Type III Secretion Contributes to the Pathogenesis of the Soft-Rot Pathogen *Erwinia carotovora*: Partial Characterization of the *hrp* Gene Cluster

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Submitted 24 October 2000; Accepted 3 April 2001.

The virulence of soft-rot *Erwinia* species is dependent mainly upon secreted enzymes such as pectinases, pectin lyases, and proteases that cause maceration of plant tissue. Some soft-rot *Erwinia* spp. also harbor genes homologous to the hypersensitive reaction and pathogenesis (*hrp*) gene cluster, encoding components of the type III secretion system. The *hrp* genes are essential virulence determinants for numerous nonmacerating gram-negative plant pathogens but their role in the virulence of soft-rot *Erwinia* spp. is not clear. We isolated and characterized 11 *hrp* genes of *Erwinia carotovora* subsp. *carotovora*. Three putative σ^L -dependent Hrp box promoter sequences were found. The genes were expressed when the bacteria were grown in Hrp-inducing medium. The operon structure of the *hrp* genes was determined by mRNA hybridization, and the results were in accordance with the location of the Hrp boxes. An *E. carotovora* strain with mutated *hrcC*, an essential *hrp* gene, was constructed. The *hrcC*⁻ strain was able to multiply and cause disease in Arabidopsis, but the population kinetics were altered so that growth was delayed during the early stages of infection.

Additional keywords: *hrcC* mutation, plant–microbe interaction.

Soft-rot *Erwinia* species are plant pathogens with a wide host range (Pérombelon and Kelman 1980). The primary virulence factors are secreted enzymes such as pectin lyases, cellulases, and proteases that unspecifically macerate plant tissues (Andro et al. 1984; Collmer and Keen 1986). The presence of the potential secondary pathogenesis determinants hypersensitive reaction and pathogenicity (*hrp*) genes has been revealed by Southern hybridization in the soft-rot *Erwinia* species (Laby and Beer 1992). In plant pathogens, the *hrp* genes encode a type III secretion machinery, which is believed to transport bacterial effector proteins directly into the host cell. The type III protein secretion system is wide-

spread in animal and plant pathogenic gram-negative bacteria (Galán and Collmer 1999). A *hrp* cluster typically consists of approximately 20 genes, organized in approximately eight operons. Nine of the genes (termed *hrc*) are conserved between animal and plant pathogens and, except for *hrcC*, have homologs in the flagellar export machinery (Alfano and Collmer 1997). The *hrcC* gene encodes an outer membrane constituent and is homologous with several secretion-associated genes (Deng et al. 1998; Hobbs and Mattick 1993). In addition to the core secretion machinery genes and regulatory genes, the *hrp* clusters encode several *hrp*-associated genes. These include genes for harpins (Bauer et al. 1995; He et al. 1993; Mukherjee et al. 1997); a gene for the Hrp-pilin, which in *Pseudomonas* and *Ralstonia* spp. is required for type III secretion to function (Roine et al. 1997; Van Gijsegem et al. 2000; Wei et al. 2000); avirulence (*avr*) genes (Leach and White 1996); and putative open reading frames (ORFs) with unknown functions. In plant-pathogenic, nonmacerating bacteria of the genera *Ralstonia*, *Pseudomonas*, and *Xanthomonas* and the fire blight-causing pathogen *Erwinia amylovora*, *hrp* genes are essential for virulence and elicitation of a hypersensitive reaction (HR), which is a specific defense response of nonhost plants that results in the localized death of plant tissue and leads to the elimination of the bacteria (He 1998). Type III secreted avirulence proteins are responsible for HR elicitation in resistant plants and are recognized inside the plant cell in a gene-for-gene-specific manner (Leach and White 1996). In susceptible hosts, the transported virulence proteins apparently modify the host cell response in favor of the pathogen (Alfano and Collmer 1997; Galán and Collmer 1999). The genes for type III secretion and at least part of the Avr proteins are located in pathogenicity islands (PAIs), which are often associated with mobile DNA elements (Jackson et al. 1999; Kim et al. 1998a). Although the role of the mobile elements associated with PAIs is still unclear, they are assumed to promote the spreading of PAIs to other bacterial strains (Groisman and Ochman 1996).

The *hrpC* and *hrpN* operons of *Erwinia chrysanthemi* (Kim et al. 1998b) and the *hrpN* operon of the soft-rotting *Erwinia carotovora* subsp. *carotovora* (Mukherjee et al. 1997) have been identified. On the basis of hybridization analysis, it was suggested that the organization of the *E.*

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carotovora subsp. *carotovora* *hrpC* operon could be similar to that of other *Erwinia* species (Kim et al. 1998b). In *E. amylovora* and *E. chrysanthemi*, the *hrpN* gene is thought to encode a harpin protein secreted through the type III system (Bauer et al. 1995; Kim et al. 1997). For *E. carotovora* subsp. *carotovora* strain 71, it has been shown that inactivation of regulation genes *rsmA* or *kdgR_{Ecc}* led to the overproduction of the harpin HrpN, and elicitation of a HR (Cui et al. 1996; Liu et al. 1999). An *E. chrysanthemi* strain with impaired maceration ability was shown to induce a HR in resistant plants, and a mutation in an essential *hrp* gene reduced the ability of the strain to incite successful infections (Bauer et al. 1994). It also was shown that the HrpN of *E. chrysanthemi* was responsible for the HR elicitation and reduced virulence (Bauer et al. 1995). This implies that a *hrp* cluster is active and functional, at least in some strains of soft-rot *Erwinia* spp., but its effect may be masked in wild-type bacteria by the massive production of macerating enzymes. Although the harpin has been shown to play a role in the pathogenesis of *E. chrysanthemi* (Bauer et al. 1995), no definite role for these genes in virulence of *E. carotovora* species has been demonstrated. Moreover, despite the seeming similarity in disease development, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* differ markedly with regard to many important aspects, including variations in the structure and function of extracellular enzymes produced, gene regulation, or the requirement of certain factors for pathogenicity (Barras et al. 1994; Expert 1999; Nasser et al. 1998; Pirhonen et al. 1993).

The purpose of this work was to characterize the *hrp* genes of *E. carotovora* subsp. *carotovora* and to study their role in virulence. We determined the organization of *hrpA* and *hrpC* operons in *E. carotovora* subsp. *carotovora* strain and their position relative to the previously identified *hrpN* operon (Mukherjee et al. 1997) and characterized the gene organization by transcript analysis. An *hrcC*-deficient mutant strain was less virulent than the wild-type in *Arabidopsis thaliana*. The *hrp* locus was flanked by a transposon-like element that might participate in the movement of *hrp* genes.

RESULTS

Isolation and structural characterization of the *hrp* genes.

A lambda library of the genomic DNA of the *E. carotovora* subsp. *carotovora* strain SCC1 was constructed. The *hrpN* gene of SCC1 was amplified by polymerase chain reaction (PCR) with *hrpN*-specific primers. The labeled *hrpN* PCR fragment was used as a probe in hybridization to isolate lambda clones containing the *hrpN* gene. On the basis of the organization of other *hrp* operons, we expected to find other *hrp* genes near the *hrpN* gene. DNA from several positive clones was purified, and the inserts were roughly mapped. The clone, λN, harbored a 13.7-kb insert and was chosen for further characterization. The DNA derived from λN was subcloned as a 13.7-kb fragment into pBluescript SK+, and the resulting plasmid was named pAR1. Sequence analysis of the insert revealed 11 genes that were similar to previously sequenced *hrp* genes, and all of these genes were in the same orientation (Fig. 1). On the basis of sequence homology to *E. amylovora* and *Pseudomonas syringae*, three putative HrpL-type promoters were found in front of the putative operons (Kim et al. 1997) (Fig. 2). The *E. carotovora* subsp. *carotovora* *hrp* genes were named *hrpA*, *hrpB*, *hrcJ*, *hrpD*, *hrpE*, *hrpF*, *hrpG*, *hrcC*, *hrpT*, *hrpV*, and *hrpN* on the basis of their homology to previously characterized genes. The organization of the genes was similar to that of the *E. amylovora* *hrpC*, *hrpA*, and *hrpN* operons (Kim et al. 1997). The *hrpC* and *hrpN* operons also were similar to the *E. chrysanthemi* *hrpC*

<i>E. carotovora</i>	
<i>hrpA</i>	CTTTTAGTTGGAACCTCACTGAC . CGGCTCTCCCACTTAATGAATGAA
<i>hrpN</i>	CTGCCACTGGGAACCTGCAC . GCTTAGGTTAACCACCTCACTATTGAGT
<i>hrpC</i>	CGTACGATGGGAACCTGAGC . AGGCAAGAAAATCACTTAATGGGGGAG
<i>E. amylovora</i>	
<i>hrpA</i>	CGCCGCGTGGGAACCGATCGAA . ACTGCCGCCCACTTAATTAACGAA
<i>hrpN</i>	CACCTCGCCGGAAC . CAGAGCGGAATAACCAGCACTCAATATAAGCT
<i>hrpC</i>	CTTCACCACGGAACCTCGCCACGCGCCGAACCCCACTCAAGACAGGA
Consensus	GGAAC CACTCAAT

Fig. 2. Similarities and consensus sequence of hypersensitive reaction and pathogenesis (Hrp) L-type promoters of *Erwinia amylovora* (Kim et al. 1997) and *Erwinia carotovora*. *hrp* boxes are underlined.

Fig. 1. Organization of the hypersensitive reaction and pathogenesis (*hrp*) cluster in *Erwinia carotovora* subsp. *carotovora* SCC1. The operons are represented by thick, open arrows with the names, positions, and relative sizes of the genes indicated. Operon genes are *hrpN* (*hrpN* operon); *hrpT*, *hrpV*, *hrcC*, *hrpG*, and *hrpF* (*hrpC* operon); and *hrpE*, *hrpD*, *hrcJ*, *hrpB*, and *hrpA* (*hrpA* operon). Black arrows above operons represent transcripts deduced from mRNA analysis. Arrow thickness corresponds to the relative abundance of the transcript as indicated by band strength in the hybridization. HrpL-type promoter regions are marked with upward arrowheads. Small, dotted arrows below the operons indicate the position of the probes used in transcript analysis. The *rhs* element is shown as a thick, striped arrow. Cluster size is 13.7 kb.

and *hrpN* operons. In *E. chrysanthemi*, however, other pathogenicity-related genes (Kim et al. 1998b) border the *hrpC* and *hrpN* operons, whereas in SCC1, the operon organization is similar to that of *E. amylovora* where *hrpC* is next to the *hrpA* and *hrpN* operons. When comparing the putative Hrp proteins in the three *Erwinia* species, *E. carotovora* subsp. *carotovora* strain SCC1 exhibited more similarity to *E. chrysanthemi* than it did with *E. amylovora* (Table 1), even though the organization of the *hrp* cluster is more similar between *E. amylovora* and *E. carotovora* subsp. *carotovora*. The organization of the *hrp* clusters in different *Erwinia* species probably reflects the evolution of the *hrp* cluster. The *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* *hrp* clusters are more closely related to each other than either of them were with the *E. amylovora* *hrp* cluster. The fact that the *E. chrysanthemi* gene organization is different can be explained by genetic rearrangements. Possibly the *hrpA* operon of *E. chrysanthemi* has moved into another location in the genome. A *vgr*-homologous fragment

Fig. 3. Analysis of hypersensitive reaction and pathogenesis (*hrp*) transcripts. Lanes are named according to the probes used. In the *hrcC* operon with the *hrcC* probe, bands are approximately 1.6 and 4 kb. The 4-kb band corresponds to the whole operon. The 1.6-kb band is concluded to be a fragmentation product. In the *hrpA* operon, the *hrpA* probe gave a strong signal, corresponding to a 350-bp band that apparently contains the *hrpA* mRNA. Weaker signals were found in two additional bands, of which the smaller, < 1.6-kb band probably corresponds to the *hrpA*, *hrpB*, and *hrcJ* genes, whereas the larger, approximately 2.7-kb band corresponds to all genes in the operon. The *hrcJ* probe hybridized to the < 1.6-kb band, and a weak signal, not visible in the figure, could be seen occasionally at the 2.7-kb position. The *hrpE* probe gave a weak signal at the 2.7-kb band (data not shown).

(57.9% identity in a 959-nucleotide [nt] overlap) of a transposon-like *rhs* element (Wang et al. 1998) was in SCC1, downstream of *hrpN*, possibly marking the “shore” of the PAI. Whether more than one copy of this element is present in the SCC1 genome is unknown.

Characterization of *hrp* gene expression.

Initially, expression of the *hrp* genes in various media was studied. A rich medium such as L medium caused only poor expression of the genes, whereas cultivation in Hrp medium, described for *P. syringae* (Huynh et al. 1989), resulted in the proper induction of *hrp* gene expression. Therefore, the bacteria were grown in Hrp medium in further experiments. Expression of *hrp* genes and the structure of the *hrp* operon were studied by mRNA hybridization with several labeled SCC1 *hrp* genes as probes (Fig. 1). The mRNA analysis with probes from the *hrpA* operon revealed that transcription gives rise to several mRNAs of different lengths. A 350-nt transcript, which was recognized with the *hrpA* probe and presumably spans only *hrpA*, was present in relatively high amounts. A weaker band of 1.4 to 1.5 kb was recognized with *hrpA* and *hrcJ* probes and was presumably carrying *hrpA*, *hrpB*, and *hrcJ* more weakly than the 350-nt transcript. A 2.7-kb transcript, which apparently also contains the downstream genes, was barely visible with the *hrpA* and *hrcJ* probes. In Figure 3, this band is below the detection limit in the *hrcJ* lane. We conclude that the operon was transcribed as multiple mRNA molecules resulting from partial internal termination signals in the operon, one after the *hrpA* gene and another after the *hrcJ* gene. With the *hrpN* probe, only one transcript of approximately 1.2 kb was detected. Hence, *hrpN* is transcribed as monocistronic mRNA, which is in accordance with a previous report on *E. carotovora* subsp. *carotovora* strain 71 (Mukherjee et al. 1997). In the *hrpC* operon, a transcript presumably spanning the *hrpG* and *hrpF* genes was seen as a weak single band of approximately 1 kb when a *hrpF*-specific probe was used (data not shown). The downstream region was present in a 4-kb transcript that was detected with a *hrcC*-specific probe, which is long enough to cover all of the operon genes from *hrpG* to *hrpV* (Fig. 3).

The *hrp* genes affect onset of disease.

To elucidate the function of the *hrp* cluster in *E. carotovora* subsp. *carotovora* SCC1 during infection, the *hrcC* gene was inactivated by inserting a chloramphenicol resistance mini-transposon at base 42 in the chromosomal *hrcC* gene by homologous recombination, as described below. The *hrcC* gene encodes an outer-membrane, pore-forming protein that is essential for type III secretion (Deng et al. 1998). The virulence of wild-type *E. carotovora* subsp. *carotovora* strain SCC1 and the *hrcC* mutant was tested by infecting young *A. thaliana* plants. We measured the infectivity of the pathogen as the

Table 1. Identities/similarities of putative hypersensitive reaction and pathogenesis (Hrp) protein amino acid sequences between *Erwinia amylovora* (*Eam*), *Erwinia chrysanthemi* (*Ech*), and *Erwinia carotovora* subsp. *carotovora* (*Ecc*)

	HrpE	HrcJ	HrpA	HrpB	HrpD	HrcC	HrpF	HrpG	HrpT	HrpV	HrpN
<i>Eam/Ecc</i>	38/48	66/73	68/75	48/53	35/41	70/76	61/68	35/43	57/63	29/38	57/61
<i>Ech/Ecc</i>						74/79	71/75	51/57	50/60	32/41	56/62
<i>Ech/Eam</i>						70/76	62/69	39/44	55/62	43/50	53/57

proportion of macerated leaves from total leaves infected and the ability of the pathogen to multiply in planta. The establishment of the infection and infection kinetics were altered for the *hrcC* mutant. When leaves were infected with equal

amounts of bacteria by placing a 1- μ l droplet on the leaf, the initial in planta growth of the wild-type bacterium was faster than that of the *hrcC* mutant (Fig. 4A), whereas when grown in L medium, the growth rate was identical between the two

Fig. 4. Virulence and maceration of the *Ecc1* wild-type SCC1 and of the *hrcC* mutant *Erwinia carotovora* subsp. *carotovora* SCC1. **A**, Bacterial growth as measured by the number of CFU/plant. The wild type shows faster growth in the plant, but after 48 h, the *hrcC* mutant reached the level of wild-type growth (see text). **B**, Percentage of infected leaves in which maceration is visible. At the 24-h time point, a higher portion of leaves infected with the wild type exhibits macerated tissue. After 48 h, the portion of macerated leaves is close to even.

strains (data not shown). The difference in bacterial amounts in favor of the wild type at the 1-day time point was consistently four- to sixfold in five separate experiments. In each of the experiments, with $n = 3$ or 4, the difference at this time point of the log-transformed population sizes (Hirano et al. 1982) was significant ($p < 0.05$). At the approximately 12-h time point, there also was a slight advantage of the wild-type strain, although the difference was not significant. The differences observed did not persist, however, because 48 h after infection, the *hrcC* mutant had reached the level of the wild type in the amount of bacteria (CFU/plant) (Fig. 4A) and in maceration symptoms in the infected leaf (Fig. 4B). Additionally, systemic spreading of the infection from the infected leaf to the whole plant was delayed in the mutant, as deduced by visual inspection of maceration (data not shown).

DISCUSSION

The role of the Hrp system, according to current consensus, is to translocate proteinaceous avirulence–virulence determinants (Avr) into the plant cell. In most of the bacteria in which a Hrp system has been characterized, *avr* genes also have been found, as is the case in *E. amylovora* (Bogdanove et al. 1998), *P. syringae*, *R. solanacearum*, and *X. campestris* (Leach and White 1996). With the wild-type soft-rot *Erwinia* species, normally no HR is seen and, perhaps, why no *avr* genes have been identified. It has been shown, however, that the type III translocated protein harpin is responsible for HR elicitation and contributes to virulence in soft-rotting *E. chrysanthemi* (Bauer et al. 1994). In the case of *E. carotovora* subsp. *carotovora*, no knowledge exists, as far as we know, of the role of harpin in virulence, although it has been shown to induce the HR (Mukherjee et al. 1997). Our finding that a *hrcC* mutant of *E. carotovora* subsp. *carotovora* strain SCC1 was delayed in symptom development indicates that type III secretion is involved in the virulence of *E. carotovora* subsp. *carotovora*, plausibly via the virulence determinants delivered by it. Hence, this indirectly suggests that *E. carotovora* subsp. *carotovora* strain SCC1 also carries *avr*–*vir* genes.

The *hrp* genes of *E. carotovora* subsp. *carotovora* SCC1 were active transcriptionally in *Pseudomonas* spp. Hrp-inducing medium. The results of the transcript analysis fits well with the location of the HrpL-type promoters predicted from the sequence analysis. The relative level of expression of the *hrp* genes in the same operon may be regulated by partial termination of the transcription between different ORFs. The relative intensity of the *hrpA* operon-specific bands declined as the distance from the promoter increased. This is consistent with the known function of the gene homologs in other bacteria containing a Hrp system. For example, *hrpA* in *P. syringae* encodes the major structural component of a pilus, and the gene product is therefore needed in large quantities. The presence of part of a transposon-like *rhs*-element in the vicinity of the *hrp* cluster of *E. carotovora* subsp. *carotovora* suggests that this element might have been involved in mediating the translocation of the *hrp* cluster to its present location in *E. carotovora* subsp. *carotovora*. In *Escherichia coli*, there are several copies of the *rhs* that generate targets for a high frequency of recombination (Wang et al. 1998).

Our data indicate that the *hrp* cluster plays a role in the virulence at the early stages of infection. The *hrcC* mutant

strain still retained the ability to cause disease, but the onset of the disease and initial bacterial growth in planta was slower. The putative type III secretion-dependent effector proteins may hinder or delay the plant defense response, thus giving the bacteria time to multiply before starting the concentrated enzymatic attack leading to plant tissue maceration. A similar conclusion was made by Bauer et al. (1995) on the role of the *E. chrysanthemi* type III secreted harpin in virulence. In comparison with other plant-pathogenic bacteria in which the function of the Hrp system has been studied, there is a difference in the effect of the Hrp system. In nonmacerating bacteria, a mutation in the essential *hrp* genes such as *hrcC* results in the inability to cause disease (Deng et al. 1998), whereas in the case of *E. carotovora* subsp. *carotovora*, the mutation in *hrcC* only delayed the infection. The different strategies of the HR-inducing and soft-rot pathogens seem to be reflected in the host range: Whereas the former are host specific, *E. carotovora* subsp. *carotovora* has a wide host range. The macerating enzymatic bombardment launched by the necrotrophic *E. carotovora* subsp. *carotovora* is a robust method of invasion compared with biotrophic bacterial species, which depend fully upon the *hrp* gene functions in their virulence. Although these biotrophic pathogens also carry additional virulence determinants such as toxin or extracellular polysaccharide genes, they are nonvirulent without the Hrp system, at least within the laboratory conditions used in most experiments performed so far. It has been shown in field conditions, however, that the biotrophic pathogen *P. syringae* occasionally causes disease symptoms in the absence of a functional type III secretion system (Hirano et al. 1999). The authors propose that the type III system already plays a role during early phyllosphere colonization. On the basis of these observations and on our data presented here, the question is raised as to whether the *hrp* gene cluster is needed to enable bacteria to multiply faster at early stages of infection, thus reaching population densities high enough for the successful incitement of disease in biotrophs and necrotrophs, rather than being required for symptom development.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Erwinia carotovora subsp. *carotovora* wild-type strain SCC1 (Saarilahti and Palva 1986) and *E. coli* DH5 α (Stratagene, La Jolla, CA, U.S.A.) were used in this study. Plasmid pAM34 (Gil and Bouché 1991) has an isopropyl- β -D-thiogalactopyranoside (IPTG)-dependent origin of replication and is phenotypically ampicillin (Ap)- and spectinomycin (Sp)-resistant phenotypes.

Culture conditions.

E. coli and *E. carotovora* subsp. *carotovora* were grown in L medium or M9 medium (Miller 1972), *E. coli* at 37°C, and *E. carotovora* subsp. *carotovora* at 27°C. For RNA analysis, *E. carotovora* subsp. *carotovora* was grown overnight as a dilution series in L medium and then cells from cultures with optical density (OD) at 600 nm of 0.2 to 0.4 were transferred to Hrp-inducing conditions for 3 h at 18°C (Huynh et al. 1989).

Recombinant DNA techniques.

Plasmids and DNA fragments were purified with the appropriate kits (Qiagen, Hilden, Germany). Electroporation was

used to transform plasmids into *E. coli* and *Erwinia* spp. strains with a Bio-Rad gene pulser (Hercules, CA, U.S.A.). Restriction enzymes were obtained from Promega (Madison, WI, U.S.A.) and ligase was obtained from New England Biolabs (Beverly, MA, U.S.A.). Purification of chromosomal DNA and the ligation of DNA fragments were performed according to standard methods (Sambrook et al. 1989). All PCR reactions were performed with a gene cycler (Bio-Rad Laboratories), following a program of initial denaturation of 4 min at 94°C, followed by 28 repeats of the cycle 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min.

Determination of the nucleotide sequence of the *hrp* operons.

Total DNA from *E. carotovora* subsp. *carotovora* SCC1 was isolated and digested partially by *Sau3AI*. Samples were run on an 0.7% agarose gel, and bands corresponding to 12 to 20 kb were collected and purified. The digested total DNA was ligated to lambda DASH II DNA (Stratagene) and cut with *Bam*HI. These ligation products constituted the lambda library. Primers to be used to detect the *hrpN* gene of *E. carotovora* subsp. *carotovora* were designed to fit conserved regions on the basis of an alignment of several *hrpN* genes (forward: GGC AAC GGC CTG AGC CAG; reverse: CGC TTT AGC CCA GGA TTT ATC). With the use of these primers, a 300-bp fragment was multiplied from *E. carotovora* subsp. *carotovora* strain SCC1. This *hrpN* gene fragment was then labeled with the DIG-PCR kit (Roche, Basel, Switzerland). The labeled *hrpN* DNA was used as a probe in hybridization analysis for locating *hrpN* positive plaques in the λ -library with a commercial DIG kit (Roche). Hybridizations were performed in an hybridization oven (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.) at 68°C with a commercial hybridization and detection kit (Roche). Several positive plaques were purified and partially characterized. One of the positive clones was chosen for sequencing and named λ N. The insert of the λ N plaque was cloned into pBluescript KS II+ vector (Stratagene) with the use of a *NorI* restriction enzyme, and the construct was named pAR1. The insert carried on pAR1 was sequenced with an Applied Biosystems prism device (Foster City, CA, U.S.A.) at the Institute of Biotechnology (Helsinki, Finland). All sequence comparisons were performed with Wisconsin software package, version 10.1 (Genetics Computer Group, Madison, WI, U.S.A.), and default parameters were used.

Mutating the *E. carotovora* subsp. *carotovora* SCC1 *hrcC* gene.

A 5-kb *EcoRI* fragment containing *hrcC* from pAR1 was ligated to the pAM34 plasmid. *E. coli* DH5 α transformed with the plasmid was grown in L agar containing 1 mM IPTG. A Mu-like mini-transposon encoding chloramphenicol resistance was introduced in vitro to the plasmid with a commercial template generation system, following the manufacturer's instructions (Finnzymes, Espoo, Finland). The mutated plasmid library was electroporated into *E. coli*, and individual clones were purified. The transposon insertion was confirmed by PCR and sequence analysis. A plasmid with pAR2, a transposon near the beginning of the *hrcC* gene, was selected for further use. Plasmid pAR2 was electroporated into *E. carotovora* subsp. *carotovora* SCC1, and transformants were selected on L plates containing 40 μ g of chloramphenicol

(Cm) per ml and 0.2% glucose. Single colonies were streaked on L-Cm (40) and L-Ap (150)–Cm (40) plates and grown overnight. A transformant that could grow on Cm but showed limited growth on Ap agar was selected. A clone with the 1-kb transposon in the upstream part of the *hrcC* gene was identified with PCR analysis. By mRNA analysis, it was shown that no *hrcC*-corresponding mRNA was produced in this mutated *E. carotovora* subsp. *carotovora* SCC1 strain.

Determination of *hrp* gene transcription.

After culturing *E. carotovora* subsp. *carotovora* bacteria in Hrp-inducing conditions for 3 h at 18°C, mRNA was isolated and analyzed by gel electrophoresis, as described by Von Gabain et al. (1983). RNA was blotted in 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer (Sambrook et al. 1989) overnight on a nylon membrane (Roche). Hybridizations were performed as above. The probes were constructed by amplifying a fragment of a specific *hrp* gene by PCR with two primers. The product of this reaction was purified, and one of the primers was used in a PCR reaction to label the antisense DNA in question with a commercial PCR labeling kit (Roche).

Virulence tests.

A. thaliana cv. Columbia plants were grown in vitro on 1/2 MS (Murashige and Skoog 1962) plates for 1 week in an incubation chamber at 22°C and 14 h of daylight. Plants were then moved into standard 12-well microtiter plates. The plates were covered with Parafilm and incubated at 22°C, with 12 h of daylight in a miniature greenhouse in a humid atmosphere. Bacteria were grown in L medium at 28°C to stationary phase in a shaker, diluted to 1/50 in M9–0.4% glycerol, and an additional incubation was performed at 28°C for 24 h. The bacteria were then diluted by 1/10 in 0.9% NaCl to yield approximately 2×10^5 cells per μ l. Each plant was infected by pipetting 1 μ l of the bacterial suspension on the upper side of the leaf. Thereafter, samples were taken at various intervals with three or four plants at each time point. The aerial parts of the plants were homogenized with a microhomogenizer in 200 μ l of 0.9% NaCl in an Eppendorf tube. Homogenized samples were suspended in 1 ml of 0.9% NaCl–0.1% Tween-20 and dilution plated to L plates to determine a viable count. The virulence also was assessed as a percentage of infected leaves that showed visible maceration symptoms and the spread of maceration in inoculated plants. The virulence was determined as a percentage of macerated leaves after 24 and 48 h. Also, the percentage of leaves in which the maceration had spread into neighboring leaves was recorded. Maceration measurements were conducted with 60 plants, where the infection progress was monitored.

ACKNOWLEDGMENTS

We thank A. Ikävalko for excellent technical assistance. This study was supported by the Academy of Finland and the Viikki Graduate School of Helsinki University Biocenter.

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