Technical Advance

Use of a Pooled Transposon Mutation Grid to Demonstrate Roles in Disease Development for *Erwinia carotovora* subsp. *atroseptica* Putative Type III Secreted Effector (DspE/A) and Helper (HrpN) Proteins

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Soft rot *Erwinia* spp., like other closely related plant pathogens, possess a type III secretion system (TTSS) (encoded by the *hrp* gene cluster) implicated in disease development. We report the sequence of the entire *hrp* gene cluster and adjacent *dsp* genes in *Erwinia carotovora* subsp. *atroseptica* SCRI1039. The cluster is similar in content and structural organization to that in *E. amylovora*. However, eight putative genes of unknown function located within the *E. carotovora* subsp. *atroseptica* cluster do not have homologues in the *E. amylovora* cluster. An arrayed set of Tn5 insertional mutants (mutation grid) was constructed and pooled to allow rapid isolation of mutants for any given gene by polymerase chain reaction screening. This novel approach was used to obtain mutations in two structural genes (*hrcC* and *hrcV*), the effector gene *dspE/A*, and the helper gene *hrpN*. An improved pathogenicity assay revealed that these mutations led to significantly reduced virulence, showing that both the putative *E. carotovora* subsp. *atroseptica* TTSS-delivered effector and helper proteins are required for potato infection.

Additional keywords: functional genomics.

Erwinia carotovora subsp. *atroseptica* is a gram-negative bacterial phytopathogen with a host-range limited to potato in temperate regions (Pérombelon 2002). Its close relatives, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi*, also cause disease on potato but have wide host ranges, mainly in subtropical and tropical regions, respectively (Pérombelon 2002). The major pathogenicity determinants of these “soft rot” *Erwinia* spp. include plant cell-wall-degrading enzymes (PCWDEs) that break down the middle lamella and plant cell walls in the latter stages of infection (Thomson et al. 1999). Because of the importance of these enzymes in pathogenesis, research has focused largely on them, building the impression that the soft rot *Erwinia* spp. act simply as “brute-force” pathogens (Py et al. 1998; Thompson et al. 1999). However, as other pathogenicity mechanisms are discovered in these organisms, this view is changing to recognize pathogens of greater sophistication. A central reason for this reevaluation is the occurrence of active type III secretion systems (TTSSs) (Toth et al. 2003).

The TTSS is present in a wide range of gram-negative bacterial plant and animal pathogens, and is used to translocate “effector” proteins across the host plasma membrane or secrete “helper” proteins that remain outside the host cell and assist translocation of the true effectors. The effectors appear to interact directly with host cell components to alter defense pathways (Collmer et al. 2002; Innes 2003). In phytopathogens, the TTSS is encoded by hypersensitive response and pathogenicity (*hrp*) and *hrp* conserved (*hrc*) genes, and has been implicated in both the establishment of disease during compatible interactions and the elicitation of plant defenses, leading to resistance in incompatible interactions (Bogdanove et al. 1996). Within the *Erwinia* spp., the necrogenic fireblight pathogen *E. amylovora* has become a model for studies on the *hrp* gene cluster. Of the three characterized proteins secreted by the *E. amylovora* TTSS (HrpN, HrpW, and DspE/A), the helpers HrpN (or harpin) (Wei et al. 1992) and HrpW (Gaudriault et al. 1998; Kim and Beer 1998) elicit the hypersensitive response (HR), while HrpN and the disease-specific effector DspE/A are required for pathogenicity (Barny 1995; Bogdanove et al. 1998a,b; Gaudriault et al. 1997; Wei et al. 1992). The *E. amylovora* DspE/A protein recently has been shown to interact with apple receptor-like protein kinases, implying a role in suppression of host defenses (Meng et al. 2002). A decade ago, Bauer and associates (1994) demonstrated the presence of *hrp* genes in *E. chrysanthemi*, suggesting that soft rot pathogenesis is more sophisticated than previously thought. HrpN^{Ech} was shown to elicit an HR in nonhost tobacco and to be involved in soft rot pathogenesis in witloof chicory (Bauer et al. 1995). Soon after, an *hrpN* gene was identified in *E. carotovora* subsp. *carotovora* and shown also to elicit an HR in tobacco (Cui et al. 1996; Mukherjee et al. 1997). Further characterizations of *hrp* genes in *E. chrysanthemi* (Ham et al. 1998; Yang et al. 2002) and *E. carotovora* subsp. *carotovora* (Chatterjee et al. 2002a,b; Rantakari et al. 2001) have strengthened the evidence supporting a role for the Hrp system in soft rot pathogenesis.

The *hrp* gene cluster and adjacent *dspEF* operon have been completely sequenced in *E. amylovora*, and at least partial characterization of the *hrp* gene clusters has been reported in *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* (Fig. 1) (Bogdanove et al. 1998b; Chatterjee et al. 2002b; Ham et al. 1998; Kim and Beer 1998; Lehtimäki et al. 2003; Rantakari et al. 2001; Rojas et al. 2002; Wei et al. 2000). Recently, we demonstrated the presence of an intact *hrp* gene cluster, includ-

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ing genes encoding HrpN, HrpW, HrpW_{chaperone}, DspE/A, and its chaperone DspF/B, in the narrow host-range soft rot *Erwinia* sp., *E. carotovora* subsp. *atroseptica* (Bell et al. 2002). This was the first indication that *dspE* was present in the soft rot *Erwinia* spp.; its role, or that of any other true effector, has not been demonstrated in this group of pathogens. A mutation in the *E. carotovora* subsp. *atroseptica* *hrpJ* gene has since been shown to inhibit HR elicitation in the nonhost *Vicia faba* and affect virulence on the host, potato (Ageichik et al. 2002).

Transposon mutagenesis has been used to analyze gene function in a range of bacteria (Hayes 2003). Transposon-based methods, including signature-tagged mutagenesis (Hensel et al. 1995) and transposon footprinting (Akerley et al. 1998) are commonly used for phenotypic screening of genes. Indeed, large-scale phenotypic screening based on transposon mutant libraries has been carried out to identify pathogenesis-related genes in *E. carotovora* subsp. *atroseptica* (Hinton et al. 1989). Transposon libraries have been constructed to identify proteins with specific properties (e.g., with transmembrane domains) (Becker et al. 2004) or showing substrate-dependent induction (Judson and Mekalanos 2000). Following the advent of whole-genome sequencing, transposon libraries also have been sequenced on a large scale to create genome-wide collections of mapped mutants for global mapping of gene function (Geoffroy et al. 2003; Jacobs et al. 2003; McAdam et al. 2002).

In this article, we describe the complete sequence and characterization of the *E. carotovora* subsp. *atroseptica* SCRI1039

hrp gene cluster and adjacent *dspEF/AB* operon. We also introduce a novel approach to investigate bacterial gene function by generating an arrayed and pooled set of Tn5 insertional mutants (mutation grid), which can be screened by polymerase chain reaction (PCR) for mutations in any gene of interest. PCR screening of the grid allowed rapid isolation of mutants in two TTSS structural genes (*hrcV* and *hrcC*), the effector gene *dspE/A*, and the helper gene *hrpN*. These mutants were significantly reduced in virulence, demonstrating a role for the TTSS and, importantly, for both TTSS-secreted effector and helper proteins in establishing disease on potato.

RESULTS

Sequence analysis and gene prediction.

We were able to use the *E. amylovora* *hrp-dsp* gene cluster as a template for reconstruction of the *E. carotovora* subsp. *atroseptica* SCRI1039 *hrp-dsp* cluster (Fig. 1) due to the high degree of sequence conservation observed previously (Bell et al. 2002). The *hrp-dsp* region in *E. carotovora* subsp. *atroseptica* SCRI1039 (approximately 39,803 bp) has a G+C content of 54.68%; greater than the average across the *E. carotovora* subsp. *atroseptica* SCRI1043 genome sequence (50.96%; unpublished data).

The complete sequence of the *E. carotovora* subsp. *atroseptica* *hrp-dsp* cluster and flanking regions (43,270 bp, accession number AY496066) (Fig. 1) was viewed in Artemis and, using

Fig. 1. Genetic organization of the type III secretion system in *Erwinia carotovora* subsp. *atroseptica* SCRI1039 and its comparison with *E. amylovora*, *E. chrysanthemi*, and *E. carotovora* subsp. *carotovora*. Protein coding regions are represented by large arrows (delineated by a dotted line for partially sequence genes; gradient filled for *hrp/hrc/dsp* genes; fully filled for *hec*, *vgr*-like, and *plcA* genes; unfilled for uncharacterized genes). Gene names appear below the genes and those with the same prefix (e.g., *hrc*) appear in the same line. Thin arrows above the genes show the putative transcriptional organization of the clusters. The genetic maps are based on the following references and are not necessarily to scale: Bogdanove et al. 1998b; Chatterjee et al. 2002b; Ham et al. 1998; Kim and Beer 1998; Lehtimäki et al. 2003; Rantakari et al. 2001; Rojas et al. 2002; and Wei et al. 2000.

GeneMark software, the sequence was searched for putative protein coding regions (CDS). These analyses predicted 42 CDS in the sequenced region. All *hrp* genes were named after their homologues in *E. amylovora* based on the unified nomenclature (Bogdanove et al. 1996). Four CDS flanked the *hrp-dsp* cluster, including *hecB* and a sequence highly similar (4e-39) to a transcriptional regulator of the GntR-family. Although the majority of the remaining CDS were highly similar to the *hrp* genes of *E. amylovora*, eight CDS within the *hrp-dsp* cluster showed no homologues in the *E. amylovora* cluster. These included four CDS similar to conserved hypothetical proteins (CDS 12 to 15), one weakly similar to *hrpG* from *Xanthomonas oryzae* (CDS 8), one similar to *vgrG* (CDS 7), and two novel sequences (CDS 9 and 21) (Fig. 1) (Bogdanove et al. 1998a). As anticipated from previous sample sequencing (Bell et al. 2002), intact genes encoding the TTSSs HrpN, HrpW (and its chaperone), and DspE/A (and its chaperone DspF/B) were also present.

A transposon mutation grid allowed rapid identification of mutations in *hrpN*, *dspE/A*, and TTSS structural genes *hrcC* and *hrcV*.

To analyze the functions of any gene in the *E. carotovora* subsp. *atroseptica*SCRI1039 genome, an arrayed set of insertional mutants (mutation grid) was constructed. In vivo transposon mutagenesis was performed using a conditional Tn5 cassette donor plasmid hosted in *Escherichia coli* S17-1 λ pir. The number of transconjugants (4,608) that constituted the mutation grid was approximately equivalent to the number of genes predicted to be in the recently sequenced *Erwinia carotovora* subsp. *atroseptica* SCRI1043 genome (approximately

4,520 genes; *unpublished data*) and, thus, was considered a onefold genome coverage mutation grid.

The mutation grid was pooled in five dimensions (discussed below) (Fig. 2) to allow mutants in any given gene to be identified in only 31 PCR reactions, using a primer annealing to the gene of interest in combination with a primer annealing to the Tn5 insertion cassette. In this way, mutations were identified rapidly in the TTSS structural genes *hrcC* and *hrcV*, and also the *hrpN* and *dspE/A* genes encoding TTSS secreted helper and effector proteins, respectively. In each case, sequencing the PCR products confirmed the insertion of Tn5 in the gene of interest. Southern analysis confirmed only single-transposon insertion events in each of the mutants into *PvuI* DNA fragments of expected size (results not shown). Transcripts from each of the genes *hrpN*, *DspE/A*, *hrcC*, and *hrcV* could be detected by reverse-transcriptase (RT)-PCR, using gene-specific primers, in the wild-type strain but not in the corresponding mutant strains (results not shown), due to the insertion of the transposon cassette.

The Hrp system, the effector protein DspE/A, and helper protein HrpN are required for virulence on potato.

To investigate the roles of the *hrp* cluster and, in particular, of the effector or helper proteins secreted by it in the potato-*E. carotovora* subsp. *atroseptica* interaction, the virulence of the *E. carotovora* subsp. *atroseptica* Tn5 mutants (*E. carotovora* subsp. *atroseptica* *hrcC*::Tn5, *hrcV*::Tn5, *hrpN*::Tn5, and *dspE/A*::Tn5) was tested. Potato plant stems were inoculated with 10^2 CFU per inoculation site and compared with the equivalent level of the wild-type strain daily over 17 days. Two

Fig. 2. Graphical representation of pooling within the mutation grid. Twelve 384-well microtiter plates (P) were placed one on top of the other. Each plate was divided into six sectors (S) and each sector into four quadrants (Q). Each quadrant was divided into four rows (R) and four columns (C).

randomly selected transposon mutants were also tested as controls. In all cases, transposon mutants showed the same growth kinetics in Luria-Bertani (LB) culture medium as the wild-type *E. carotovora* subsp. *atroseptica* strain.

The length of disease lesions was considerably reduced for the TTSS structural and effector or helper gene mutants relative to the wild-type *E. carotovora* subsp. *atroseptica* (Fig. 3), but the two randomly selected transposon mutants showed no significant difference to the wild-type strain (results not shown). Whereas symptoms were noted for the wild-type and control mutant *E. carotovora* subsp. *atroseptica* strains as early as 2 days postinoculation (dpi), those for each of the

TTSS structural and effector or helper gene mutants were not clearly visible until 4 dpi and remained restricted up to 17 dpi.

DISCUSSION

The role of the TTSS in pathogenesis is becoming increasingly clear in both plant and animal bacterial pathogens (Galán and Collmer 1999; Innes 2003). The soft rot *Erwinia* spp., like their necrogenic relative *E. amylovora*, require the TTSS both for triggering the HR in nonhosts and causing disease in host plants (Ageichik et al. 2002; Bauer et al. 1994; Cui et al. 1996; Rantakari et al. 2001). In this study, we examined the structure

Fig. 3. Lesion development between **A**, wild-type and *Erwinia carotovora* subsp. *atroseptica* mutants **B**, *E. carotovora* subsp. *atroseptica* *hrc V::Tn5*, **C**, *E. carotovora* subsp. *atroseptica* *hrc hrcC::Tn5*, **D**, *E. carotovora* subsp. *atroseptica* *hrc dspE/A::Tn5*, and **E**, *E. carotovora* subsp. *atroseptica* *hrc hrpN::Tn5* at 10^2 cells per inoculation site together with a vertical bar representing their LSD value. **F**, Bar graph showing length of rot (mm) between wild-type and mutants.

of the complete *hrp-dsp* cluster of *E. carotovora* subsp. *atroseptica*, and used a novel transposon mutation pooling and PCR strategy to determine the role of the structural genes *hrcC* and *hrcV*, the helper gene *hrpN*, and the effector gene *dspE/A* in the disease process on potato.

Overall, the *hrp-dsp* clusters from both *E. carotovora* subsp. *atroseptica* and *E. amylovora* were highly conserved (Fig. 1). However, in both cases, unrelated CDS of unknown function were present, being more numerous in *E. carotovora* subsp. *atroseptica* than *E. amylovora*. The functions of these CDS are currently being investigated. *Hrp* gene cluster sequences of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi*, where known, also appear to be similar in structure to *E. amylovora* and *E. carotovora* subsp. *atroseptica*, and contain additional CDS (Fig. 1). Interestingly, in contrast to *E. carotovora* subsp. *atroseptica* and *E. amylovora*, equivalent regions upstream of *hrpN* in *E. herbicola* pv. *gypsophylae* and *Pantoea stewartii* appear not to contain the putative helper gene *hrpW*, its chaperone gene, or additional CDS (Frederick et al. 2001; Mor et al. 2001). Such differences between species may be important in determining differences in host range or disease symptoms.

To investigate the function of any gene or genes in *E. carotovora* subsp. *atroseptica*SCRI1039, a onefold genome coverage transposon mutation grid was generated. Mutants were pooled in five dimensions to allow screening for mutations in any given gene using only 31 gene-specific PCR amplifications (Fig. 2). This allowed mutations in *hrpN*, *dspE/A*, *hrcC*, and *hrcV* to be identified rapidly. The pooled mutation grid differs from other published transposon library approaches in that it allows rapid, high-throughput, gene-specific, and relatively low-cost PCR amplification to target mutations in any selected gene. This is in contrast to i) expensive, large-scale sequencing of transposon libraries, which is used for comprehensive global mapping of gene function (Geoffroy et al. 2003; Jacobs et al. 2003; McAdam et al. 2002); ii) phenotypic screening of transposon libraries, which does not allow the selection of specific gene mutations and is highly dependent on the bioassay used to identify gene function; and iii) mutagenesis of individual genes as required, which is low throughput. This approach is particularly useful in the era of functional genomics, because full genome sequences of bacterial plant

pathogens are available, revealing hitherto unpredicted candidate pathogenicity genes. The genome sequences of *E. carotovora* subsp. *atroseptica*SCRI1043 and *E. chrysanthemi* 3937 have recently been completed and mutation grids for these strains will accelerate functional analyses of novel genes within each. Mutation grids also could be layered with different transposons, offering alternative reporter genes and antibiotic resistances that allow the effects of multiple mutations to be investigated simultaneously.

Mutations in *dspE/A*_{Eca} and *hrpN*_{Eca}, and structural genes *hrcC*_{Eca} and *hrcV*_{Eca} involved in the formation of the TTSS, resulted in significantly reduced virulence on potato (Fig. 3). These observations were achieved using an improved method to assess pathogenicity, by inoculating potato stems with low bacterial concentrations and measuring the progress of lesion development.

Although the putative effector *dspE/A* has been shown to play a role in necrogenic disease development of *E. amylovora* on pear (Bogdanove et al. 1998a,b), this is the first demonstration that *dspE/A* has a role in soft rot pathogenesis in the *Erwinia* spp. Thus, despite the different modes of disease development in *E. amylovora* and the soft rot *Erwinia* spp., *DspE/A* appears to play a role in establishing infection in both. This is of fundamental importance in the study of the soft rot *Erwinia* spp., long considered to be "brute force" pathogens that lack more subtle interactions with the host.

HrpN proteins from *E. carotovora* subsp. *carotovora*, *E. chrysanthemi*, and *E. amylovora* have all been shown to elicit the HR in nonhosts (Bauer et al. 1994, 1995; Mukherjee et al. 1997; Wei et al. 1992). However, their roles in pathogenesis are less clear. Mutants in *hrpN*_{Eam} have been demonstrated to be nonpathogenic (Wei et al. 1992) or weakly pathogenic (Barney 1995) on pear. In the soft rot pathogens, mutants in *hrpN*_{Ech} were weakly pathogenic on chicory (Bauer et al. 1994, 1995), whereas *hrpN*_{Ecc} mutants were fully pathogenic on host plants (Cui et al. 1996). However, some of these differences may have been due to variation in inoculum concentrations adopted in these studies. The effects of *hrp* genes on soft rot pathogenesis may be difficult to assess when inoculating the host with higher bacterial cell densities, due to the prolific production of PCWDEs. In this work, an *E. carotovora* subsp. *atroseptica* inoculum (10² CFU per inoculation site) was used that

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype, characteristics ^a	Source or reference
Strains		
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>		
<i>E. carotovora</i> subsp. <i>atroseptica</i> SCRI1039	Wild type, isolated from potato	SCRI bacterial collection
<i>E. carotovora</i> subsp. <i>atroseptica</i> <i>hrcC</i> ::Tn5	SCRI1039 with insertion of the Tn5 cassette in <i>hrcC</i>	This work
<i>E. carotovora</i> subsp. <i>atroseptica</i> <i>hrcV</i> ::Tn5	SCRI1039 with insertion of the Tn5 cassette in <i>hrcV</i>	This work
<i>E. carotovora</i> subsp. <i>atroseptica</i> <i>hrpN</i> ::Tn5	SCRI1039 with insertion of the Tn5 cassette in <i>hrpN</i>	This work
<i>E. carotovora</i> subsp. <i>atroseptica</i> <i>dspE/A</i> ::Tn5	SCRI1039 with insertion of the Tn5 cassette in <i>dspE</i>	This work
<i>Escherichia coli</i>		
ElectroMAX DH10B	Electro-competent cells used for electroporation and the construction of the SCRI1039 BAC library	Invitrogen
2B8	<i>E. coli</i> DH10B with pBeloBAC11 carrying, in a 125-kb insert, the <i>hrp</i> cluster and part of the <i>dspE</i> gene; clone, from the SCRI1039 BAC library, Cm ^r	Bell et al. 2002
1C22	<i>E. coli</i> DH10B with pBeloBAC11 carrying, in a 90-kb insert, part of the <i>hrp</i> cluster and the complete <i>dsp</i> cluster; clone from the SCRI1039 BAC library, Cm ^r	Bell et al. 2002
S17- λ pir	<i>recA</i> , <i>pro</i> , <i>hsdR</i> , RP4-2-Tc::Mu-Km::Tn7, λ pir, Sm ^r , Tp ^r , S17- λ pir carrying the mTn5-gus-pgfp21 cassette	SCRI collection, Simon et al. 1983
S17- λ pir (mTn5-gus-pgfp21)	('Tn5 cassette') in pUT, Km ^r , Ap ^r	Xi et al. 1999
Plasmids		
PUT	Plasmid vector, Ap ^r	Xi et al. 1999
pGEM-T-easy	Plasmid vector (3,003 bp), Ap ^r	Promega
pBeloBAC11	BAC vector (7,378 bp), Cm ^r	Caltech Genome Research Laboratory

^a Cm = chloramphenicol, Sm = streptomycin, Tp = trimethoprin, = kanamycin, and Ap = ampicillin.

is below the quorum-sensing level at which such enzyme production proliferates (Whitehead et al. 2001) and clear differences in virulence were observed between the *hrp/hrc* or *dspE/A* mutants and the wild-type strain. These differences may reflect a role for the TTSS early in disease development, before quorum sensing concentrations of bacterial cells trigger PCWDE production. However, further work is required to validate this possibility.

In summary, we have sequenced and annotated the entire *hrp* cluster and adjacent *dspEF/AB* operon of a soft rot *Erwinia* sp. The use of a novel, pooled transposon mutation grid led to the rapid isolation of mutants for *hrcC*, *hrcV*, the effector gene *dspE/A*, and helper gene *hrpN*. When inoculated into potato plants at low inoculum levels, a significant reduction in disease development was seen for all mutants. Thus, we provide the first data to show that the TTSS-delivered helper, *hrpN*, and effector, *DspE/A*, are unambiguously involved in potato infection by *E. carotovora* subsp. *atroseptica* and, in the latter case, the first evidence of a role in soft rot pathogenesis. The mutation grid now is being used for a wider functional genomics program to identify all components contributing to the pathogenic lifestyle of this important bacterial plant pathogen.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1. In general, bacterial strains were grown in LB medium or M9 minimal medium (M9) (Sambrook et al. 1989) with *Escherichia coli* grown at 37°C and *Erwinia* spp. at 27°C. When required, the following antibiotics were used: chloramphenicol at 12.5 µg ml⁻¹, kanamycin (Km) at 50 µg ml⁻¹, and ampicillin (Ap) at 100 µg ml⁻¹. Media and reagents were purchased from Difco (Detroit) and Sigma (St. Louis).

General molecular biology procedures.

Genomic and plasmid DNA isolation was carried out using the DNeasy tissue kit (Qiagen, Chatsworth, CA, U.S.A.) and the Wizard Plus Minipreps DNA purification system (Promega, Lyon, France), respectively. BAC DNA isolation was carried out as described by Bell and associates (2002). All other molecular biology procedures and recombinant DNA techniques were carried out according to Sambrook and associates (1989). For PCR, *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.) was used, unless otherwise stated. Purification of DNA from agarose gels (Nusieve GTG agarose; Flowgen, McLean, VA, U.S.A.) was carried out using the Wizard PCR Preps DNA purification system (Promega). For Southern blotting, genomic DNA was digested with *PvuI* and 750 ng was electrophoresed on a 1% agarose gel with appropriate DNA size markers. A portion of the *gfp* gene in the Tn5 cassette was used as a probe for the transposon mutants.

Total RNA was isolated from bacterial strains grown in M9 using the RNeasy Mini Kit (Qiagen) and contaminating DNA was eliminated by DNase treatment (DNA-free; Ambion, Austin, TX, U.S.A.). A mixture of 10 11-mer oligonucleotide primers (Eal to Eal0) was used for cDNA synthesis as described by Dellagi and associates (2000). First-strand cDNA synthesis was carried out using the First-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The following gene-specific primers were used for RT-PCR: 5'-TGCCGTCGATAAACAGGGTG-3' and 5'-AAGAGAATG CCGTCAGCGTG-3' (*dspE/A*); 5'-CGGCGTAAGGTGATGATGA TTTTC-3' and 5'-ACACATAGCGCGTAGGATCG-3' (*hrcC*); 5'-GTTAACGCCTTGGGTATACGC-3' and 5'-TAATTCTCTT GGTGGCGGTAC-3' (*hrpN*); 5'-TTGCGTCGCTGGTTATCG TG-3' and 5'-TCAAACATGGTGTCTTCCTATG-3' (*hrcV*).

Sequencing the *hrp* gene cluster.

Generation of *hrp* gene subclone sequences of *Erwinia carotovora* subsp. *atroseptica* SCRI1039 BAC clones 2B8 and 1C22 is described by Bell and associates (2002). These sequences were compared with those in the GenBank nr database using BLASTX and BLASTN (Altschul et al. 1997) and the results used to position the subclones onto the *E. amylovora* *hrp-dsp* cluster (Bogdanove et al. 1998a). PCR was then used to bridge sequence gaps using 169 oligonucleotide primers designed to anneal to the ends of the subclone sequences flanking the gaps. The length of these products ranged from approximately 90 bp to 6.4 kb with "hot start" PCR and Expand High Fidelity PCR System (Roche Molecular Systems, Branchburg, NJ, U.S.A.) being used for larger PCR products. The purified PCR products were sequenced in both directions using the ABI PRISM Big dye terminator cycle sequencing ready reaction kit (v2.0; PE Biosystems, Foster City, CA, U.S.A.).

Sequence assembly and analysis.

Sequences were assembled into a single contig using Phred-Phrap-Consed (release 10.0) (Gordon et al. 2001). Artemis (v4) was used as a general sequence viewer and annotation tool (Rutherford et al. 2000).

GeneMark was used to identify putative CDS and operons were identified based on i) the presence of putative transcription promoters and terminators, ii) short distances (–20 to +30 nucleotides [nt]) between the genes of the same operon, iii) an average distance between operons of 190 nt; iv) conservation of adjacent genes in the same strand; and v) the presence of the operon in a gene cluster conserved in other microbial genomes.

Transposon mutation grid and screening by PCR.

Escherichia coli S17-1 λ pir, carrying the Tn5 cassette (mTn5-*gusA*-*pgfp*) in the pUT vector (donor strain) and *Erwinia carotovora* subsp. *atroseptica* SCRI1039 (recipient strain) were grown to exponential phase in LB broth, supplemented with Km in the case of *Escherichia coli*. Donor and recipient strains were conjugated and transconjugants recovered on M9 Km at 27°C. In all, 4,608 transconjugants were selected and grown in 12 384-well microtiter plates (M9 Km broth plus 4% glycerol), constituting the mutation grid. All transconjugants were tested for resistance to Km and sensitivity to Ap, confirming insertion of the transposon and loss of pUT.

Each clone or well within the mutation grid was given a unique address, *xyz*, where (by visualizing the plates as a stack) $24 \geq x \geq 1$, $16 \geq y \geq 1$, and $12 \geq z \geq 1$ (Fig. 2). The grid then was subdivided into five pool categories (plate, sector, quadrant, row, and column) as follows: i) 12 plate pools (P1 to P12, each containing the clones of an entire plate); ii) six sector pools, S1 to S6, each containing clones from one sixth of a plate from all 12 plates (i.e., addresses $8 \geq x \geq 1$, $8 \geq y \geq 1$, $12 \geq z \geq 1$, S1; $16 \geq x \geq 9$, $8 \geq y \geq 1$, $12 \geq z \geq 1$, S2; $24 \geq x \geq 17$, $8 \geq y \geq 1$, $12 \geq z \geq 1$, S3; $8 \geq x \geq 1$, $16 \geq y \geq 9$, $12 \geq z \geq 1$, S4; $16 \geq x \geq 9$, $16 \geq y \geq 9$, $12 \geq z \geq 1$, S5; $24 \geq x \geq 16$, $16 \geq y \geq 9$, $12 \geq z \geq 1$, S6); iii) four quadrant pools, Q1 to Q4, where each group contains clones from a square within each of the sector pools (i.e., addresses $x = 1, 2, 3, 4, 9, 10, 11, 12, 17, 18, 19, 20$, $y = 1, 2, 3, 4, 9, 10, 11, 12$, and $12 \geq z \geq 1$, Q1; $x = 5, 6, 7, 8, 13, 14, 15, 16, 21, 22, 23, 24$, $y = 1, 2, 3, 4, 9, 10, 11, 12$, and $12 \geq z \geq 1$, Q2; $x = 1, 2, 3, 4, 9, 10, 11, 12, 17, 18, 19, 20$, $y = 5, 6, 7, 8, 13, 14, 15, 16$, and $12 \geq z \geq 1$, Q3; $x = 5, 6, 7, 8, 13, 14, 15, 16, 21, 22, 23, 24$, $y = 5, 6, 7, 8, 13, 14, 15, 16$, and $12 \geq z \geq 1$, Q4); iv) four column pools, C1 to C4, each group containing clones from every fourth column from all 12 plates (i.e., addresses $x = 1, 5, 9, 13, 17, 21$, $16 \geq y \geq 1$ and $12 \geq z \geq 1$, C1; $x = 2, 6, 10, 14, 18, 22$, $16 \geq y \geq 1$ and $12 \geq z \geq 1$, C2; $x =$

3,7,11,15,19,23, $16 \geq y \geq 1$ and $12 \geq z \geq 1$, C3; $x = 4,8,12, 16, 20,24$, $16 \geq y \geq 1$ and $12 \geq z \geq 1$, C4); and v) four row pools, R1 to R4, each containing clones from every fourth row from all 12 plates (i.e., addresses $24 \geq x \geq 1$, $y = 1,5,9,13$ and $12 \geq z \geq 1$, R1; $24 \geq x \geq 1$, $y = 2,6,10,14$ and $12 \geq z \geq 1$, R2; $24 \geq x \geq 1$, $y = 3,7,11,15$ and $12 \geq z \geq 1$, R3; $24 \geq x \geq 1$, $y = 4,8,12,16$ and $12 \geq z \geq 1$, R4). Additionally, all clones in the grid were pooled to give a library pool (Fig. 4).

DNA was extracted from each group of clones to give 31 pools of DNA for PCR screening using primers designed to anneal to the gene of interest in combination with a primer (Tn5R: 5'-ATCCTCCTTAGCTAGTCAGG-3') designed to anneal to one end of the transposon cassette. Initially, the total library DNA pool was screened to ascertain whether a mutant was present in the library before screening the remaining pools to identify the mutant strain. Primers used to identify mutants were: 5'-TACTCAGCATGATGATCTGGC-3' (*hrcV*), 5'-TCA TCCACGCTTACCCTGCTG-3' (*hrcC*), 5'-AGCCTGACCTG CGCAATGG-3' (*hrpN*), and 5'-ATTGCTATCCAGATTGAAG CTG-3' (*dspE/A*). The purified PCR products were sequenced in both directions to confirm the location of the transposon.

Pathogenicity test.

E. carotovora subsp. *atroseptica* susceptible potato micro-propagated plants cv. Estima (Gentech, Dundee, U.K.) were grown in compost to a height of approximately 20 cm. *E. carotovora* subsp. *atroseptica* SCRI1039 cells were grown to log phase (overnight) in LB (supplemented with Km for mutants), and cells harvested by centrifugation at $4,000 \times g$ for 10 min were resuspended in the same volume of 10 mM MgSO₄ (pH 7.4). A dilution in MgSO₄ of 10^4 cells/ml was prepared from these suspensions and potato plant stems were inoculated under the second fully expanded leaf using a micropipette tip with 10 µl of bacterial suspension (equivalent to 10^2 cells per inoculation site). The inoculation site then was covered with parafilm to avoid desiccation. The parafilm was kept throughout the incubation to maintain a microaerophilic environment. However, tests were performed to ensure that the parafilm did not act as a reservoir for bacteria to grow. Five replica stems for each strain and each inoculum level were used and each test was conducted on at least two occasions with reproducible results. The inoculated plants were kept in the glasshouse under controlled temperature conditions (approximately 22°C) and high humidity and the presence and length (mm) of disease lesions recorded daily over a 17-day period. Genstat for Windows (version 6.1.0.200; Lawes Agricultural Trust, Rothamsted, U.K.) was used for statistical analyses ($P < 0.05$).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- The Sanger Institute's *Erwinia carotovora* subsp. *atroseptica* webpage: www.sanger.ac.uk/Projects/E_carotovora
- The Scottish Crop Research Institute *Erwinia* genomics webpage: www.scri.sari.ac.uk/erwinia
- University of Wisconsin-Madison Department of Animal Health and Biomedical Sciences *Erwinia* index: www.ahabs.wisc.edu/~pernalab/erwinia/index.html
- The Georgia Institute of Technology Bioinformatics Group's GeneMark gene prediction programs: opal.biology.gatech.edu