Eop1 from a *Rubus* Strain of *Erwinia amylovora* Functions as a Host-Range Limiting Factor

J. E. Asselin, J. M. Bonasera, J. F. Kim, C.-S. Oh, and S. V. Beer

First, second, and fifth authors: Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, NY 14853; third author: Laboratory of Microbial Genomics and Systems/Synthetic Microbiology, Industrial Biotechnology and Bioenergy Research Center, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yuseong, Daejeon 305-600, Republic of Korea; and fourth author: Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, and Department of Horticultural Biotechnology, Kyung Hee University, Yong-In 446-701, Korea.

Accepted for publication 14 March 2011.

ABSTRACT

Asselin, J. E., Bonasera, J. M., Kim, J. F., Oh, C.-S., and Beer, S. V. 2011. Eop1 from a *Rubus* strain of *Erwinia amylovora* functions as a host-range limiting factor. *Phytopathology* 101:935-944.

Strains of *Erwinia amylovora*, the bacterium causing the disease fire blight of rosaceous plants, are separated into two groups based on host range: *Spiraeoideae* and *Rubus* strains. *Spiraeoideae* strains have wide host ranges, infecting plants in many rosaceous genera, including apple and pear. In the field, *Rubus* strains infect the genus *Rubus* exclusively, which includes raspberry and blackberry. Based on comparisons of

limited sequence data from a *Rubus* and a *Spiraeoideae* strain, the gene *eop1* was identified as unusually divergent, and it was selected as a possible host specificity factor. To test this, *eop1* genes from a *Rubus* strain and a *Spiraeoideae* strain were cloned and mutated. Expression of the *Rubus*-strain *eop1* reduced the virulence of *E. amylovora* in immature pear fruit and in apple shoots. Sequencing the *orfA-eop1* regions of several strains of *E. amylovora* confirmed that forms of *eop1* are conserved among strains with similar host ranges. This work provides evidence that *eop1* from a *Rubus*-specific strain can function as a determinant of host specificity in *E. amylovora*.

Erwinia amylovora, a bacterial pathogen of rosaceous plants, causes the devastating disease fire blight. *E. amylovora* infects apple (*Malus* spp.), pear (*Pyrus* spp.), hawthorn (*Crataegus* spp.), and raspberry and blackberry (*Rubus* spp.), among others. Bacteria enter the host plant through natural openings in blossoms or leaves or through wounds, advance to infect shoots or canes, and, if able to infect the base of the plant, girdle and kill it. As the name suggests, shoots and canes stricken with fire blight appear as if scorched by fire, becoming blackened and desiccated, with dry, crumpled leaves clinging to the plant. Infections of immature fruit and shoots can reduce a season's yield (46). However, due to the perennial nature of the hosts of *E. amylovora*, severe infections create additional costs by killing fruit-bearing structures, sometimes to the extent that heavily blighted trees and brambles must be removed and new plants established.

Strains of *E. amylovora* are often separated into two groups based on host range: strains isolated from *Rubus* spp. (hereinafter *Rubus* strains), and wide host range strains isolated from non-*Rubus* hosts, including species in the genera *Malus*, *Pyrus*, *Prunus*, *Fragaria*, and *Crataegus* (hereinafter *Spiraeoideae* strains, after a Rosaceous subfamily containing many hosts) (19, 30,34,51). In previous studies, *Rubus* strains repeatedly failed to cause disease when inoculated into apple shoots, but some strains caused disease when inoculated into immature apple and pear fruit (5,34,45). Although at least some *Spiraeoideae* strains seem

capable of causing fire blight in *Rubus* hosts in response to artificial inoculation, only *Rubus*-specific strains have been isolated and characterized from naturally infected *Rubus* spp. (5).

In order to understand how *Rubus* and *Spiraeoideae* strains differ, previous studies have examined whether they differ in exopolysaccharide structure, serological properties, carbon source utilization, and RNA expression (5,16,18,45). Although differences between the two groups were identified, more work is required to demonstrate whether these observed differences affect the host ranges of *Rubus* or *Spiraeoideae* strains.

Comparisons of genome sequences of *Rubus* strains with those of pathogenic *E. amylovora* strains with different host ranges might suggest candidate host specificity factors; however, sequence data of multiple *E. amylovora* strains have become available only recently (32,37,39). Indications from comparison of limited sequences of single *Rubus* and *Spiraeoideae* strains, performed early in this work, led to the present studies.

In the present work, part of a region of *E. amylovora* genomic DNA containing important pathogenicity determinants was sequenced and compared from a *Spiraeoideae* strain, Ea273, and a *Rubus* strain, Ea246, to seek differences that might contribute to host specificity. Strain Ea246 is synonymous with American Type Culture Collection (ATCC) BAA-2158 and Ea273 is synonymous with ATCC 49946. The names Ea246 and Ea273 are used herein to maintain shorter names for strains and their derivatives. This region, termed the *hrp* pathogenicity island (PAI), encodes a type III secretion system (T3SS), its regulatory components, and proteins that traverse the T3SS to be delivered into host cell cytoplasm (25). Based on a comparison of the *hrp* PAI, the region surrounding and including the genes *eop1* (formerly called *orfB* or *eopB*), *orfA*, and *orfC* was selected as especially divergent. *orfA* and *orfC* encode putative chaperones, and *eop1* encodes a type III effector protein (24). *OrfA* is a putative chaperone for Eop1; however, it appears dispensable for the secretion of Eop1 (1). Eop1 is both secreted and translocated into plant cells by the

Corresponding author: S. V. Beer; E-mail address: svb1@cornell.edu

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains two supplemental figures and one supplemental table. Figure 1 appears in color online.

doi:10.1094/PHYTO-12-10-0339

© 2011 The American Phytopathological Society

T3SS in Ea273 (23,24). However, no role of Eop1 in virulence of *E. amylovora* has been demonstrated.

Eop1 is a member of the YopJ/AvrRxv family of type III secreted proteins, which is widespread in both plant and animal pathogens (40). YopJ, a well-studied member of this family from human pathogenic *Yersinia* spp., originally was described as a cysteine protease and later shown to have acetyltransferase activity (17,22,26). Whether YopJ and other members of this family have one or both of these activities is still unclear, because they are not necessarily mutually exclusive (49). However, either catalytic activity requires a conserved catalytic triad consisting of histidine, aspartic acid or glutamic acid, and cysteine (22).

As virulence factors, YopJ family members whose functions have been characterized are involved in the suppression of host defense responses. Among these, YopJ targets several pathways to reduce local inflammatory responses in animals (28,36,43), and AvrBsT from *Xanthomonas campestris* pv. *vesicatoria* (the pathogen causing bacterial spot of pepper) is involved in suppressing effector-triggered plant immunity (44). However, numerous members of the YopJ/AvrRxv protein family from plant-pathogenic bacteria also have avirulence effects, eliciting plant defense responses when expressed in resistant hosts or non-host plants. For example, PopP1 from *Ralstonia solanacearum* acts as a host-specificity factor at the race level and also negatively affects the virulence of this bacterium in some hosts (14). Also, HopZ1a from *Pseudomonas syringae* can elicit defense responses in a host-specific manner (15).

Just as YopJ/AvrRxv family members are widespread in bacterial pathogens, versions of *eop1* are present in strains of *E. pyrifoliae* and *E. tasmaniensis*, close relatives of *E. amylovora*. *E. pyrifoliae* has been isolated from pear in Japan and Korea and causes Asian pear blight, a disease similar to fire blight (8,10). The host range of *E. pyrifoliae* is limited compared with *Spiraeoideae* strains of *E. amylovora*, reportedly striking pear but with limited virulence in apple (11). *E. tasmaniensis* was isolated from apple flowers in Tasmania, Australia and is considered nonpathogenic (13).

In order to investigate the potential role of Eop1 in virulence and host specificity of *E. amylovora*, we mutated *eop1* in both *Spiraeoideae* and *Rubus* strains and analyzed the virulence of the mutants in several rosaceous plants. To determine whether *eop1* was conserved in strains with similar host ranges, we compared the predicted Eop1 proteins from several *E. amylovora* strains from different hosts, *E. pyrifoliae*, and *E. tasmaniensis*. Our results suggest that Eop1 from *Rubus* strains of *E. amylovora* is conserved and functions as a host-range-limiting factor, capable of reducing the virulence of *E. amylovora* strains in non-*Rubus* hosts.

MATERIALS AND METHODS

Bacterial strains and plasmids used in this study. Bacteria were routinely propagated in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C for *Escherichia coli* or at 28°C for *Erwinia amylovora* and *E. pyrifoliae*. Several plasmids were used to express proteins in bacteria, as listed in Table 1.

Plant inoculations. Inoculum of *E. amylovora* was prepared by growing strains overnight on LB agar plates with appropriate antibiotics at 28°C. Bacteria were lifted using sterile cotton swabs and suspended into 5 mM potassium phosphate buffer, pH 6.5, to an optical density at 600 nm (OD_{600}) = 0.2, corresponding to 5×10^8 CFU/ml.

Immature Bartlett pear fruit were inoculated as described previously (41). Briefly, pear fruit were surface disinfested, cut longitudinally, and placed atop moist paper towel in lidded plastic boxes. Each pear half was punctured three times with a wide-bore pipette tip and inoculated with 5 μ l of inoculum, and the boxes were sealed with plastic wrap and placed at 28°C for 5 days. Pear

assays were repeated three times with four replicates per assay. Each box contained pear inoculated with Ea273(pSU23), Ea246(pSU23), and buffer, as controls. Photographs of pear fruit were taken daily beginning 2 days post inoculation (dpi) and 4 dpi was chosen as most illustrative of differences among symptoms caused by the strains. Percent lesion area was assessed using photographs of pear fruit taken 4 dpi by measuring the percentage of the visible pear surface covered with necrotic lesions and opaque bacterial ooze with the computer program Photoshop CS4 Extended (Adobe, San Jose, CA).

Actively growing apple shoots and raspberry canes were placed in a controlled environment chamber at 26°C and >65% relative humidity. For inoculations, shoot apices were pierced by the multiple pins of an inoculum-dipped circular florist's frog having 127 pins over a diameter of 4.6 cm (3). Shoot lesions were measured several times over the course of 10 days. All strains were inoculated into at least five shoots.

Statistical analyses. Statistical analysis was performed using the JMP 8.0 statistical software package (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) models were fit to the data from immature pear fruit and apple shoot assays. Mixed-effect models were fit using REML to account for the random variability due to blocking in the immature pear assay. In the pear assay, the outcome variable was transformed using log (% symptomatic area + 0.1) to remedy the unequal variances among strains. For the apple assays with 12 strains in 'Gala', outliers were detected from the standardized residual plots. Those that were more than two standard deviations from the average were removed from the analysis. For apple shoot inoculations using multiple cultivars, the outcome variable was transformed using the power transformation proposed by Box and Cox (4). For each experiment, model diagnostics were assessed by plotting residuals versus predicted values and Q-Q plots of studentized residuals, and visually inspecting the plots. *P* values < 0.05 were considered significant.

Sequence analysis of the *eop1*-spanning region in Ea246. Nucleotide sequencing of portions of the Ea246 *hrp* PAI, including the region between *hrpN* and *dspE* of Ea246 that includes full-length *orfA*, *eop1* (*orfB*), *orfC*, and *hrpW*, was performed on an ABI 373A automated DNA sequencer (Perkin-Elmer, Norwalk, CT) at the Cornell University Biotechnology Program DNA Sequencing Facility with oligonucleotide primers synthesized at the same facility. The sequence was deposited in GenBank with the accession number AF083620. DNA and deduced amino acid sequences were analyzed with programs in the GCG software package (version 7.3; Genetics Computer Group, Madison, WI) and DNASTar (DNASTar, Madison, WI).

Alignment of genes from published nucleotide sequences. The genes *eop1*, *rpoD*, *gyrB*, and *hrcC* from *E. amylovora* Ea273 (NC_013971.1) and Ea246 (AF083620, FR719186.1, and FR719198.1), *E. pyrifoliae* Ep1/96 (NC_012214), DSM 12163 (FN392235.1), and Ejp617 (CP002124), *E. tasmaniensis* Et1/99 (NC_010694), and *P. syringae* pv. *syringae* B728a (NC_007005) were collected from GenBank and entered into the program MegAlign (DNASTar). For *eop1* of Ejp617, we used a start codon upstream of the annotated start site but similar to the start sites of the other annotated *eop1* genes. For *gyrB* of DSM 12163, we used a start codon slightly downstream of the annotated site but similar to that of the other annotated *gyrB* genes. DNA sequences and predicted Eop1 protein sequences were compared using the ClustalW alignment method and visualized as phylogenetic trees.

Cloning and mutagenesis. Chromosomal *eop1*_{Ea273} was mutated using pCPP1509 (1), a phagemid harboring a copy of *eop1*_{Ea273} interrupted by the transposon EZ:TN<KAN-2> (Epicentre, Madison, WI). The transposon encodes resistance to kanamycin, and the vector backbone, pBC SK⁻ (Stratagene, La Jolla, CA), encodes resistance to chloramphenicol. pCPP1509 was transformed by electroporation into Ea273, and a single

transformed colony was serially propagated into LB broth without antibiotics daily for 5 days and then plated. Kanamycin-resistant, chloramphenicol-susceptible colonies were then checked by polymerase chain reaction (PCR), using primer sets that produced amplicons of different sizes for wild-type, plasmid-carrying strains, and *eop1* transposon mutants. The resulting mutated strain was named Ea273 *eop1*::Tn.

Chromosomal *eop1*_{Ea246} was deleted via marker exchange mutagenesis. Approximately 2 kb of DNA immediately up- and downstream of *eop1*_{Ea246} was amplified by PCR and ligated into pBC SK⁻ (Stratagene). The Ω cassette from pHP45 Ω (33), which contains transcription and translation termination sequences and a spectinomycin/streptomycin resistance gene, was ligated between these regions, creating pCPP1778. The deletion mutation was integrated into the Ea246 genome and confirmed in a manner similar to that used for Ea273 *eop1*::Tn.

Plasmid construction with *eop1* genes. The *eop1* genes from both Ea273 and Ea246 were cloned without their putative chaperones into the vector pSU23 (2) to create pCPP1776 and pCPP1777, respectively. To ensure similar expression of Eop1 protein from all constructs, each insert was amplified with the same forward primer, which contains the putative ribosome binding site for *eop1*_{Ea246}. These plasmids were transformed into appropriate strains of *E. amylovora* by electroporation.

Raising antibody to Eop1_{Ea273}. The gene *eop1*_{Ea273} was cloned into pET-24a(+) (Novagen) in frame with an N-terminal T7 tag to produce pCPP1799. T7-tagged Eop1_{Ea273} protein was expressed in *Escherichia coli* BL21(DE3) and purified using the T7 Tag Affinity Purification Kit (Novagen). Protein was dialyzed against physiological saline solution or repeatedly concentrated and resuspended in physiological saline using a Centrprep-30 concentrator (Millipore, Billerica, MA) according to the manufacturer's directions. Protein was quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) and sent to the Center for Research Animal Resources and Laboratory Animal Services at Cornell University to be injected into rabbits. Immunoglobulin g (IgG) was purified from raw serum by ammonium sulfate precipitation, modified from Page and Thorpe (27). Antibodies were cross-absorbed to *E. coli* BL21(DE3)[pET-24a(+)] (35). Antibody was checked against known amounts of purified N-terminally His-tagged Eop1_{Ea273} and Eop1_{Ea246}. The antibody detected both proteins but its affinity toward Eop1_{Ea273} was higher (data not shown).

Expression of Eop1. Secretion assays were performed to detect Eop1 protein expressed from pCPP1776 and pCPP1777. Bacteria were grown in LB broth with appropriate antibiotics to OD₆₀₀ = 0.8 to 1.0, centrifuged at 3,400 $\times$ g for 10 min, and washed twice in 10 ml of Huynh's minimal medium (HMM) (9). Washed cells were used to inoculate 50-ml cultures of HMM with

TABLE 1. Bacterial strains and plasmids used in this study

Strains, plasmids	Description or host, location of isolation ^a	Alternate names ^b	Source or reference
Strain			
<i>Escherichia coli</i>			
DH5 α	F- ϕ 80lacZAM15 Δ (lacZYA-argF) U169 <i>recA1 endA1 hsdR17</i> (rk ⁻ , mk ⁺) <i>gal- phoA supE44 λ- thi-1 gyrA96 relA1</i>	...	Invitrogen (Carlsbad, CA)
<i>Erwinia amylovora</i>			
From non- <i>Rubus</i> hosts			
Ea110	Pear blossom, California, United States	Y 33	S. V. Thomson
Ea262	Unknown, Netherlands	259	H. P. Maas-Geesteranus
Ea321	Hawthorn, France	CFBP 1367, ATCC 49947	50
Ea273	Apple, New York, United States	ATCC 49946	37
Ea273 <i>eop1</i> ::Tn	Ea273 derivative with <i>eop1</i> gene mutated by transposon insertion. Km		This study
Ea566	Strawberry, Germany	Ea-Eb	51
Ea587	Japanese plum, Idaho, United States	PFB-4, ATCC 51852	20
Ea629	Cotoneaster, Australia		Hale via Vanneste
From <i>Rubus</i> hosts			
Ea246 Δ <i>eop1</i>	Ea246 derivative with <i>eop1</i> gene deleted and replaced with a cassette encoding spectinomycin resistance	...	This study
Ea246	Blackberry, Illinois, United States	Blackberry 1, ATCC BAA-2158	42
Ea510	Raspberry, Alberta, Canada	BR89-FR41	21
Ea528	Raspberry, Maine, United States	ICMP1539, AFRS 1637, ICPB Ea124	21,31
Ea592	Raspberry, Alberta, Canada	...	I. R. Evans via G. Bonn
Ea644	Raspberry, Massachusetts, United States	...	J. M. Bonasera
<i>E. pyrifoliae</i>			
Ep1/96	Asian pear, South Korea	CFBP 4171, DSM 12162	10
Ep4/97	Asian pear, South Korea	DSM 12394	10
Plasmid			
pSU23	Medium copy number vector with p15A replicon and constitutive promoter Sp6. Cm	...	2
pBC SK ⁻	High copy number vector. Cm	...	Stratagene
pCPP461	Contains portion of <i>hrp</i> PAI from Ea246 including <i>orfA</i> and <i>eop1</i>	...	R. J. Laby, unpublished
pCPP1509	Contains ~5.9 kb of the <i>hrp</i> PAI from Ea321 including a transposon insertion in <i>eop1</i> in pBC SK ⁻ . Km, Cm	...	1
pCPP1776	<i>eop1</i> from Ea273 cloned into pSU23 under control of SP6 promoter. Cm	...	This work
pCPP1777	<i>eop1</i> from Ea246 cloned into pSU23 under control of SP6 promoter. Cm	...	This work
pCPP1778	Deletion construct for <i>eop1</i> from Ea246 in pBC SK ⁻ . Contains DNA flanking <i>eop1</i> and omega cassette from pHP45 Ω . Sp/Sm, Cm	...	This work
pCPP1799	<i>eop1</i> _{Ea273} cloned into pET-24a(+) to express an N-terminally T7-tagged Eop1 _{Ea273}	...	This work

^a Km, Cm, Sp, and Sm = resistant to kanamycin, chloramphenicol, spectinomycin, and streptomycin, respectively.

^b NCPPB = National Collection of Plant Pathogenic Bacteria, United Kingdom; ATCC = American Type Culture Collection, United States; DSM = from DSMZ culture collection, Germany; CFBP = Collection Française de Bactéries Phytopathogènes, France; ICMP = International Collection of Micro-organisms from Plants, New Zealand; AFRS = Appalachian Fruit Research Station, United States; ICPB = International Collection of Phytopathogenic Bacteria, Davis, CA.

antibiotics at OD₆₀₀ = 0.1. Cultures were grown at 18°C with shaking for 36 h and normalized to OD₆₀₀ = 0.5. Cells and media containing secreted proteins were separated by centrifugation at 9,150 × g for 15 min. Supernatants were poured through 0.22-μm polyethersulfone membrane bottle-top filters (Corning Inc., Corning, NY) to exclude bacterial cells; phenylmethylsulfonyl fluoride was added to 500 μM to inhibit protease activity.

Proteins were precipitated using 4.5 ml of 100% trichloroacetic acid per 30 ml of supernatant liquid, mixed vigorously, precipitated on ice overnight, and harvested by centrifugation at 17,200 × g at 4°C for 40 min. Pellets were washed twice with ice-cold acetone, dried, and resuspended in 1× sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) loading dye for subsequent analysis.

Secreted proteins were loaded onto a Duramide PAGER 4 to 20% Tris-glycine gel, resolved by electrophoresis, and blotted onto an Immobilon-P transfer membrane according to the manufacturer's directions (Millipore) using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Loading volumes were 15 μl of Ea273, Ea273 *eop1*::Tn(pSU23), and Ea273 *eop1*::Tn(pCPP1776); a 1:10 dilution of Ea273 *eop1*::Tn(pCPP1777); and 20 μl of Ea246 and Ea246Δ*eop1*. Uneven loading volumes were necessary to compensate for uneven affinity of the Eop1 antibody for *Rubus* and *Spiraeoideae* strain Eop1 and for overexpression of Eop1 from the plasmids. Membranes were reacted with Eop1_{Ea273} primary antibody and secondary antibody of goat anti-rabbit IgG conjugated to alkaline phosphatase (Molecular Probes, Eugene, OR).

Development was according to the kit instructions for the Western-Star Chemiluminescent Detection System (Tropix, Bedford, MA) using CSPD, ready-to-use (Roche Diagnostic, Indianapolis, IN) in place of CDP-Star.

Characterization of *eop1* from environmental strains. Three primer sets (Supplementary Table 1) were designed to amplify the *orfA-eop1* region based on sequences from *Erwinia amylovora* strains Ea246 and Ea273 and *E. pyrifoliae* strain WT3. The region was amplified from 11 strains of *E. amylovora* and *E. pyrifoliae* from various hosts and regions. Bacteria were picked directly from overnight plates and swirled into sterile water. Reaction mixtures were amplified with Dynazyme (Finnzymes) or Pfu Turbo (Stratagene) according to standard reactions as defined by the manufacturers. PCR products were visualized by agarose gel electrophoresis and purified. Purified PCR products were sent directly for sequencing or blunt-end ligated into pBS KS⁺ (Stratagene), transformed into *Escherichia coli*, isolated using the Genejet plasmid miniprep kit (Fermentas, Glen Burnie, MD), and submitted for sequencing to the BioResource Center at Cornell University. Alignments of Eop1 amino acid sequences were performed using MegAlign, and percent identities were calculated.

RESULTS

The *eop1* genes of a *Rubus* and a *Spiraeoideae* strain of *Erwinia amylovora* are significantly divergent. Comparison of

Fig. 1. Comparison of a portion of the *hrp* pathogenicity island (PAI) from a *Spiraeoideae* strain (Ea273) and a *Rubus* strain (Ea246) of *Erwinia amylovora*. The divergent portion of the Hrp elicitor and effector (HEE) region between these two strains begins in *hrpN* and continues through *orfA*, *eop1*, and *orfC*. **A**, GenBank accessions, including the region of interest in Ea246 and Ea273. Arrowheads indicate two deletions in Ea246 relative to Ea273. **B**, Nucleotide sequence identity plot with a sliding window of 100 nucleotides. **C**, Gene organization of Ea246 and Ea273, in which *orfA*, *eop1*, and *orfC* are highly variable. DNA sequence identity between Ea246 and Ea273 is shown below each gene. Omega (Ω) indicates an inverted repeat that might function as a terminator. **D**, The *hrp* PAI of Ea273 (≈42 kb). Homologous regions of Ea246 were sequenced over the deeply shaded portions. The boxed region is presented in B and C. HES-2, Hrp effector secretion 2; HER, regulation of *hrp* gene expression; HES-1, Hrp effector secretion 1; HAE, Hrp-associated enzymes. The bar below represents 5 kb.

approximately 20 kb of the *hrp* PAI of the *Rubus* strain Ea246 compared with *Spiraeoideae* strain Ea273 indicated that portions of the core *hrp* PAI (including parts of the *hrp* effectors and elicitors [HEE], Hrp effector secretion 2 [HES-2], regulation of *hrp* gene expression [HER], Hrp effector secretion 1 [HES-1], the Hrp-associated enzyme [HAE] regions, and the *hrpK* gene) are highly similar, with amino acid sequence identities of 98 to 100%. Nevertheless, parts of the *hrp* PAI were highly dissimilar. In particular, the *eop1* genes were highly divergent between Ea246 and Ea273 compared with neighboring genes in the HEE region. The DNA sequence identity of the *eop1* genes is 73% (67% identity at the amino acid level), while those of *hrpN*, *orfA*, *orfC*, and *hrpW* are 99, 86, 83, and 99%, respectively (Fig. 1). Ea246 has a 60-bp deletion in the intergenic DNA sequence between *hrpN* and *orfA* and a 21-bp deletion in *eop1* compared with Ea273 that contributes to the low sequence identity of *eop1* between these strains. Although *orfA* and *orfC* are relatively dissimilar between Ea246 and Ea273, as putative chaperone genes, they are not expected to travel the T3SS and interact with plant cells directly. Additionally, the amino acid sequence of the Eop1 protein from *E. pyrifoliae* EpWT3, which has a host range that overlaps *Spiraeoideae* strains of *E. amylovora* but is more restricted, is more similar to Ea273 (the *Spiraeoideae* strain) at 80% identity than to Ea246 (the *Rubus* strain) at 68% (Table 2).

Furthermore, *eop1*, *rpoD*, *gyrB*, and *hrcC* DNA sequences from *E. amylovora* strains Ea246 and Ea273; *E. pyrifoliae* strains Ep1/96, DSM 12163, and Ejp617; and *E. tasmaniensis* strain Et1/99 were aligned with genes from *P. syringae* pv. *syringae* B728a (Psy B728a) as an outgroup. Phylogenetic trees were constructed based on these alignments to reveal relationships among strains (Fig. 2). The housekeeping genes *rpoD* and *gyrB*, and *hrcC*, a well-conserved component of the *hrp* T3SS, were used to estimate the relatedness of the strains in question. Psy B728a was chosen as an outgroup because it encodes versions of these well-conserved genes as well as *hopZ3*, which has 60% nucleotide identity to *eop1*_{Ea273}. Based on the trees generated by aligning *rpoD*, *gyrB*, and *hrcC*, Ea246 and Ea273 appear more closely related to each other than to *E. pyrifoliae* or *E. tasmaniensis*; however, the placement of *E. tasmaniensis* to the other strains is not consistent in all trees. In contrast, in the *eop1* tree, strains of *E. pyrifoliae* grouped with the *E. amylovora* *Spiraeoi-*

deae strain Ea273, while the *E. amylovora* *Rubus* strain Ea246 grouped in a separate clade with *E. tasmaniensis*.

The *eop1* sequences are well conserved among *E. amylovora* and *E. pyrifoliae* strains with similar host ranges. To determine whether the divergence in Eop1 protein sequence is a general feature among *Rubus* and *Spiraeoideae* strains and is well conserved in strains with similar host ranges, *eop1* genes from four additional *Rubus* strains, five *Spiraeoideae* strains, and two *E. pyrifoliae* strains originating from different geographic locations were sequenced. These and publicly available DNA sequences from Ea273, Ea246, three strains of *E. pyrifoliae*, and one strain of *E. tasmaniensis* were compared.

Sequencing of the *orfA-eop1* region of 11 strains confirmed that the sequences from *Rubus* strains were nearly identical to each other, as were those of *E. amylovora* strains isolated from non-*Rubus* hosts. The deduced amino acid sequences of Eop1 from *Erwinia* strains expected to have similar host ranges based on host of isolation have 93 to 100% sequence identity, whereas Eop1 among strains expected to have different host ranges have only 67 to 81% sequence identity (Table 2). Accordingly, when visualized as a phylogenetic tree, *eop1* sequences from these various strains cluster according to host of isolation (Supplementary Figure 1). The observed differences in Eop1 sequences were distributed throughout the proteins, although each version of the protein retained an intact catalytic triad, as demonstrated by an alignment of a subset of Eop1 sequences from these strains (Supplementary Figure 2).

Expression of Eop1 from a *Rubus* strain reduced the virulence of both *Rubus* and *Spiraeoideae* strains in immature pear fruit. To evaluate the role of Eop1 in host specificity and virulence more directly, we generated *eop1* mutants from Ea246 and Ea273, as representative *Rubus* and *Spiraeoideae* strains, and compared their virulence in immature Bartlett pear fruit, which is a highly susceptible host of *Spiraeoideae* strains. The wild-type strain Ea273 typically causes symptoms, including necrosis around the inoculated area and bacterial ooze formation 1 to 2 days after inoculation. In contrast, symptom development by the wild-type Ea246 is delayed 2 to 3 days compared with Ea273. Thus, compared with Ea273, symptoms caused by Ea246 appear severely reduced at 4 dpi, with ooze and tissue necrosis barely visible around puncture wounds (Fig. 3A). Surprisingly, the *eop1*

TABLE 2. Comparison of Eop1 from strains of *Erwinia amylovora* and *E. pyrifoliae*^a

Strains ^b	Group	Length	Strains (%) ^c								
			Ea273	Ea110	EpWT3	Ep1/96	Ejp617	Ea246	Ea510	Ea644	Et1/99
Ea273	S	401	100.0	99.3	80.3	81.0	80.0	66.6	67.1	66.8	69.1
Ea110	S	401	99.3	100.0	80.0	80.8	79.6	66.8	67.3	67.1	69.3
Ea262	S	401	100.0	99.3	80.3	81.0	80.0	66.6	67.1	66.8	69.1
Ea566	S	401	100.0	99.3	80.3	81.0	80.0	66.6	67.1	66.8	69.1
Ea587	S	401	100.0	99.3	80.3	81.0	80.0	66.6	67.1	66.8	69.1
Ea629	S	401	100.0	99.3	80.3	81.0	80.0	66.6	67.1	66.8	69.1
Ep WT3	P	401	80.3	80.0	100.0	98.5	93.0	67.6	67.8	67.8	69.3
DSM 12163	P	401	81.0	80.8	98.5	100.0	94.3	68.3	68.6	68.6	70.3
Ep1/96 ^d	P	401	81.0	80.8	98.5	100.0	94.3	68.3	68.6	68.6	70.3
Ep4/97	P	401	81.0	80.8	98.5	100.0	94.3	68.3	68.6	68.6	70.3
Ejp617	P	399	80.0	79.6	93.0	94.3	100.0	68.2	68.4	68.4	70.4
Ea246	R	393	66.6	66.8	67.6	68.3	68.2	100.0	99.2	99.7	82.3
Ea510	R	393	67.1	67.3	67.8	68.6	68.4	99.2	100.0	99.5	83.1
Ea528	R	393	67.1	67.3	67.8	68.6	68.4	99.2	100.0	99.5	83.1
Ea592	R	393	67.1	67.3	67.8	68.6	68.4	99.2	100.0	99.5	83.1
Ea644	R	393	66.8	67.1	67.8	68.6	68.4	99.7	99.5	100.0	82.6
Et1/99	T	396	69.1	69.3	69.3	70.3	70.4	82.3	83.1	82.6	100.0

^a The *orfA-eop1* regions from several *Erwinia* strains were sequenced, and the deduced Eop1 sequences were compared with select *Erwinia* strains. Eop1 was found to be well-conserved among strains with similar host ranges.

^b Strains are grouped as *Spiraeoideae* (S), *Rubus* (R), *E. pyrifoliae* (P), or as *E. tasmaniensis* (T) based on species, host, and geographical region of isolation.

^c Genbank accession numbers for *eop1* sequences: Ea273, FN666575; Ea110, JF912499; Ea262, JF912500; Ea566, JF912503; Ea587, JF912504; Ea629, JF912506; Ep WT3, DQ180962.2; DSM 12163, FN392235.1; Ep1/96, NC_012214; Ep4/97, JF912508; Ejp617, CP002124; Ea246, AF083620; Ea510, JF912501; Ea528, JF912502; Ea592, JF912505; Ea644, JF912507; Et1/99, NC_010694.

^d *eop1* was independently sequenced and matches *eop1* from the Ep1/96 genome sequence (FP236842.1).

deletion mutant of Ea246 (Ea246 Δ eop1) exhibited a gain-of-virulence phenotype in pear, eliminating the delay in symptom development compared with Ea273 (Fig. 3A and B). However, a transposon insertion mutant in the *eop1* gene of Ea273 (Ea273 *eop1*::Tn) caused symptoms similar to those of the wild-type strain (Fig. 3A and B).

To confirm that the observed phenotype of Ea246 Δ eop1 was due to the missing *eop1* gene, this mutant strain was transformed with plasmid pCPP1777, which harbors *eop1*_{Ea246}. The presence of plasmid pCPP1777 restored the delay in symptom development relative to Ea246 Δ eop1 (Fig. 3A and B). These results indicated that the *eop1*_{Ea246} gene was responsible for the change in phenotype. In contrast, *eop1*_{Ea273}, harbored by plasmid pCPP1776, failed to complement Ea246 Δ eop1. Additionally, expression of Eop1_{Ea246} in Ea273 backgrounds consistently caused a delay in symptom development compared with wild-type Ea273 (Fig. 3A and B).

Expression and secretion of Eop1 encoded by pCPP1776 and pCPP1777 was confirmed in an Ea273 *eop1*::Tn background by Western blotting with antibodies raised against Eop1_{Ea273} (Fig. 3C). However, we failed to detect Eop1_{Ea246} from wild-type Ea246, presumably because the levels produced were below the threshold of detection for the primary antibody, which seems to

have a 5- to 10-fold greater affinity for Eop1_{Ea273} than Eop1_{Ea246} (data not shown). Previously, Eop1 was visualized after staining proteins resolved by two-dimensional electrophoresis of type III-secreted proteins from Ea246 (Nissinen and Beer, *personal communication*). Additionally, we confirmed expression of Eop1 from pCPP1776 and pCPP1777 in an Ea246 Δ eop1 background by performing Western blot analysis of cell lysates (data not shown).

These combined results indicated that Eop1 from a *Rubus* strain but not Eop1 from a *Spiraeoideae* strain reduced virulence of *E. amylovora* in immature pear fruit.

Eop1_{Ea273} confers virulence activity when expressed in a *Rubus* strain. Whereas the expression of Eop1_{Ea246} functioned to reduce the virulence of *E. amylovora* in immature pear fruit, we might expect Eop1_{Ea273} to contribute to virulence, because it is a type III effector from a *Spiraeoideae* strain that is highly virulent on immature pear fruit. To test the role of Eop1_{Ea273}, we transformed plasmid pCPP1776, which harbors *eop1*_{Ea273}, into Ea246, Ea273, and derivatives of these strains, and assayed their virulence in immature pear fruit. Interestingly, expression of Eop1_{Ea273} in Ea246 resulted in significantly greater virulence relative to the wild-type Ea246 (Fig. 3B). Consistently, virulence also increased over the background strain when Eop1_{Ea273} was expressed in Ea246 Δ eop1. In contrast, increased expression of Eop1_{Ea273} in Ea273 derivatives did not change the virulence of Ea273 significantly (Fig. 3B). These data indicate that Eop1_{Ea273} may have virulence activity that is masked in Ea273 but is more clearly evident in other backgrounds.

Expression of *Rubus*-strain Eop1 reduces the virulence of *Spiraeoideae* strains in apple shoots. To determine whether Eop1_{Ea246} also delays symptom onset induced by *E. amylovora* in apple shoots, the same strains as assayed for virulence in immature pear fruit were inoculated into apple shoots (Gala). There were no significant differences in virulence between wild-type Ea273 and the Ea273 *eop1*::Tn mutant strain. Furthermore, overexpression of *eop1*_{Ea273} did not significantly alter virulence of any strain. However, expression of *eop1*_{Ea246} reduced the virulence of Ea273 derivatives. At 7 dpi, symptoms in shoots inoculated with Ea273 derivatives expressing Eop1_{Ea246} had not caused measurable shoot lesions. In contrast, shoots inoculated with other strains of Ea273 had caused lesions that involved an average of 25 to 40% of the shoot length (Fig. 4A). After prolonged incubation, some shoots inoculated with Ea273 derivatives expressing Eop1_{Ea246} caused sizable lesions in Gala. This may have been due to plasmid loss, because some plasmid-free bacteria were recovered from these lesions (data not shown). *Rubus* strains Ea246 and Ea246 Δ eop1 typically did not cause symptoms on Gala apple shoots even after inoculation and incubation for many days under favorable conditions for disease development. In the most severe cases, inoculated leaves and petioles blackened with necrosis but the lesions did not advance into the shoot (data not shown). Therefore, although *Rubus*-strain Eop1 was sufficient to reduce the virulence of a *Spiraeoideae* strain in apple shoots and pear fruit, it seems that additional factors are involved to prevent *Rubus* strain Ea246 from becoming an aggressive pathogen of apple.

In order to determine whether the effect of Eop1_{Ea246} on Gala apple was cultivar specific, Ea273 and Ea273(pCPP1777), carrying *eop1*_{Ea246}, were inoculated into shoots of five other cultivars ('Granny Smith', 'Idared', 'Fuji', 'Jonagold', and 'Rome'), using five or six replicates per strain. Symptom development was delayed in all cultivars in response to inoculation with Ea273(pCPP1777), relative to inoculation with wild-type strain Ea273 or Ea273(pSU23) (Fig. 4B). The effect of cultivar was not statistically significant as determined by ANOVA. However, the effect of strain on lesion length was significant, with a *P* value < 0.0001. Thus, expression of Eop1 from a *Rubus* strain reduced the virulence of a *Spiraeoideae* strain of *E. amylovora*, regardless of apple cultivar.

Fig. 2. Phylogenetic analyses with **A**, *eop1* from strains of *Erwinia* spp. and *hopZ3* from *Pseudomonas syringae* pv. *syringae* strain B728a (Psy B728a); two housekeeping genes **B**, *gyrB* and **C**, *rpoD*; and **D**, *hrcC* gene from the *hrp* pathogenicity island. Phylogenetic trees were generated from ClustalW alignments of DNA sequences from *Erwinia amylovora* strains Ea273 and Ea246, Korean *E. pyrifoliae* strains Ep1/96 and DSM 12163, Japanese *E. pyrifoliae* strain Ejp617, and *E. tasmaniensis* strain Et1/99. Each gene homolog from Psy B728a was used as an outgroup. The X-axis indicates percent nucleotide divergence. Bootstrap values are derived from 1,000 trials and a seed value of 111.

Eop1 protein is not necessary for virulence of *E. amylovora* in raspberry. To examine the effect of Eop1 on the virulence of *E. amylovora* in raspberry, which is a natural host of *Rubus* strains, we inoculated Ea273, Ea246, and their *eop1*-altered derivatives in ‘Boyne’ and ‘Titan’ raspberry canes. Strains of Ea273 were consistently poor pathogens of Boyne raspberry regardless of their *eop1* genotype. The Ea246 strains altered in *eop1* (mutated *eop1* or overexpressing Eop1_{Ea246} or Eop1_{Ea273}) caused symptoms similar to those of the wild-type strain (data not shown). Thus, we detected no differences in the virulence of *E. amylovora* strains in raspberry canes, based on the presence or level of expression of *eop1*.

DISCUSSION

To our knowledge, *Rubus*-strain Eop1_{Ea246} is the first factor from an *E. amylovora* strain shown to alter the virulence of the fire blight pathogen in a host-specific manner. Eop1_{Ea246} seems to

behave as an avirulence protein in *Spiraeoideae* hosts; as a member of the YopJ/AvrRxv protein family, Eop1 belongs to a group that includes many avirulence proteins (6,7,14,47). PopP1, for example, can modulate the aggressiveness of *R. solanacearum* in plants, delaying symptom onset in *Arabidopsis thaliana* ecotype Col-5 by 1 to 2 days, a reduction in aggressiveness similar to that which we observed in *E. amylovora* strains expressing *Rubus*-strain *eop1* in immature pear fruit (14).
The *eop1* genes from a *Rubus* and a *Spiraeoideae* strain are unusually divergent compared with nearby genes. Additionally, the trees generated by comparing the conserved genes *rpoD*, *gyrB*, and *hrcC* show that the *Spiraeoideae* and *Rubus* strains of *E. amylovora* we assessed are closely related. However, phylogenetic trees generated from *eop1* sequences place the *Spiraeoideae* strain in a separate clade from the *Rubus* strain. Thus, the similarity of *eop1* genes does not seem to reflect the overall relatedness of strains. This suggests that *eop1* genes in some strains are accumulating nucleotide changes faster than others, even

Fig. 3. Effect of expression of *eop1* on virulence of *Erwinia amylovora* in immature pear fruit. The *eop1* gene from a *Spiraeoideae* strain (Ea273) or a *Rubus* strain (Ea246) was expressed from a plasmid (in trans) in wild-type (WT) or *eop1* mutants of Ea246 and Ea273. Fruit were inoculated, and percentage of the fruit surface that was symptomatic was assessed. Each bacterial suspension of 10⁸ CFU/ml was inoculated at three places per each pear half; inoculated pear halves were kept in sealed boxes at 26°C. **A**, Representative assays at 4 days postinoculation (dpi). Necrosis and appearance of bacterial ooze at the inoculation sites are typical symptoms in immature pear fruit. **B**, *E. amylovora* strains altered in Eop1 expression were assessed for virulence in immature pear fruit at 4 dpi. Bars represent least square means. Error bars indicate 95% confidence intervals. Letters refer to statistical groupings as determined by analysis of variance. **C**, Western blot of secreted proteins confirms that Eop1 is both expressed and secreted. The antibody was raised against and has higher affinity for Eop1_{Ea273}. EV, empty vector; L, ladder of protein size markers. Black bar in lane L indicates location of 49-kDa marker.

though *eop1* seems to be well conserved among strains expected to have similar host ranges. We speculate that the diversification of *E. amylovora eop1* genes may be driven by selective pressure from host plants which may mount defense responses against certain forms of Eop1.

The gain of virulence in immature pear fruit, caused by deletion of *eop1* from Ea246, is especially striking. The presence of this deletion phenotype in a *Rubus* strain suggests that the contribution of Eop1_{Ea246} as a host-range-limiting factor is biologically relevant. However, the deletion of *eop1* did not convert the Ea246

mutant to an aggressive pathogen of apple shoots. Therefore, *Rubus* strains must harbor other avirulence genes or lack factors necessary to effectively infect apple shoots. Deletion of a single gene from a bacterial plant pathogen is rarely sufficient to alter its host range at the species level. To our knowledge, only one such case has been documented: deletion of a single type III effector gene, *hopQ1-1*, allowed a strain of *P. syringae* pv. *tomato* strain DC3000 to expand its host range to infect *Nicotiana benthamiana* (48).

The increased virulence of *Rubus* strains expressing the *Spiraeoideae*-strain Eop1_{Ea273} in immature pear fruit suggests that Eop1_{Ea273} has a virulence role in *Spiraeoideae* hosts. However, Ea273 mutated in *eop1* or overexpressing *eop1*_{Ea273} did not differ significantly in virulence from the wild-type strain. Ea273 may harbor other virulence factors that render *eop1* redundant that are absent or altered in Ea246. We expect that Eop1_{Ea273} may play a role in suppressing defenses in host plants, as do other members of the YopJ/AvrRxv protein family. If so, greater or altered elicitation of these defenses by the *Rubus* strain Ea246 (which is an efficient pathogen of brambles but not fruit trees) compared with a *Spiraeoideae* strain might explain the greater apparent benefit of expression of *eop1*_{Ea273} for Ea246 in immature pear fruit. Additional work will be needed to distinguish these possibilities.

The *eop1* gene was found in every strain of *E. amylovora* and *E. pyrifoliae* assessed. The amino acid sequences of Eop1 were well-conserved in strains of similar host range, suggesting that the experimental results from our representative strains are likely to be broadly applicable to other *Rubus* and *Spiraeoideae* strains of *E. amylovora*. The differences among Eop1 amino acid sequences from strains predicted to have different host specificities were distributed throughout the proteins. However, in each case, the putative catalytic triad was conserved, suggesting that the proteins are functional. Due to the distribution of differences among Eop1 proteins, it is difficult to predict which regions of *Rubus*-strain Eop1 might be responsible for the apparent recognition of the protein or its activity by non-*Rubus* rosaceous plants. Additional mutants and protein chimeras will be needed to assess the importance of specific regions of the protein and whether the activities of putative catalytic triads are necessary for virulence and avirulence functions.

This research commenced with limited available sequence information. However, the genomes of several strains of *Erwinia* have been completely sequenced recently. These include the *Rubus* strain Ea246 (32); two *Spiraeoideae* strains of *E. amylovora*: Ea273 (37) and CFBP 1430 (39); three strains of *E. pyrifoliae*: Ep1/96 (12), DSM 12163 (38), and Ejp 617(29); and one strain of *E. tasmaniensis*: Et1/99 (13). The availability of these whole genomes should facilitate future research into host-range-determining factors in *E. amylovora* and *E. pyrifoliae*. Comparison of strains with different host ranges should help researchers to identify genes that are unique or polymorphic among these strains. Thus, it is likely that the molecular bases for host specificity among strains inducing fire blight symptoms soon will be further clarified.

ACKNOWLEDGMENTS

This work was supported, in part, by USDA CSREES Special Grants to support work on fire blight of apple in New York and by the Department of Plant Pathology and Plant-Microbe Biology, Cornell University. J. E. Asselin was supported, in part, by funds from the Dorothy I. Willets Endowment of the Department of Plant Pathology and Plant-Microbe Biology, Cornell University. We thank undergraduate research assistants E. Viña, C. Gant, W. Ewart, K. N. Yip, N. Cooney, and S. Walker for their help with plant inoculations and PCR; Nourse Farms of South Deerfield, MA for gifts of raspberry plants; H. Aldwinckle for providing and C. Watkins and J. Nock and storing immature pear fruit for use throughout the year; and S. Carpenter for a critical reading of the manuscript.

Fig. 4. Effect of *eop1* expression on the virulence of Ea273 in apple shoots. **A**, Mean shoot length infected after 7 days in ‘Gala’ apple shoots. Strains not causing lesions were not included in statistical analysis. Percentages of shoot lengths infected by strains causing lesions did not differ significantly from each other. Each data point represents the mean of five or six apple shoots; error bars represent standard errors of the means. **B**, Virulence assay of *Spiraeoideae* strain Ea273 harboring *eop1* from a *Rubus* strain (Ea246) versus wild-type Ea273. Bars represent the least squares means from statistical analysis. Error bars represent 95% confidence intervals. Letters over bars define statistical groupings of strains based on analysis of variance.

LITERATURE CITED

- Asselin, J. E., Oh, C.-S., Nissinen, R. M., and Beer, S. V. 2006. The secretion of EopB from *Erwinia amylovora*. Acta Hort. 704:409-416.
- Bartolomé, B., Jubete, Y., Martinez, E., and de la Cruz, F. 1991. Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene 102:75-78.
- Bonaser, J. M., Kim, J. F., and Beer, S. V. 2006. PR genes of apple: identification and expression in response to elicitors and inoculation with *Erwinia amylovora*. BMC Plant Biol. 6:23.
- Box, G. E. P., and Cox, D. R. 1964. An analysis of transformations. J. R. Stat. Soc. Ser. B 26:211-252.
- Braun, P. G., and Hildebrand, P. D. 2005. Infection, carbohydrate utilization, and protein profiles of apple, pear, and raspberry isolates of *Erwinia amylovora*. Can. J. Plant Pathol. 27:338-346.
- Ciesiolka, L. D., Hwin, T., Gearlds, J. D., Minsavage, G. V., Saenz, R., Bravo, M., Handley, V., Conover, S. M., Zhang, H., Caporgno, J., Phengrasamy, N. B., Toms, A. O., Stall, R. E., and Whalen, M. C. 1999. Regulation of expression of avirulence gene *avrRxv* and identification of a family of host interaction factors by sequence analysis of *avrBsT*. Mol. Plant-Microbe Interact. 12:35-44.
- Collier-Hyams, L. S., Zeng, H., Sun, J., Tomlinson, A. D., Bao, Z. Q., Chen, H., Madara, J. L., Orth, K., and Neish, A. S. 2002. Cutting edge: *Salmonella* AvrA effector inhibits the key proinflammatory, anti-apoptotic NF- κ B pathway. J. Immunol. 169:2846-2850.
- Geider, K., Auling, G., Jakovljevic, V., and Volksch, B. 2009. A polyphasic approach assigns the pathogenic *Erwinia* strains from diseased pear trees in Japan to *Erwinia pyrifoliae*. Lett. Appl. Microbiol. 48:324-330.
- Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.
- Kim, W.-S., Gardan, L., Rhim, S.-L., and Geider, K. 1999. *Erwinia pyrifoliae* sp. nov., a novel pathogen that affects Asian pear trees (*Pyrus pyrifolia* Nakai). Int. J. Syst. Bacteriol. 49:899-906.
- Kim, W. S., Jock, S., Paulin, J.-P., Rhim, S.-L., and Geider, K. 2001. Molecular detection and differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. Plant Dis. 85:1183-1188.
- Kube, M., Migdoll, A. M., Gehring, I., Heitmann, K., Mayer, Y., Kuhl, H., Knaust, F., Geider, K., and Reinhardt, R. 2010. Genome comparison of the epiphytic bacteria *Erwinia billingiae* and *E. tasmaniensis* with the pear pathogen *E. pyrifoliae*. BMC Genomics 11:393.
- Kube, M., Migdoll, A. M., Muller, I., Kuhl, H., Beck, A., Reinhardt, R., and Geider, K. 2008. The genome of *Erwinia tasmaniensis* strain Et1/99, a non-pathogenic bacterium in the genus *Erwinia*. Environ. Microbiol. 10:2211-2222.
- Lavie, M., Shillington, E., Eguiluz, C., Grimsley, N., and Boucher, C. 2007. PopP1, a new member of the YopJ/AvrRxv family of type III effector proteins, acts as a host-specificity factor and modulates aggressiveness of *Ralstonia solanacearum*. Mol. Plant-Microbe Interact. 15:1058-1068.
- Ma, W. B., Dong, F. F. T., Stavrinides, J., and Guttman, D. S. 2006. Type III effector diversification via both pathoadaptation and horizontal transfer in response to a coevolutionary arms race. PLoS Genet. 2:2131-2142.
- Maes, M., Orye, K., Bobev, S., Devreese, B., Van Beeumen, J., De Bruyn, A., Busson, R., Herdewijn, P., Morreel, K., and Messens, E. 2001. Influence of amylovoran production on virulence of *Erwinia amylovora* and a different amylovoran structure in *E. amylovora* isolates from Rubus. Eur. J. Plant Pathol. 107:839-844.
- Mittal, R., Peak-Chew, S. Y., and McMahon, H. T. 2006. Acetylation of MEK2 and I κ B kinase (IKK) activation loop residues by YopJ inhibits signaling. Proc. Natl. Acad. Sci. USA 103:18574-18579.
- Mizuno, A., Sato, S., and Kawai, A. 2002. Serological differences among *Erwinia amylovora* biovars. J. Gen. Plant Pathol. 68:350-355.
- Mohan, S. K., and Bijman, V. P. 1999. Susceptibility of *Prunus* species to *Erwinia amylovora*. Acta Hort. 145-148.
- Mohan, S. K., and Thomson, S. V. 1996. An outbreak of fire blight in plums. Acta Hort. 411:73-76.
- Momol, E. A., Momol, M. T., Norelli, J. L., Beer, S. V., Burr, T. J., and Aldwinckle, H. S. 1999. Relatedness of *Erwinia amylovora* strains based on amplified 16S-23S ribosomal DNA restriction enzyme analysis - ARDREA. Acta Hort. 489:55-59.
- Mukherjee, S., Keitany, G., Li, Y., Wang, Y., Ball, H. L., Goldsmith, E. J., and Orth, K. 2006. *Yersinia* YopJ acetylates and inhibits kinase activation by blocking phosphorylation. Science 312:1211-1214.
- Nissinen, R. M., Ytterberg, A. J., Bogdanove, A. J., Van Wijk, K. J., and Beer, S. V. 2007. Analyses of the secretomes of *Erwinia amylovora* and selected hrp mutants reveal novel type III secreted proteins and an effect of HrpJ on extracellular harpin levels. Mol. Plant Pathol. 8:55-67.
- Oh, C.-S., and Beer, S. V. 2005. Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. FEMS Microbiol. Lett. 253:185-192.
- Oh, C.-S., Kim, J. F., and Beer, S. V. 2005. The Hrp pathogenicity island of *Erwinia amylovora* and identification of three novel genes required for systemic infection. Mol. Plant Pathol. 6:125-138.
- Orth, K., Xu, Z., Mudgett, M. B., Bao, Z. Q., Palmer, L. E., Bliska, J. B., Mangel, W. F., Staskawicz, B., and Dixon, J. E. 2000. Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. Science 290:1594-1597.
- Page, M., and Thorpe, R. 1996. Purification of IgG by precipitation with sodium sulfate or ammonium sulfate. Pages 721-722 in: The Protein Protocols Handbook. J. M. Walker, ed. Humana Press, Totowa, NJ.
- Palmer, L. E., Hobbie, S., Galan, J. E., and Bliska, J. B. 1998. YopJ of *Yersinia pseudotuberculosis* is required for the inhibition of macrophage TNF- α production and downregulation of the MAP kinases p38 and JNK. Mol. Microbiol. 27:953-965.
- Park, D. H., Thapa, S. P., Choi, B. S., Kim, W. S., Hur, J. H., Cho, J. M., Lim, J. S., Choi, I. Y., and Lim, C. K. 2011. Complete genome sequence of Japanese *Erwinia* strain Ejp617, a bacterial shoot blight pathogen of pear. J. Bacteriol. 193:586-587.
- Potter, D., Eriksson, T., Evans, R. C., Oh, S., Smedmark, J. E. E., Morgan, D. R., Kerr, M., Robertson, K. R., Arsenault, M., Dickinson, T. A., and Campbell, C. S. 2007. Phylogeny and classification of Rosaceae. Plant Syst. Evol. 266:5-43.
- Powney, R., Beer, S. V., Plummer, K., Luck, J., and Rodoni, B. 2011. The specificity of PCR-based protocols for detection of *Erwinia amylovora*. Australas. Plant Pathol. 40:87-97.
- Powney, R., Smits, T. H., Sawbridge, T., Frey, B., Blom, J., Frey, J. E., Plummer, K. M., Beer, S. V., Luck, J., Duffy, B., and Rodoni, B. 2011. Genome sequence of an *Erwinia amylovora* strain with pathogenicity restricted to *Rubus* plants. J. Bacteriol. 193:785-786.
- Prentki, P., and Krusch, H. M. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29:303-313.
- Ries, S. M., and Otterbacher, A. G. 1977. Occurrence of fire blight on thornless blackberry in Illinois. Plant Dis. Rep. 61:232-235.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schesser, K., Spiik, A. K., Dukuzumuremyi, J. M., Neurath, M. F., Pettersson, S., and Wolf-Watz, H. 1998. The *yopJ* locus is required for *Yersinia*-mediated inhibition of NF- κ B activation and cytokine expression: YopJ contains a eukaryotic SH2-like domain that is essential for its repressive activity. Mol. Microbiol. 28:1067-1079.
- Sebahia, M., Bocsanczy, A. M., Biehl, B. S., Quail, M. A., Perna, N. T., Glasner, J. D., DeClerck, G. A., Cartinhou, S., Schneider, D. J., Bentley, S. D., Parkhill, J., and Beer, S. V. 2010. Complete genome sequence of the plant pathogen *Erwinia amylovora* strain ATCC 49946. J. Bacteriol. 192:2020-2021.
- Smits, T. H., Jaenicke, S., Rezzonico, F., Kamber, T., Goesmann, A., Frey, J. E., and Duffy, B. 2010. Complete genome sequence of the fire blight pathogen *Erwinia pyrifoliae* DSM 12163^T and comparative genomic insights into plant pathogenicity. BMC Genomics 11:2.
- Smits, T. H., Rezzonico, F., Kamber, T., Blom, J., Goesmann, A., Frey, J. E., and Duffy, B. 2010. Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. Mol. Plant-Microbe Interact. 23:384-393.
- Staskawicz, B. J., Mudgett, M. B., Dangel, J. L., and Galan, J. E. 2001. Common and contrasting themes of plant and animal diseases. Science 292:2285-2289.
- Steinberger, E. M., and Beer, S. V. 1988. Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. Mol. Plant-Microbe Interact. 1:135-144.
- Steinberger, E. M., Cheng, G. Y., and Beer, S. V. 1990. Characterization of a 56-kb plasmid of *Erwinia amylovora* Ea322: Its noninvolvement in pathogenicity. Plasmid 24:12-24.
- Sweet, C. R., Conlon, J., Golenbock, D. T., Goguen, J., and Silverman, N. 2007. YopJ targets TRAF proteins to inhibit TLR-mediated NF- κ B, MAPK and IRF3 signal transduction. Cell Microbiol. 9:2700-2715.
- Szczesny, R., Buttner, D., Escobar, L., Schulze, S., Seifert, A., and Bonas, U. 2010. Suppression of the AvrBs1-specific hypersensitive response by the YopJ effector homolog AvrBsT from *Xanthomonas* depends on a SNF1-related kinase. New Phytol. 187:1058-1074.
- Triplett, L. R., Zhao, Y., and Sundin, G. W. 2006. Genetic differences between blight-causing *Erwinia* species with differing host specificities, identified by suppression subtractive hybridization. Appl. Environ. Microbiol. 72:7359-7364.
- van der Zwet, T., and Beer, S. V. 1999. Fire blight--its nature, prevention, and control: a practical guide to integrated disease management. U. S. Dep. Agric. Agric. Inf. Bull. No. 631.

47. Vinatzer, B. A., Teitzel, G. M., Lee, M. W., Jelenska, J., Hotton, S., Fairfax, K., Jenrette, J., and Greenberg, J. T. 2006. The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. *Mol. Microbiol.* 62:26-44.
48. Wei, C. F., Kvitko, B. H., Shimizu, R., Crabill, E., Alfano, J. R., Lin, N. C., Martin, G. B., Huang, H. C., and Collmer, A. 2007. A *Pseudomonas syringae* pv. *tomato* DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant *Nicotiana benthamiana*. *Plant J.* 51:32-46.
49. Wiley, D. J., Shrestha, N., Yang, J., Atis, N., Dayton, K., and Schesser, K. 2009. The activities of the *Yersinia* protein kinase A (YpkA) and outer protein J (YopJ) virulence factors converge on an eIF2 α kinase. *J. Biol. Chem.* 284:24744-24753.
50. Wilson, W. J., Wiedmann, M., Dillard, H. R., and Batt, C. A. 1994. Identification of *Erwinia stewartii* by a ligase chain reaction assay. *Appl. Environ. Microbiol.* 60:278-284.
51. Zhang, Y., and Geider, K. 1997. Differentiation of *Erwinia amylovora* strains by pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* 63:4421-4426.