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Homology and functional similarity of an *hrp*-linked pathogenicity locus, *dspEF*, of *Erwinia amylovora* and the avirulence locus *avrE* of *Pseudomonas syringae* pathovar tomato

(plant disease resistance/coevolution/Hrp pathway)

ADAM J. BOGDANOVE*, JIHYUN F. KIM, ZHONGMIN WEI†, PETER KOLCHINSKY, AMY O. CHARKOWSKI, ALISON K. CONLIN, ALAN COLLMER, AND STEVEN V. BEER‡

Department of Plant Pathology, Cornell University, Ithaca, NY 14853

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ABSTRACT **The "disease-specific" (*dsp*) region next to the *hrp* gene cluster of *Erwinia amylovora* is required for pathogenicity but not for elicitation of the hypersensitive reaction. A 6.6-kb apparent operon, *dspEF*, was found responsible for this phenotype. The operon contains genes *dspE* and *dspF* and is positively regulated by *hrpL*. A BLAST search revealed similarity in the *dspE* gene to a partial sequence of the *avrE* locus of *Pseudomonas syringae* pathovar tomato. The entire *avrE* locus was sequenced. Homologs of *dspE* and *dspF* were found in juxtaposed operons and were designated *avrE* and *avrF*. Introduced on a plasmid, the *dspEF* locus rendered *P. syringae* pv. glycinea race 4 avirulent on soybean. An *E. amylovora dspE* mutant, however, elicited a hypersensitive reaction in soybean. The *avrE* locus *in trans* restored pathogenicity to *dspE* strains of *E. amylovora*, although restored strains were low in virulence. DspE and AvrE are large (198 kDa and 195 kDa) and hydrophilic. DspF and AvrF are small (16 kDa and 14 kDa) and acidic with predicted amphipathic α helices in their C termini; they resemble chaperones for virulence factors secreted by type III secretion systems of animal pathogens.**

Erwinia amylovora causes fire blight of apple, pear, and other rosaceous plants and elicits plant defense responses in nonhost plants. Required for these interactions are the clustered bacterial *hrp* genes, encoding regulatory proteins (ref. 1; Z.M.W., B. J. Sneath, and S.V.B., unpublished data), a large set of proteins broadly conserved among plant and animal pathogens and constituting a type III secretion pathway (known as the "Hrp pathway" in phytopathogenic bacteria; refs. 2 and 3), and at least two proteins secreted via the Hrp pathway (4, 5). *hrp* genes, present in all Gram-negative necrogenic plant pathogens, were discovered by transposon mutagenesis of *Pseudomonas syringae* pathovars and were named for the "*h*ypersensitive *r*eaction" (HR) and "*p*athogenicity" (reviewed in ref. 6). The HR is a manifestation of plant defense characterized by rapid necrosis at the site of pathogen ingress.

Pathogen avirulence (*avr*) genes (for a review see ref. 7) generate signals that trigger defense responses leading to disease resistance in plants with corresponding resistance (*R*) genes. Typically, *avr* genes are isolated by expressing a cosmid library from one pathogen in another pathogen and screening for narrowed host range. *avr* genes traditionally have been considered as negative determinants of host specificity at the race-cultivar level, but some, including the *avrE* locus from the bacterial speck pathogen *Pseudomonas syringae* pathovar (pv.) tomato (8), may restrict host range at the pathovar–species or species–species level (9, 10). Many *avr* genes, including *avrE*, are *hrp* regulated. *avrE* and *avrPphE* (11) are physically linked to *hrp* genes. Only a few *avr* genes (such as *avrE*), however, play detectable roles in pathogen fitness or in virulence in hosts tested (12–16), and the selective force driving the maintenance in pathogen genomes of many of these host-range-limiting factors has remained a mystery.

When expressed *in trans*, the *avrE* locus renders *P. syringae* pv. glycinea, which causes bacterial blight of soybean, avirulent in each of 10 tested cultivars (17). The locus comprises two convergent transcription units, one preceded by a putative σ^{54} promoter and the other by a *hrp* box (17, 18), a sequence found upstream of many *hrp* and *avr* genes that are positively regulated by the alternate sigma factor HrpL (1, 18). Expression of both transcripts requires *hrpL*. The *avrE* locus contributes quantitatively to the virulence in tomato leaves of *P. syringae* pv. tomato strain PT23, but not of strain DC3000 (15, 17).

Transposon mutagenesis of *E. amylovora* revealed, linked to the *hrp* gene cluster, a "*d*isease *sp*ecific" (*dsp*; see ref. 19) region required for pathogenicity but dispensable for HR elicitation. Through sequencing and further mutagenesis, we have defined a two-gene apparent operon, the *dspEF* locus, responsible for this phenotype. Here, we present an analysis of the genes, including the finding that they are homologous with genes in the *avrE* locus. In addition, we show that the *dspEF* locus converts *P. syringae* pv. glycinea to avirulence in soybean, and that *avrE* restores pathogenicity to *dsp* mutant strains of *E. amylovora*. We discuss the implications of these findings with respect to the nature, evolution, and potential usefulness of bacterial genes encoding proteins involved in infection of plants.

MATERIALS AND METHODS

Recombinant DNA Techniques. DNA was isolated, cut by using restriction enzymes, and ligated, and transformed into *Escherichia coli* according to procedures described by Sambrook *et al*. (20). A *P. syringae* pv. tomato DC3000 genomic library was constructed and screened by using colony hybridization also as described (20). The library was constructed by

Abbreviations: *dsp*, disease specific; *hrp*, hypersensitive reaction and pathogenicity; HR, hypersensitive reaction; *R* gene, resistance gene; pv., pathovar; cfu, colony-forming units; GUS, β-glucuronidase; LB, Luria–Bertani; Hrp MM, *hrp*-gene-inducing minimal medium.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. U97504 (*dspEF* locus and flanking DNA) and U97505 (*avrE* and *avrF*)].

*Present address: Department of Agronomy, Purdue University, West Lafayette, IN 47907.

†Present address: Eden Bioscience Corp., 11816 North Creek Parkway, Bothell, WA 98011.

‡To whom reprint requests should be addressed. e-mail: svb1@cornell.edu.

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using pCPP47, a low-copy-number, broad-host-range cosmid (21). Except where noted, *E. coli* DH5 and *E. coli* DH5α were used as hosts for DNA clones, and pBluescript or pBC plasmids (Stratagene) were used as vectors. *E. amylovora* was transformed by electroporation as described (22). Plasmids were mobilized into *E. amylovora* and *P. syringae* by using pRK2013 (23).

Nucleotide Sequencing and Analysis. The nucleotide sequence of the *dsp* region of *E. amylovora* strain Ea321 was determined by using subclones of pCPP430 (24). The nucleotide sequence of the *avrE* locus was determined by using subclones of pCPP2357, a clone selected from a *P. syringae* pv. tomato DC3000 genomic cosmid library based on hybridization with the *hrpRS* operon of *P. syringae* pv. syringae, and the finding, based on partial sequencing, that it contained the *avrE* locus. Nucleotide sequencing was performed by the Cornell Biotechnology Sequencing Facility on a Model 377 Sequencer (Perkin–Elmer/Applied Biosystems Division, Foster City, CA). Sequence analyses were performed by using the programs of the GCG 7.1 software package (Genetics Computer Groups, Madison, WI) and DNASTAR (DNAstar, Madison, WI). Database searches were performed by using BLAST (25).

Expression of DspE and DspE′ in *E. coli*. The *dspEF* locus was cloned in two pieces into pCPP50, a derivative of pINIII^{113}-A2 (26) with an expanded polylinker (D. W. Bauer and A.J.B., unpublished data), yielding pCPP1259. Expression in pCPP1259 is driven by the *lpp* promoter of *E. coli*, under the control of the *lac* operator. An intermediate clone, pCPP1244, extending from the start of the locus to the *Bam*HI site in the middle of *dspE*, also was isolated. *E. coli* DH5α strains containing pCPP1259 and pCPP1244 were grown in Luria–Bertani (LB) medium at 37°C to an OD_{620} of 0.3. Isopropyl-thio-β-D-galactoside then was added to 1 mM, and the cells further incubated until reaching an OD_{620} of 0.5. Cells were concentrated 2-fold, lysed, and subjected to SDS/PAGE as described (20). Cells containing pCPP50 were included for comparison. Proteins were visualized by Coomassie blue staining.

Deletion Mutagenesis of *dspE*. We deleted 1,554 bp from the 5′ *Hin*dIII-*Bam*HI fragment of *dspE* in pCPP1237 by using unique *Stu*I and *Sma*I sites. The mutagenized clone then was inserted into the suicide vector pKNG101 (27) by using *E. coli* SM10λ*pir* as a host, yielding pCPP1241. The mutation, designated Δ1554, then was transferred into *E. amylovora* strains by using marker eviction as described previously (2). By using two *Bst*EII sites blunted with Klenow fragment, 1,521 bp were deleted from the 3′ *Hin*dIII fragment of *dspE* in pCPP1246. This mutation, Δ1521, was transferred into *E. amylovora* strains as above.

Pathogenicity Assays. For *E. amylovora* strains, cell suspensions of 5×10^8 colony-forming units (cfu) per ml were pipetted into wells cut in immature Bartlett pear fruit, or stabbed into Jonamac apple and cotoneaster shoot apices, and assays were carried out as described previously (28, 29). For *P. syringae* pv. glycinea strains, panels of primary leaves of 2-week-old soybean seedlings (*Glycine max*, cultivar Norchief) were infiltrated with bacterial suspensions of 8×10^5 cfu/ml as for the HR assay, below. Plants were then covered with clear plastic bags for 1 day and incubated under fluorescent lights (16 hr/day) at 22°C for 5–7 days. Leaves were scored for necrosis and chlorosis.

Bacterial Population Assays. Cotoneaster shoot tips, 10 cm long, that had been inoculated with *E. amylovora* strains were homogenized in 5 mM KPO_4 buffer, pH 6.8, at 5 days postinoculation. Inoculated pear fruits were homogenized at 7 days postinoculation. Homogenates were plated in a dilution series on LB agar with antibiotics (rifampicin, 25 μg/ml; tetracyclin, 10 μg/ml; kanamycin, 50 μg/ml) as appropriate to determine bacterial populations. Triplicate shoots or fruits were assayed individually for each strain tested.

HR Assays. Tobacco leaf panels (*Nicotiana tabacum* L. 'xanthi') were infiltrated with bacterial cell suspensions as described previously (4, 30). Primary leaves of 2-week-old soybean seedlings (secondary leaves emerging) were infiltrated with bacterial cell suspensions as for tobacco. Plants were scored for HR (tissue collapse) after 24–48 hr on the laboratory bench. *E. amylovora* strains were suspended in 5 mM KPO_4 buffer, pH 6.8, and *P. syringae* strains in 10 mM $MgCl_2$.

GUS Assays. Cells were (*i*) grown in LB to an OD_{620} of 0.9–1.0; (*ii*) grown in LB to an OD_{620} of 0.5, then washed and resuspended in an *hrp*-gene-inducing minimal medium (Hrp MM; ref. 31) to an OD_{620} of 0.2 and incubated at 21°C for 36 hr to a final OD_{620} of 0.9–1.0; or (*iii*) grown in LB to an OD_{620} of 0.5, washed and concentrated 2-fold in 5 mM KPO_4 buffer, pH 6.8, and then transferred to freshly cut wells in pear halves and incubated as for the pathogenicity assay for 36 hr. Cells were assayed for β-glucuronidase (GUS) activity essentially according to Jefferson (32). For the cells in LB or Hrp MM, 50 μl were mixed with 200 μl GUS extraction buffer (50 mM $NaHPO_4$, pH 7.0/10 mM 2-mercaptoethanol/10 mM Na_2EDTA/0.1% sodium lauryl sarcosine/0.1% Triton X-100) containing 2 mM 4-methylumbelliferyl β-D-glucuronide as substrate and incubated at 37°C for 100 min. For cells in pear fruit, the tissue surrounding the well was excised by using a #4 cork borer and homogenized in 5 mM KPO_4 buffer, pH 6.8. Two hundred microliters of homogenate was mixed with 800 μl of GUS extraction buffer with substrate and incubated as above. Reactions were stopped by adding Na_2CO_3 to a final concentration of 0.2 M in a total volume of 2 ml. Fluorescence was measured by using a TKO 100 Mini-Fluorometer (Hoefer). For all samples, cell concentration was estimated by dilution plating, and fluorometric readings were converted to pmol of substrate hydrolyzed per 10^8 cfu/min, after Miller (33).

RESULTS

The "Disease-Specific" (*dsp*) Region of *E. amylovora* Consists of a 6.6-kb, Two-Gene Apparent Operon. Mapping of previous transposon insertions (ref. 34; C. H. Zumoff, D. W. Bauer, B. J. Sneath, Z.M.W., and S.V.B., unpublished data) that abolish pathogenicity but not HR-eliciting ability confirmed the presence of the "disease-specific" (*dsp*) region downstream of the *hrpN* gene in strain Ea321 as reported in strain CFBP1430 (19). The sequence of approximately 15 kb of DNA downstream of *hrpN* from Ea321 was determined, revealing several ORFs (Fig. 1). One large ORF was found that encompassed the region to which all our *dsp* insertions mapped. This ORF was present in an apparent 6.6-kb operon containing another, smaller ORF downstream. The two ORFs were designated *dspE* and *dspF*, and the operon, the *dspEF* locus. *dspE* is preceded (beginning 70 bp upstream of the initiation codon) by the sequence $GGAACCN_{15}CAACATAA$, which matches the HrpL-dependent promoter consensus sequence, or "*hrp* box" of *E. amylovora* (1, 3) and strongly resembles the *hrp* box of *P. syringae hrp* and *avr* genes (18). Immediately downstream of *dspF* is A/T-rich DNA, followed by an ORF highly similar to the *Salmonella typhimurium* gene *spvR*, a member of the *lysR* family of regulatory genes (35). Immediately upstream of the *dspEF* locus is an Hrp-regulated gene, *hrpW*, encoding a second harpin (5).

The deduced product of *dspE* contains 1,838-aa residues and is hydrophilic. The predicted molecular mass, 198 kDa, was confirmed by expression in *E. coli* (Fig. 2). Expression of an intermediate clone containing only the 5′ half of *dspE* yielded a protein of corresponding predicted mobility, suggesting that the N-terminal half of the protein might form an independently stable domain. DspF, predicted to be 16 kDa, acidic (pI, 4.45), and predominantly α-helical, with amphipathic α-helices

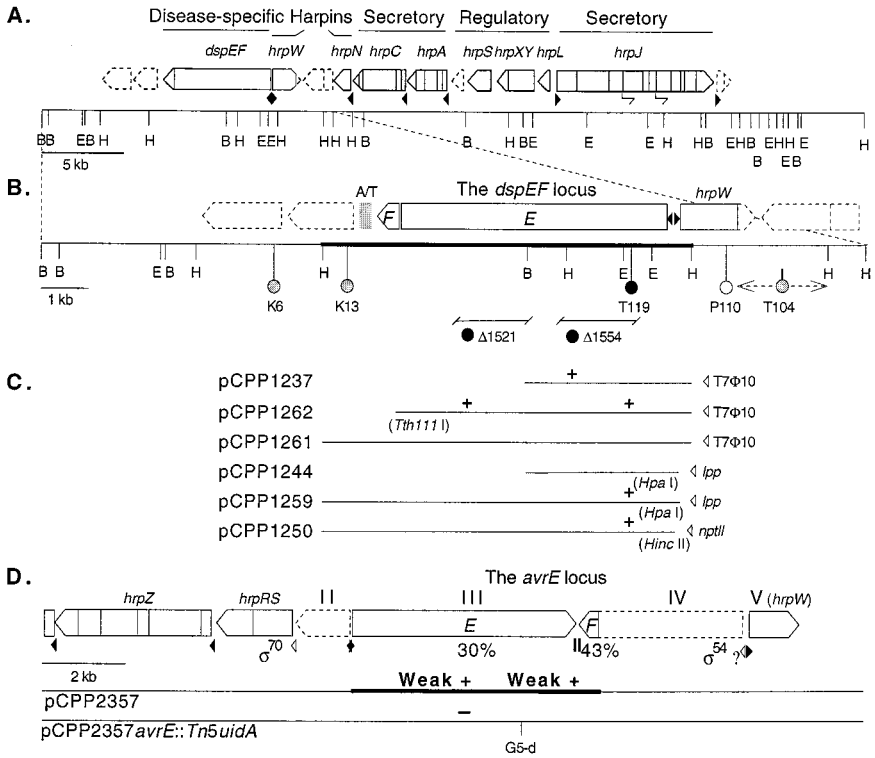

FIG. 1. The *dspEF* locus of *E. amylovora*: mutagenesis, complementation and heterologous expression constructs, and homology with and restoration of mutants by the *avrE* locus of *P. syringae*. Dashed boxes are uncharacterized ORFs; a solid triangle indicates an *hrp* box, and an open triangle indicates another promoter. Thick lines delineate the DNA for which sequence was accessioned. (*A*) The *dsp/hrp* gene cluster of *E. amylovora* in pCPP430. Operon names and types of proteins encoded are indicated at the top. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. Half-arrows indicate internal promoters without similarity to the *hrp* box consensus. (*B*) The region downstream of *hrpN* containing the *dspEF* locus. Circles mark deletion mutations and representative transposon insertions: black, nonpathogenic and HR^+ or HR-reduced (*dsp*); gray, reduced virulence and HR; white, wild type. T104 lies within the area marked by the dashed double arrow. K, Tn*5*miniKm; P, Tn*5phoA*; T, Tn*10*tet^r; Δ, deletion mutation. The gray box is A/T-rich DNA. (*C*) Clones and subclones of the *dspEF* locus. Plasmid designations are indicated at the left, and vector-borne promoters are indicated at the right. Restriction sites used for subcloning not shown above are shown in parentheses. A "+" aligned with a circle representing a mutation in *B* indicates that the subclone complements that mutation. (*D*) The *avrE* locus (transcription units III and IV) of *P. syringae* pv. tomato DC3000 in pCPP2357. Percent amino acid identity of the predicted proteins AvrE and AvrF to DspE and DspF, respectively, are indicated. Solid rectangles are transcriptional terminators (inverted repeats). Ability to restore mutations depicted in *B* are indicated, aligned as for complementation data in *C*.

in its C terminus, is physically similar to virulence factor chaperones of animal-pathogenic bacteria (36).

***dspE* Is Required for Fire Blight.** Two in-frame deletions within *dspE* (Fig. 1) were made in Ea321 and Ea273 (low- and high-virulence strains, respectively). The first (Δ1554) corresponds to amino acid residues G_{203} to G_{720} and the second (Δ1521) to amino acid residues T_{1064} to V_{1570}. Each deletion abolished the ability of both strains to generate fire blight symptoms (necrosis) and bacterial ooze when inoculated to immature pear fruit (Fig. 3). The mutants also failed to cause fire blight when inoculated to apple and cotoneaster shoots (not shown). Populations of *dsp* mutant strains isolated from cotoneaster shoots after 5 days were equivalent to that of a *hrpL* regulatory mutant strain ("K49"; ref. 1). The Δ1554 deletion mutants of Ea321 and Ea273 were restored to full virulence by pCPP1237, a clone carrying only the overlapping 5′ half of *dspE*, further suggesting that the N terminus of the protein forms a stable domain (Figs. 1 and 3).

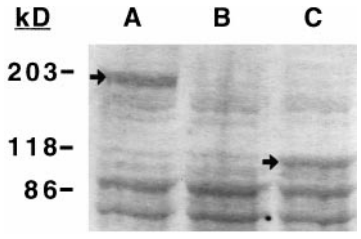

FIG. 2. Expression of the full-length and the N-terminal half of DspE in recombinant *E. coli* DH5α. Lysates of cells carrying either pCPP1259, containing the entire *dspEF* locus (lane A); pCPP50, the cloning vector (lane B); or pCPP1244, containing only the 5′ half of the *dspE* gene (lane C), were subjected to SDS/PAGE (7.5% acrylamide) followed by Coomassie staining. Bands corresponding to DspE (lane A) and the N-terminal half of DspE (lane C) are marked by arrows. Migration of molecular mass markers is indicated on the left.

The *dspEF* Locus Contributes Quantitatively and in a Strain-Dependent Fashion to HR Elicitation by *E. amylovora* in Tobacco and Is Not Required for HR Elicitation by *E. amylovora* in Soybean. Transposon insertions in the *dsp* region of *E. amylovora* strain Ea321 reduce the ability of this strain to elicit the HR in tobacco (data not shown). Dilution series of suspensions of *dspE*Δ1554 mutant strains of Ea321 and Ea273 were infiltrated into tobacco leaves alongside their wild-type parents to define precisely the role of *dspE* in HR elicitation (Fig. 3). All strains were capable of eliciting the HR, but Ea321*dspE*Δ1554, on a per-cell basis, was roughly one-tenth as effective as the wild type. pCPP1237 restored full HR-eliciting ability to this strain (not shown). There was no noticeable difference in HR-eliciting ability in tobacco between Ea273 and Ea273*dspE*Δ1554. Ea321*dspE*Δ1554, infiltrated at a standard concentration, elicited wild-type HR in Acme, Centennial, Harasoy, and Norchief soybean leaves (Fig. 3).

The *dspEF* Locus Is Hrp-Regulated. A promoterless *uidA* gene construct (D. W. Bauer) was cloned downstream of the

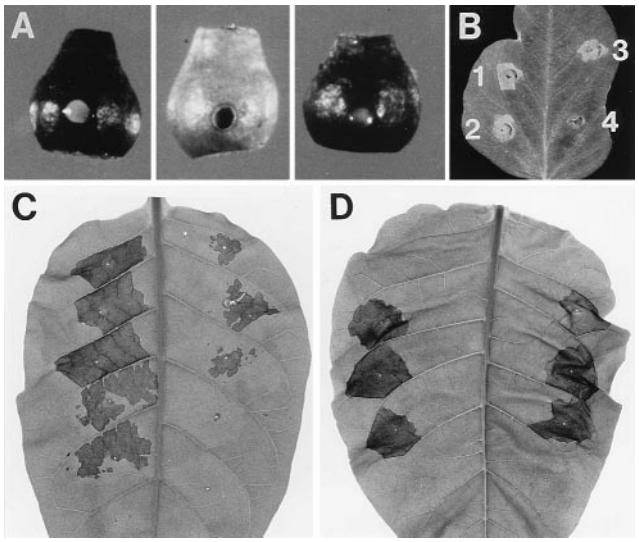

FIG. 3. The role of the *dspE* gene in pathogenicity and HR elicitation. (*A*) Immature pear fruit 4 days after inoculation with (left to right) strains Ea321, Ea321*dspE*Δ1554, or Ea321*dspE*Δ1554 harboring the 5′ half of *dspE* on pCPP1237. (*B*) Norchief soybean leaf 24 hr after infiltration with 5×10^8 cfu/ml suspensions of (1) Ea321, (2) Ea321*dspE*Δ1554, (3) Ea321*hrpN*::Tn*5* (ref. 4), and (4) Ea321*hrpL*::Tn*5* (ref. 1). (*C*) Tobacco leaf 48 hr after infiltration with parallel dilution series of suspensions of strains Ea321 (*Left*) and Ea321*dspE*Δ1554 (*Right*). The concentrations infiltrated (top to bottom) are 1×10^{10}, 1×10^9, 5×10^8, 1×10^8, and 5×10^7 cfu/ml. (*D*) As for *C*, except the more virulent strain, Ea273, and corresponding mutant Ea273*dspE*Δ1554 were used, and concentrations ranged from 5×10^9 to 5×10^5 cfu/ml in log increments.

dspE fragment in pCPP1241 that was used to introduce the Δ1554 mutation (Fig. 1) into wild-type strains of *E. amylovora* (this construct consists of a 3′-truncated *dspE* gene with the internal deletion). The resulting plasmid, pCPP1263, was mobilized into Ea321 and Ea273. Pathogenic strains, in which plasmid integration had preserved an intact copy of *dspE*, and nonpathogenic strains, in which the native copy of *dspE* had been mutated, were isolated. All strains were assayed for GUS activity in LB and in Hrp MM, and pathogenic strains were assayed for activity in pear fruit. High levels of activity were obtained from strains incubated in Hrp MM and pear, but not LB. The level of expression in Hrp MM was equivalent to that of a *hrcV-uidA* fusion ("G73"; ref. 1) used as a positive control. There were no significant differences in levels of expression of the *dspE-uidA* fusion in the wild-type and *dspE* mutant backgrounds (data not shown), indicating that *dspE* likely is not autoregulated. Expression of the *dspE-uidA* fusion in *hrpL* mutants of Ea321 and Ea273 in Hrp MM was two orders of magnitude lower than that in $HrpL^+$ strains. Data for Ea273 and derivatives are shown in Fig. 4.

***dspE* and *dspF* Are Homologous with Genes in the *avrE* Locus of *Pseudomonas syringae* pv. Tomato.** A BLAST (37) search of the genetic databases revealed similarity in the *dspE* gene to a partial sequence of the *avrE* locus of *P. syringae* pv. tomato (17). A cosmid library of *P. syringae* pv. tomato DC3000 genomic DNA was constructed, and a clone overlapping the *hrp* gene cluster and containing the *avrE* locus was isolated (pCPP2357). The complete nucleotide sequence of the *avrE* locus was determined, revealing homologs of *dspE* and *dspF* (Fig. 1). The *dspE* homolog, alone in an operon previously designated transcription unit III, encodes a 195-kDa, 1,795-aa protein 30% identical to DspE. The *dspF* homolog, at the end of the opposing operon previously designated transcription unit IV, encodes a 14-kDa, 129-aa protein 43% identical to DspF. We designate these genes *avrE* and *avrF*, respectively. The aligned C-terminal halves of DspE and AvrE (starting from V_{845} of DspE) show greater conservation (33% identity) than the N-terminal halves (26% identical). AvrE contains an ATP-/GTP-binding-site motif ("P-loop"; ref. 38) at residues A_{450} to T_{457} and a putative leucine zipper at residues L_{1772} to L_{1793}. These features are not present in DspE, however, and their functional significance in AvrE, if any, is unclear. Amino acid identities are distributed equally throughout the DspF and AvrF alignment, and AvrF shares the predicted physical characteristics of DspF. Upstream of *avrF*, completing the operon, is a 2.5-kb gene with no similarity to sequences in the genetic databases.

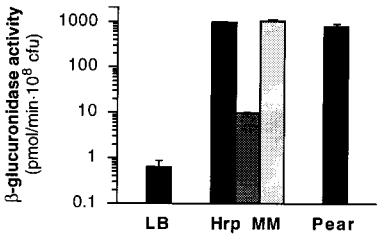

FIG. 4. Expression of a promoterless GUS construct fused to *dspE* in *E. amylovora* Ea273. Ea273 and Ea273*dspE*::*uidA* (a merodiploid containing both a wild-type *dspE* and a truncated *dspE* fused to the *uidA* gene; solid bars) were grown in LB or Hrp MM, or inoculated to immature pear fruit. Ea273*dspE*::*uidA*Δ*hrpL*::Tn*5* (darkly shaded bar) and Ea273*hrcV*::Tn*5uidA* (lightly shaded bar) were also grown in *hrp* MM. Values shown represent means of triplicate samples normalized for bacterial cell concentration. Standard deviations are represented by lines extending from each bar. The mean values for three samples of Ea273 in each assay were subtracted from, and standard deviations added to, the corresponding values obtained for the other strains.

The *dspEF* Locus Functions as an Avirulence Locus. The *dspEF* locus was cloned into pML122 (39) downstream of the *nptII* promoter, and this construct, pCPP1250, was mobilized into *P. syringae* pv. glycinea race 4 (gift of N. T. Keen, Univ. of California, Riverside). The resulting strain, but not a control strain containing pML122, elicited the HR in soybean cultivars Acme, Centennial, Harasoy, and Norchief; in Norchief plants incubated under conducive conditions, race 4 harboring pCPP1250 did not cause symptoms of disease, whereas the control strain caused necrosis and chlorosis that spread from the point of inoculation (Fig. 5).

The *avrE* Locus Restores Pathogenicity to *dspE* Mutants. Cosmids pCPP2357 (carrying the *avrE* locus) and pCPP2357*avrE*::Tn*5uidA* (Fig. 1) were mobilized into *dspE*Δ1521 mutants of Ea273 and Ea321, and the resulting transconjugants, and wild-type strains, were inoculated to immature pear fruit (Fig. 5). Ea273*dspE*Δ1521(pCPP2357) cells increased in number 10-fold over 7 days, generating ooze, water soaking, and slight necrosis in and immediately surrounding the sites of inoculation. Virulence was much lower than that of wild-type cells, which increased 5×10^3-fold, resulting in copious ooze and necrosis throughout the fruit. Ea273*dspE*Δ1521(pCPP2357*avrE*::Tn*5uidA*) cells did not increase in number and generated no symptoms, indicating that the observed restoration of pathogenicity was *avrE*-specific. Similar results were observed for transconjugants of Ea321*dspE*Δ1521 (Fig. 5) and Ea321*dspE*Δ1554 (not shown).

DISCUSSION

We characterized the *dspEF* locus of *E. amylovora* and discovered that it is homologous with the *avrE* locus of *P. syringae* pv. tomato and is essential for *E. amylovora* pathogenicity. In contrast, *avrE* plays only a quantitative role in virulence in *P. syringae* pv. tomato strain PT23 and is completely dispensable in strain DC3000 (the source of the clone used here; refs. 15 and 17). We found that the *dspEF* locus and the *avrE* locus function similarly and function transgenerically: like *avrE*, the

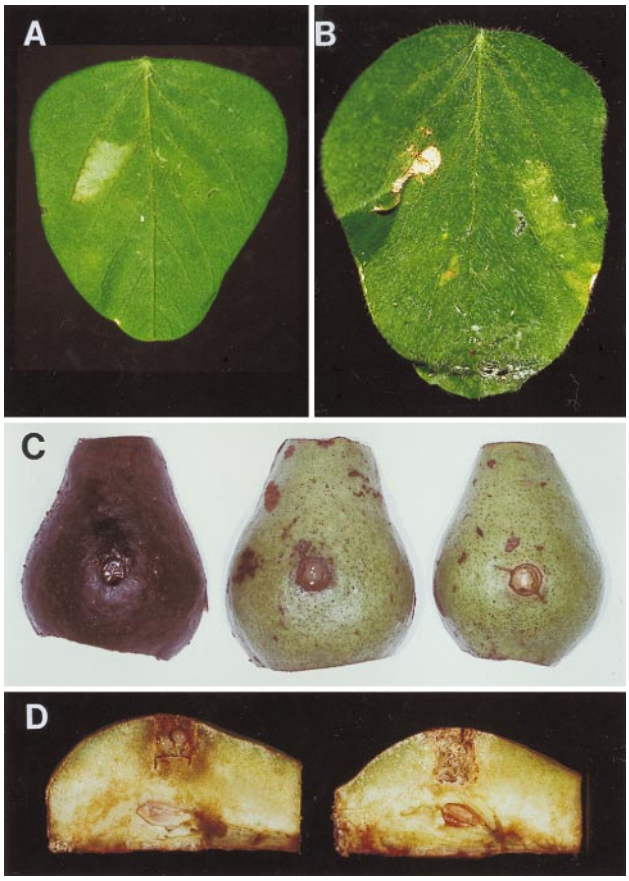

FIG. 5. Transgeneric avirulence function of the *dspEF* locus and restoration of *dspE* mutants with the *avrE* locus. Norchief soybean leaves were either (*A*) infiltrated with 1×10^8 cfu/ml suspensions of *P. syringae* pv. glycinea race 4 carrying pCPP1250 (containing the *dspEF* locus) (*Left*) or pML122 (the cloning vector) and photographed after 24 hr at room temperature (*Right*), or (*B*) infiltrated with 8×10^5 cfu/ml suspensions of the same strains and photographed after 7 days at 22°C and high relative humidity. Tissue collapse is apparent on both leaves where the strain carrying pCPP1250 was infiltrated. On the leaf incubated for 7 days, chlorosis extending beyond the infiltrated area, typical of disease, is apparent on the half infiltrated with the strain carrying the vector only. The dark section on the side of the leaf infiltrated with the strain carrying pCPP1250 is a shadow caused by a buckle in the leaf. Pear halves are shown (*C*) 10 days after inoculation with (left to right) Ea273, Ea273*dspE*Δ1521(pCPP2357, containing the *avrE* locus), or Ea273*dspE*Δ1521(pCPP2357*avrE*::Tn*5uidA*), and (*B*) cross-sectioned through the well 10 days after inoculation with Ea321*dspE*Δ1521(pCPP2357) (*Left*) and Ea321*dspE*Δ1521-(pCPP2357*avrE*::Tn*5uidA*) (*Right*). Although greatly reduced relative to wild type, water soaking and necrosis are apparent around and ooze can be seen within the wells of fruit inoculated with *dspE* strains carrying the intact *avrE* locus. Fruit inoculated with *dspE* strains carrying a disrupted clone of *avrE* is symptomless and shows no ooze.

dspEF locus confers avirulence when expressed in *P. syringae* pv. glycinea, and the *avrE* gene can partially substitute for the *dspE* gene in mutant strains of *E. amylovora*. Our findings provide a striking example of dual functionality for Avr-like effector proteins of plant pathogenic bacteria. Further, the data indicate that the relative contribution of homologous virulence/avirulence genes to disease depends on the genetic background in which they are expressed. Our results therefore suggest that many *avr* genes for which no virulence phenotype yet has been detected have functions that can promote infection.

How can differences in genetic background evolve that lead to such dramatic differences in the virulence phenotype of *avr* gene homologs in different bacteria? Alfano and Collmer (6) proposed a model in which coevolution of pathogen and host plant(s) favors proliferation and redundancy of virulence factors through modification of preexisting factors and acquisition of others from heterologous pathogens, while conserving the virulence-factor-delivery system (the Hrp secretion system). According to this model, the more coevolved a pathogen with its host(s), the less likely is any single virulence factor to be critical for pathogenicity. The phenotypic difference between a *dspE* mutation and an *avrE* mutation may result from and reflect a difference in the extent or nature of the coevolution with plant hosts experienced by *E. amylovora* and by *P. syringae*. Evolution of corresponding *R* genes and modification of targets of pathogen virulence factors (that would lead to modification, substitution, and redundancy of the factors) are likely to have occurred more over time in the numerous herbaceous hosts typically infected by *P. syringae* pathovars than in the relatively fewer and more slowly reproducing woody hosts with which *E. amylovora* presumably evolved. Alternatively or additionally, *E. amylovora* may have acquired the *dspEF* locus and the linked *hrp* gene cluster more recently than *P. syringae* acquired the *hrp*-linked *avrE* locus, allowing less time for coevolution leading to modification or the development of redundant function. In support of this idea, the harpin-encoding genes of these two bacteria show a phenotypic difference similar to that of *dspE* and *avrE*. *hrpN* mutants of *E. amylovora* are drastically reduced in virulence or are nonpathogenic (4, 40), whereas *hrpZ* mutants of *P. syringae* show little or no difference in disease-causing ability from the wild type (41). These results suggest that *E. amylovora* generally has evolved fewer redundant virulence functions than *P. syringae*.

Localization of the *dspE* and *dspF* gene products during the plant–bacterial interaction will be important, in light of the absolute requirement for the *dspEF* locus in pathogenicity. Several reports (reviewed in ref. 42) provide indirect yet compelling evidence that a number of Avr proteins are localized to the plant cell interior via the Hrp pathway in much the same way as virulence proteins of animal pathogenic bacteria are delivered into host cells (43). It remains to be determined whether the avirulence function of the *dspEF* locus depends on secretion through the Hrp pathway. This seems likely considering the physical similarity of DspF (and AvrF) to chaperones required for type III secretion of virulence factors from animal pathogenic bacteria (36).

The *dspEF* locus is the first-described avirulence locus in *E. amylovora*. We have also found a homolog of *avrRxv* from *Xanthomonas campestris* (44) near the *dspEF* locus (5). Monogenic (*R*-gene-mediated) resistance to fire blight has not been reported, but differential virulence of *E. amylovora* strains on apple cultivars has been observed (45). Also, some strains of *E. amylovora* infect *Rubus* spp. and not pomaceous plants, and vice versa (46). Whether DspE, the AvrRxv homolog, or other similar proteins play a role in these specificities awaits determination.

Although the *dspEF* locus triggers defense responses in soybean when expressed in *P. syringae* pv. glycinea, it is not required for the HR of soybean elicited by *E. amylovora*. Nor is *hrpN* required (Fig. 3). It is possible that *E. amylovora* must have either *dspE* or *hrpN* to elicit the HR in soybean. We have observed, however, that, in contrast to its effect on many other plant species (47), infiltrated harpin (HrpN) does not elicit the HR in soybean, suggesting the alternative explanation that *E. amylovora* harbors another *avr* gene recognized by this plant. A cell-free DspE and DspF preparation also failed to elicit the HR when infiltrated into soybean leaves, raising the possibility that one or both of these proteins trigger defense responses from within the plant cell (D. W. Bauer and S.V.B., unpublished data).

Recognition of *E. amylovora* avirulence signals in soybean suggests the presence of one or more specific *R* genes. A

dspEF-specific *R* gene might be useful for engineering apple and pear for resistance to fire blight. *R*-gene-mediated resistance to the apple scab pathogen *Venturia inaequalis* (48) and successful transformation of apple with attacin E for control of fire blight (49) attest the feasibility of such an approach. *R*-gene-mediated resistance to apple scab has been overcome in the field (50), but the requirement for the *dspEF* locus in disease favors relative durability of a corresponding *R* gene (12). Avirulence screening of *dspEF* and other *E. amylovora* genes in pathogens of genetically tractable plants such as *Arabidopsis* could broaden the pool of candidate *R* genes and hasten their isolation. A similar approach could be used to isolate *R* genes effective against other pathogens of woody plants. Furthermore, if the *dspEF* locus is as widely conserved as is suggested by its homology with the *avrE* locus, a corresponding *R* gene could be effective against a variety of pathogens both of woody and herbaceous plants.

Note Added in Proof. Recently, Gaudriault *et al*. (51) characterized the *dsp* locus of *Erwinia amylovora* strain 1430. They designated the genes corresponding to *dspE* and *dspF* as *dspA* and *dspB*, respectively.

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