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

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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 *hrp*-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-
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 Pantoea 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 hrpC 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. dadantii (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_{2}PO_{4} 2 g, K_{2}HPO_{4} 7.7 g, MgSO_{4}·7H_{2}O 0.1 g, (NH_{4})_{2}SO_{4} 1 g, glucose 4 g. 10 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 HindIII. The digested DNA samples were fractionated with a Bio-Rad CHEF Mapper and large fragments were cloned into the BAC cloning vector, pfndigoBAC-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 GFA TGT TGT 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
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Table 1. Primers used in this study

| Primer | Sequence |
|--------|----------|
| hrpN   | F 5'-CCA TTC ATC CAG CCT GAA AT-3' |
| hrpN-hrcC | R GAC ATT ACC CCG GTA TGC TG |
| hrcC-hrpE | F ACA GCT GTC CCG GTA TGC TG |
| hrcE-hrpF | R CGC TTT CAG TGG TTC GTG TA |
| hrcF-hrcR | F GTC AGA CAG CGA GGT GTA AC |
| hrcR-hrcT | R CGA TGC GAT GAA GAT GTA TA |
| hrcT-hrcS | F TGT GGC GTC TTC TAT GTT GG |
| hrcS-hrpH | R CGG CCT GTT CTA CAG CTA CC |
| hrpH-hrcQ | F TGG TGC GAT GAA GTA TCC |
| hrcQ-hrcN | R TGG TGC GAT GAA GAT GTA TA |
| hrcN-hrcO | F TGG TGC GAT GAA GAT GTA TA |
| hrcO-hrcP | R TGG TGC GAT GAA GAT GTA TA |
| hrcP-hrcQ | F TGG TGC GAT GAA GAT GTA TA |
| hrcQ-hrcR | R TGG TGC GAT GAA GAT GTA TA |
| hrcR-hrcN | F TGG TGC GAT GAA GAT GTA TA |
| hrcN-hrcO | R TGG TGC GAT GAA GAT GTA TA |
| hrcO-hrcP | F TGG TGC GAT GAA GAT GTA TA |
| hrcP-hrcQ | R TGG TGC GAT GAA GAT GTA 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 GCC 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 Sau3AI 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 BamHI 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, hrpL, 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 EcoRI 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 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'-TTC CGT CAG TTT CTC -3') and reverse (5'-CCC ACT GCC TCC CTG 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$^{-ΔΔC_T}$ 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 *EcoRI* fragment containing a kanamycin resistance gene from plasmid pRL648 (Elhai and Wolk, 1988) into the unique *MfeI* 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* fragment 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*.
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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 | 5'- CTTTCTGTGGAACTCAGCTTCCCACCTAATGAA-3' | Rantakari et al. (2001) |
| *hrpC* | CGTACGATGGAAGGATGACAGGGCAAGAACATCTATATGAG | Rantakari et al. (2001) |
| *hrpN* | CTGCCCACTGGGAAGACTGACAGCTTATTACCCACTACTATTGAT | Rantakari et al. (2001) |
| *hrpJ* | TACGTCGCGGGAACCACATCTTTTTCAGCCTACACCAACATACAT | Lehtima et al. (2003) |
| *hrcN* | AAAAAAGCTGGAACGATGCTCGCTCAACTCCACCAAAGTTACAG | Lehtima et al. (2003) |

| *P. carotovorum* 35 |  |  |
|---------------------|---------------------------------------------|------------|
| *hrpA* | CTTTCTGTGGAACTCAGCTTCCCACCTAATGAA-3' | This study |
| *hrpG* | TGCTAAATGGGAACCTCAGCCGGTACAACCACCTACATATTGAT | This study |
| *hrpN* | CATAACGCGGAAACTGACGCAGCAAGGAAATCACTTTATGAGGGGA | This study |
| *hrpJ* | TACGTCGCGGGAACCACATCTTTTTCAGCCTACACCAACATACAT | This study |
| *hrcN* | AAAAAAGCTGGAACGATGCTCGCTCAACTCCACCAAAGTTACAG | This study |

**Consensus**

GGAAACc ------ 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

| Clone No. | Gene or function in *E. carotovora* ssp. atroseptica | GFP with *hrpL* (RLU) | GFP without *hrpL* (RLU)$^b$ |
|-----------|-----------------------------------------------------|-----------------------|-----------------------------|
| J11B and 100 clones | *hrpJ*, type III 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, (GGAAACc →16 bp space→ cCACtcA), was identical between the SCC1 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$_{600\text{ nm}}$=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
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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 (Chatterjee 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* DC3000. In isolate 35, we observed the 35 *hrp* box, 5′-GGAAACCNA-N15-16-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 harbored a putative HrpL binding box. The expression from *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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