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Erwinia amylovora Secretes DspE, a Pathogenicity Factor and Functional AvrE Homolog, through the Hrp (Type III Secretion) Pathway

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***Erwinia amylovora* was shown to secrete DspE, a pathogenicity factor of 198 kDa and a functional homolog of AvrE of *Pseudomonas syringae* pv. tomato. DspE was identified among the supernatant proteins isolated from cultures grown in an *hrp* gene-inducing minimal medium by immunodetection with a DspE-specific antiserum. Secretion required an intact Hrp pathway.**

Erwinia amylovora causes the disease of apple, pear, and other members of *Rosaceae* known as fire blight. The *dspE* gene (4, 11) ("*dspA*" in reference 11) ("*dsp*" stands for "disease-specific") of this gram-negative bacterium encodes an essential pathogenicity factor of 198 kDa and is homologous with *avrE*, one of at least two genes in the avirulence (*avr*) locus *avrE* of *Pseudomonas syringae* pv. tomato (4, 11, 21). Immediately downstream of *dspE* is *dspF* (4, 11) ("*dspB*" in reference 11), which encodes a protein physically similar to chaperones of virulence proteins of animal pathogenic bacteria such as *Yersinia* spp. and *Shigella flexneri* (32). *dspF* also has a homolog in the *avrE* locus, *avrF* (4). *dspE* and *dspF* together constitute the *dspEF* locus, which functions as the *avrE* locus does (19, 21), conferring avirulence (an inability to cause disease due to specific elicitation of plant defense responses) when heterologously expressed in the soybean pathogen *P. syringae* pv. glycinea (4).

Bacterial *avr* genes specifically limit host range in their native organism and when expressed in pathogenic bacteria different from the native organism (16). A corresponding resistance (*R*) gene in the plant is required for *avr* gene-dependent elicitation of plant defense responses. The avirulence phenomenon historically has been observed to affect interactions of pathovars of *Pseudomonas* and *Xanthomonas* spp. with cultivars of respective host plant species (10). *avr* genes have been observed also, however, to limit host range at the host species level (19, 36). In the absence of a corresponding plant *R* gene, some *avr* genes, including *avrE* (22), have been shown to play roles in virulence or pathogen fitness (10).

To function, *avr* genes typically require the Hrp pathway (12, 15, 18, 23), a specialized, type III secretion system conserved among phytopathogenic bacteria. The Hrp pathway components are encoded by *hrp* genes, required both for bacterial elicitation of the hypersensitive reaction (HR), a plant defense reaction, and for pathogenicity. Expression of *hrp* genes can be induced in vitro by growth in low-nutrient media (15, 35). Proteins known to traverse the Hrp pathway include harpins of *P. syringae* and *Erwinia* spp. and the harpin-like protein PopA of *Ralstonia solanacearum* (see reference 2 for a review of *hrp* genes and Hrp-secreted proteins). Harpins are glycine-rich proteins that, unlike Avr proteins, elicit the HR when introduced in solution into the apoplast (leaf intercellular space) (14, 34a). Purified harpins elicit the HR, and stimulate systemic resistance to a broad array of pathogens, in many plant species (27, 33). Genetic studies suggest an important role in virulence for harpins in several phytopathogenic bacteria (2, 14, 34a).

A number of virulence proteins of animal pathogenic bacteria such as *Yersinia* spp., *Shigella flexneri*, and *Salmonella typhimurium* are transported into host cells via type III pathways in a cell contact-dependent manner (8, 24, 26, 30). Recent evidence strongly suggests that Avr proteins similarly are translocated to the plant cell interior via the Hrp pathway (for further discussion, see reference 6). Four *avr* genes have been shown to elicit the hypersensitive response when expressed in plant cells (12, 20, 25, 28, 31). However, no natively expressed Avr protein has been reported outside of bacterial cells in culture or in planta; thus, direct evidence for Avr protein transit through the Hrp pathway has been lacking.

We report here that the product of the *dspE* gene, DspE, is secreted via the Hrp pathway by *Erwinia amylovora* cells grown in an *hrp* gene-inducing minimal medium (15).

Anti-DspE antiserum was raised in rabbit to a polypeptide corresponding to the N-terminal half of DspE (DspE′) as follows. DspE′ was generated by expression of a truncated clone of the *dspE* gene in *Escherichia coli* DH5α (plasmid pCPP1244; Table 1) as previously described (4). It was isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell lysate followed by electroelution in an Elutrap apparatus (Schleicher & Schuell, Nashua, N.H.) of the band corresponding to DspE′. Eluted protein was dialyzed against buffered saline (2.5 mM KPO_4, 0.85% NaCl [pH 6.8]) and concentrated with Centricon ultrafiltration devices (Amicon Inc., Beverly, Mass.). Aliquots of 0.5 ml containing between 0.2 and 0.5 mg of protein were mixed with Freund's incomplete adjuvant and injected into a female chinchilla rabbit, one aliquot each at 0, 4, and 6 weeks. Antiserum was harvested at 8 weeks and cross-adsorbed with boiled sonicates of *E. coli* DH5α cells carrying the expression vector (pCPP50; Table 1) and with trichloroacetic acid-precipitated supernatant proteins and boiled cell sonicates of *hrp* gene-induced *dspE* mutant cells derived from *Erwinia amylovora* Ea273 (4). The titer and specificity of the resulting antiserum preparation was ascertained by Western blots of cell lysates of *E. coli* DH5α (pCPP1244) and *E. coli* DH5α(pCPP50). The antiserum bound

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Source or reference
Strains		
E. coli DH5α	F^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(r_K^- r_M^+) *deoR thi-1 supE44* λ^-*gyrA96 relA1*	Life Technologies, Inc. (Gaithersburg, Md.)
Erwinia amylovora		
Ea273	Wild type	S. V. Beer
Ea273-K178	Hrp secretion mutant; Tn*10* mini-Km insertion in *hrpJ* operon	Z.-M. Wei (34)
Ea273*dspE*Δ1521	Nonpolar *dspE* mutant; 1,521-bp in-frame deletion in 3′ half of *dspE*	4
Plasmids		
pCPP50	pINIII^{113}-A2-based expression vector; Ap^r	3
pCPP1244	3.1-kb *Hpa*I-*Bam*HI clone containing 5′ half of *dspE* in pCPP50	4
pCPP1259	7.7-kb *Hpa*I-*Hin*dIII clone containing *dspE* and *dspF* in pCPP50	4

specifically to the band corresponding to DspE′ in lysates of *E. coli* DH5α(pCPP1244) and bound quantitatively at dilutions (of antiserum) up to 1:10,000 (data not shown).

Erwinia amylovora Ea273, Ea 273-K178 (an *hrp* secretion mutant), and Ea273*dspE*Δ1521 (Table 1) were grown overnight at 28°C in Terrific Broth (29). For each strain, cells were harvested by centrifugation and resuspended to an optical density at 620 nm (OD_{620}) of 0.3 in 15 ml of an *hrp* gene-inducing minimal medium (15) and grown at 20°C with shaking for 24 h, reaching an OD_{620} of ca. 1.1. Cells were harvested by centrifugation and resuspended in 0.125 volume of high-purity water. Seventy-five microliters of the cell suspension was combined with 25 μl of 4× NuPAGE SDS sample buffer (NOVEX, San Diego, Calif.) and heated to 100°C for 4 min. Culture supernatants were filter sterilized, followed by addition of phenylmethylsulfonyl fluoride (PMSF) (a serine protease inhibitor) and EDTA to 0.2 and 25 mM, respectively. Supernatants were concentrated 230-fold by using Centriprep and Centricon ultrafiltration devices. One-quarter volume of 4× NuPAGE SDS sample buffer was added to the concentrated supernatants, and the samples were heated to 100°C for 4 min. Proteins were separated by SDS-PAGE in a 4 to 12% gradient NuPAGE polyacrylamide gel by using 1× NuPAGE morpholinepropanesulfonic acid running buffer according to the manufacturer's instructions. Transfer of the proteins to the Immobilon-P membrane (Millipore, Bedford, Mass.) was carried out by using 1× NuPAGE transfer buffer in a Bio-Rad Trans-Blot semidry transfer cell (Bio-Rad Laboratories, Hercules, Calif.), and immunodetection of blotted proteins was performed by using the Western-Star immunodetection system (Tropix, Bedford, Mass.) and anti-DspE antiserum.

DspE was evident in culture supernatants of Ea273 and was not detected in culture supernatants of the Hrp pathway mutant strain Ea273-K178 (Fig. 1). Supernatant fractions were approximately 30-fold more concentrated than cell fractions based on culture volume. Nevertheless, DspE was evident in cells of Ea273-K178 but was not detected in cells of Ea273. Thus, the absence of DspE from the supernatant of Ea273-K178 was not due to a failure of the cells to produce the protein, and possible cell lysis during culture growth could not be the source of DspE detected in the culture supernatant of Ea273. Ea273*dspE*Δ1521 cells encode a DspE derivative of 145 kDa with an internal deletion corresponding to residues T_{1064} to V_{1570}. This protein was present in the culture supernatant of this strain, suggesting that the missing residues are not required for secretion of DspE. Several faint bands of lower molecular weight were present in cell fractions. It is not clear whether these represent degradation products of DspE (and DspE′) or are due to residual cross-reactivity of the anti-DspE antiserum.

Our results contrast with results for other Avr proteins, which show detectable amounts only within the bacterial cell (7, 18, 37). Linked expression of DspE with the putative chaperone DspF (11) also sets apart DspE from characterized Avr proteins (other than AvrE). The question arises whether DspE and AvrE (and perhaps similar proteins in *Pseudomonas* and *Xanthomonas* spp.) are specifically released into the external medium, as are harpins, or Hrp pathway transit is less tightly regulated in *Erwinia amylovora*, in general, than in *Pseudomonas* and *Xanthomonas* spp. such that it can occur without the presumed requirement for contact with plant cells. Subtle differences between *Erwinia amylovora* and *P. syringae* in regulation of the Hrp secretion process are suggested by the observations that overexpression of harpin (HrpZ) in *P. syringae* shuts down Hrp secretion (1) yet overexpression of harpin (HrpN) in *Erwinia amylovora* does not (3a). Preliminary evidence suggests that, in addition to two harpins and DspE, *Erwinia amylovora* secretes several other proteins into the extracellular space via the Hrp pathway (17, 17a). If *Erwinia amylovora* indeed is unique among phytopathogenic bacteria in secreting through the Hrp pathway proteins such as DspE in

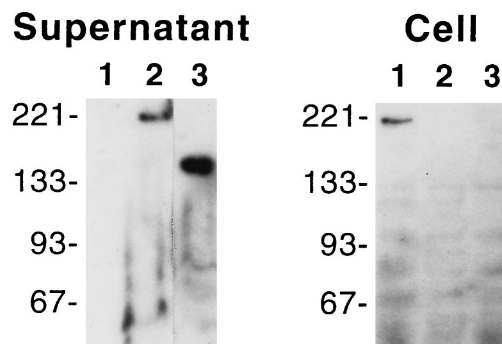

FIG. 1. Immunodetection of DspE in a Western blot of culture supernatant and cell fractions. Cultures of the *Erwinia amylovora* Hrp pathway mutant strain Ea273-K178 (lanes 1), the wild-type strain Ea273 (lanes 2), and a partial *dspE* deletion mutant strain, Ea273*dspE*Δ1521 (lanes 3), were grown under *hrp* gene-inducing conditions. Molecular weight markers (BenchMark prestained protein ladder; Life Technologies, Gaithersburg, Md.) are indicated. Migration of proteins from the culture supernatant was slightly retarded due to the presence of the extracellular polysaccharide of *Erwinia amylovora.*

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vitro, these proteins could be characterized by N-terminal sequencing. A reverse-genetic approach then should yield novel genes whose products are involved in interactions with plants.

Secretion of DspE in culture suggests that, in planta, the protein may be released into the apoplast, possibly indicating a plant extracellular or cell-surface-associated target in apple, pear, and other host plants. However, secretion of DspE in culture does not necessarily preclude Hrp-mediated translocation of the protein to the plant cell interior during an attempted colonization by *Erwinia amylovora*. Virulence proteins of animal pathogenic bacteria that are transported into host cells also are secreted into the extracellular space under certain culture conditions (8, 9); one of these, in fact, was discovered to be structurally related to AvrRxv of *Xanthomonas campestris* pv. vesicatoria (13). We determined whether a cell-free preparation of DspE and DspF would elicit the HR when introduced into soybean leaves. *E. coli* DH5α containing plasmid pCPP1259 for DspE and DspF expression (Table 1) and, as a control, *E. coli* DH5α carrying the expression vector pCPP50 (Table 1) were grown to an OD_{620} of 0.5 in Luria-Bertani medium. Isopropylthio-β-D-galactoside was added to 0.1 mM to induce expression, and cells were further incubated until reaching an OD_{620} of 1.2. Cells were then harvested by centrifugation and lysed by sonication in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.8)–0.1 mM PMSF. Following the removal of cellular debris by centrifugation, sonicates were infiltrated into the primary leaves of soybean plants (cultivar Harosoy) at full strength and dilutions ranging from 1:2 to 1:5. Despite the presence of large amounts of DspE and DspF in the sonicates (as ascertained by SDS-PAGE and staining with Coomassie blue), the HR did not occur (data not shown). Although the possibility exists that HR elicitation was blocked by interaction of DspE with the putative chaperone DspF, the data suggest that the target, or receptor, of DspE in soybean may be inside the plant cell. Determining the location of DspE (and DspF) in planta will be an important step toward understanding both the virulence and avirulence functions of the *dspEF* locus.

While an earlier version of this paper was under review, Gaudriault et al. (11) reported an independent characterization of the *dsp* locus of *Erwinia amylovora*. They reported also that when wild-type *Erwinia amylovora* cells were grown on a solid minimal medium and then washed from the plates and subsequently removed from the wash liquid by centrifugation, the liquid contained a protein corresponding in molecular weight to DspE (DspA). The protein was absent from wash liquids of both a *dspA* mutant and an Hrp pathway mutant. Although not definitive, their data provide the first published indication that DspE (DspA) is an Hrp-secreted protein. The authors also observed that the protein was absent from the wash supernatant of a *dspF* (*dspB*) mutant, bolstering the prediction that DspF serves as a molecular chaperone to DspE.

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