing 1×10^8 cfu/ml on the surfaces of tobacco leaves. The photographs were taken 48 h post inoculation. (*B*) Potato slice inoculated with 10 μl of the wild-type or *hrpL* mutant, 1×10^8 cfu/ml. Agarose plate assays of polygalaturonase (Peh), and cellulase (Cel) activities of *Pectobacterium carotovorum* 35 wild-type and *hrpL* strain. Bacteria were grown at 28ºC in *hrp*-inducing medium to stationary growth phase and cultural supernatants were used for the assay. Each well contained 10 μl of culture supernatant. The photographs were taken 4 days after inoculation.

induced under nutrient-limited conditions in *P. carotovorum*.

Transcriptional analysis of *hrp* genes in the *hrpL* mutant. Transcripts of the *hrp* operons harboring the Hrp box in their promoters, specially the *hrpJ, hrcN, hrpG,* and *hrpA* operons, were reduced significantly in the *hrpL* mutant relative to the wild-type strain (Fig. 3). Transcript levels from another potentially HrpL-regulated gene, *hrpN*, were not detectable in the wild-type or the *hrpL* mutant strains (Fig. 3). Thus, HrpL strongly regulated *hrpJ,* and was involved in expression from the *hrpG* and *hrpA* operons, but was not operative in the regulation of the *hrpN* gene.

Effect of the *hrpL* mutation on pathogenicity and secretion of enzyme activities. When the inocula of the *hrpL* mutant and wild-type strains were applied to tobacco leaf surface, the induced soft-rot symptoms were less severe in the *hrpL* mutant-treated plants relative to the wild-type treated leaves (Fig. 4A). By way of contrast, both the wild-type and *hrpL* mutant induced extensive maceration when inoculated onto sliced potato samples (Fig. 4B). Production of the

extracellular enzymes, pectin-degrading enzymes and cellulase were not different between the wild-type and *hrpL* mutant (Fig. 4B).

Discussion

P. carotovorum causes severe soft-rot in Chinese cabbage all over the world, and no effective approaches to the control of this pathogen have yet been developed. Control strategies might arise from a better understanding of mechanisms underlying the pathogenicity of this particular bacterium. The *hrp* gene cluster in plant bacterial pathogens plays a role both in pathogenicity and in the triggering of the hypersensitive response. *P. carotovorum* strain 35 harbors an *hrp* cluster with genes with high identity and order relative to that of *P. carotovorum* SCCI (Lehtimaki et al., 2003).

The expression of some *hrp* genes depends on the alternative sigma factor, HrpL (Wei and Beer, 1995), which is encoded within the *hrp* cluster. Both HrpS and another alternative sigma factor, RpoN, are known to be involved in the regulation of *hrpL* expression in *P. carotovorum* 71 (Chatterjee et al., 2002). The promoter sequence of *hrpL* in *P. carotovorum* 35 was similar to that in isolate 71 (Chartterjee et al., 2002), showing two potential RpoN-binding sites, between nucleotides -24 and -7 (GGctGGcacaagGCttGC), and an integration host factor (IHF)-binding site between nucleotides -130 and -115, TTGCAAgaacTTGCAA. Consequently, regulation in the isolate 35 may also involve RpoN and IHF, as suggested in a previous study (Chatterjee et al., 2002).

The *hrp* cluster genes have been less well characterized in the soft rot bacteria than in the biotrophic bacterial pathogens, such as *Pseudomonas syringae* pv. *tomato* DC300. In isolate 35, we observed the 35 *hrp* box, 5'-GGAACCNA-$N_{15\text{-}16}$-CCACNNA-3', located within the promoters of the *hrp* operons or genes as described for other isolates (Fouts et al., 2002; Nissan et al., 2005; Xiao and Hutcheson, 1994). Using a GFP-based promoter probe approach we determined that one gene in the *hrp* cluster, *hrpJ*, was regulated by HrpL, and that this gene harbored a putative HrpL binding box. The expression from *hrpJ* was correlated with that from *hrpL* for different growth media during the culture phase. However, two other genes selected for their possessing of HrpL-activated promoters, one encoding for a pectate lyase and the second for a large repetitive protein, lacked *hrp* boxes and other conserved sequences. So far, there is no direct evidence that the transcriptional regulation of the pectate lyase and large repetitive genes occurs in an HrpL-dependent fashion. Therefore, we are currently attempting to ascertain whether HrpL binds to the promoters of these genes. It has been previously reported that the overexpression of the *hrpL* gene may induce the atypical expression of some genes (Chang et al., 2005).

We demonstrated that the production of extracellular pectinases, cellulase, and protease enzymes was not altered in the *hrpL* mutant of *P. carotovorum* 35. Because these extracellular enzymes are key virulence factors for the development of soft-rot symptoms (Andro et al., 1984; Collmer and Keen, 1986), this finding is consistent with the observed ability of the *hrpL* mutant to cause soft rot on sliced potato samples at the wild-type level. However, the severity of soft-rot symptoms was reduced profoundly in the *hrpL* mutant when inoculated onto the tobacco leaf surface, mimicking a natural infection process. This finding was consistent with previous reports of the reduced pathogenicity of the HrpL mutant of *D. dadantii* (Shi and Cooksey, 2009) and the HrcC mutant of *P. carotovorum* (Rantakari et al., 2001) on susceptible host plants. Thus, HrpL may regulate other genes involved in the development of soft-rot symptoms. In conclusion, our results suggest that HrpL is involved in expression from the *hrp* genes harboring the *hrp* box in their promoters, and is also involved in the development of soft rot in the tobacco plant. However, the function of HrpL in isolate 35 did not appear to involve the production of extracellular plant cell wall-degrading enzymes. We are currently conducting a more detailed analysis of the regulation of the *hrp* cluster expression *P. carotovorum* 35.

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