Functional Analysis of the N Terminus of the *Erwinia amylovora* Secreted Effector DspA/E Reveals Features Required for Secretion, Translocation, and Binding to the Chaperone DspB/F

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Submitted 14 April 2009. Accepted 28 May 2009.

DspA/E is a type III secreted effector protein required for pathogenicity in the apple and pear pathogen *Erwinia amylovora*, and DspB/F is a small chaperone protein involved in DspA/E secretion. While the secretion and translocation signals of many type III secretion effector proteins in human enteric pathogens have been characterized extensively, relatively little is known about the translocation requirements of many effectors in plant pathogens, including large DspE-like proteins. In this study, we report a functional analysis of the N terminus of DspE. The minimal requirements for secretion, translocation, and chaperone binding were characterized. Translocation assays using an adenylate cyclase (CyaA) reporter indicated that the first 51 amino acids of DspE were sufficient for translocation and that 150 amino acids were required for optimal translocation levels. The minimal translocation signal corresponded with the requirements for secretion into culture media. Mutations of conserved regions in amino acids 2 through 10 and 31 through 40 were found to influence translocation levels of an N-terminal DspE-CyaA fusion. Yeast two-hybrid and in-vitro pull-down assays revealed a chaperone-binding site within amino acids 51 through 100 of DspE and binding to DspF in this region was disrupted by specific mutations. However, neither disruption of the chaperone-binding domain nor deletion of the *dspF* gene had a significant impact on translocation levels of N-terminal DspE-CyaA fusions. Our results indicate that the minimal translocation signal of DspE is not coincident with the signal for DspF binding and that translocation of the N terminus of DspE is not dependent on the N-terminal DspF-binding domain.

Type III secretion systems (T3SS) are syringe-like structures central to the infection strategy of many bacterial pathogens and symbionts. Through these systems, effector proteins are injected into host cells and then function to suppress innate defenses or induce infection or both. Although the N-terminal 15 amino acids is sufficient for the secretion of many effectors into the external medium, this minimal portion is often insufficient to elicit measurable effector translocation into host cells,

and a longer downstream translocation signal is required (Schesser et al. 1996; Sory et al. 1995). The translocation signal often coincides with a domain within the N-terminal 200 amino acids that binds to a type III secretion chaperone (Ehrbar et al. 2006; Ghosh et al. 2004; Lee and Galan 2004; Woestyn et al. 1996).

Type III secretion chaperones are small proteins that facilitate secretion of effector, translocator, and secretion apparatus proteins through the T3SS. These chaperones have been divided into several classes; of these, class I chaperones are small structurally conserved dimers that interact specifically with T3SS effector proteins, while class II and III chaperones interact with the translocator and secretion apparatus, respectively (Parsot et al. 2003). Chaperones typically bind to a single site within residues 30 through 150 of the cognate effector protein (Lilic et al. 2006). CocrySTALLIZATION studies have provided a model of the chaperone-effector interaction in which the chaperone-binding domain of the effector wraps around the exterior of the chaperone dimer, interacting with a helix-binding groove and hydrophobic surfaces on the chaperone (Birtalan et al. 2002; Stebbins and Galan 2001). In many cases, the chaperone-binding domain coincides with or is adjacent to a destabilizing, aggregation-prone, or otherwise secretion-inhibitory domain masked by chaperone binding (Boyd et al. 2000; Feldman et al. 2002; Letzelter et al. 2006). In addition to stability, chaperone binding can confer secretion system specificity to a cognate effector or modulate the order in which effectors are translocated into host cells (Birtalan et al. 2002; Buttner and Bonas 2006; Ehrbar et al. 2006). Interaction with a chaperone is necessary for substantial levels of protein translocation of many T3SS effectors (Feldman and Cornelis 2003), although chaperone-independent translocation of some effectors can be detected (Cheng et al. 1997; Elliot et al. 1999; Thomas et al. 2005).

In the past decade, it has become increasingly clear that class I T3SS chaperones have an important role in plant pathogenesis. T3SS chaperones in plant-pathogenic bacteria, like their counterparts in animal pathogens, exhibit a conserved three-helix architecture and bind directly to cognate effector proteins to confer stability and efficient secretion (Buttner and Bonas, 2006). Despite these similarities, studies focusing on the role of the effector-chaperone interaction in plant pathogenesis have yielded several novel finds. For example, while class I T3SS chaperones were previously thought to universally bind to the N terminus of their cognate effector, the *Pseudomonas syringae* effector HopO1-1 was reported to have a

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chaperone-binding site in the middle third of the protein (Guo et al. 2005). Second, while the chaperone-binding domain itself is a destabilization domain for several *Yersinia*-secreted effectors, the chaperones of *P. syringae* effectors were shown to prevent proteolytic cleavage targeted to a site outside the chaperone-binding domain (Losada et al. 2002). Third, studies of secretion signaling in plant pathogens have provided several new examples of broad specificity chaperones that facilitate the secretion of multiple partners (Buttner et al. 2004; Guo et al. 2005). Thus, additional examinations of chaperone-effector interactions in plant pathogens could greatly further our understanding of chaperone-effector specificity and secretion-system targeting.

Erwinia amylovora, the causal agent of fire blight disease of apple and pear, secretes at least four effector-like proteins in a T3SS-dependent manner (Nissinen et al. 2007). Of these, only the effector called DspA or DspE (henceforth DspE) is known to be required for pathogenicity (Asselin et al. 2008; Bogdanove et al. 1998; Gaudriault et al. 1997; Zhao et al. 2006). At 189 kDa, DspE is an unusually large secreted effector protein that interacts with at least four apple proteins (Meng et al. 2006). The functional role of DspE in apple pathogenesis is unknown, although it has been shown to suppress salicylic acid-mediated host defenses and cause necrotic cell death in host and nonhost plants (Boreau et al. 2005; Debroy et al. 2004). DspE binds directly to a class I T3SS chaperone protein, DspF (Gaudriault et al. 1997), which is also apparently required for efficient intrabacterial DspE production or stability of DspE, although it is not thought to affect transcription levels (Gaudriault et al. 2002). Likewise, the related chaperone protein WtsF from *Pantoea stewartii* subsp. *stewartii* is involved in the temperature-dependent stability and secretion of WtsE, a DspE homolog (Ham et al. 2006). Thus, initial analyses of DspF-like chaperones have been performed; however, to our knowledge, a detailed analysis of the chaperone-effector interaction and of sequences in the effector important to chaperone binding have not been conducted.

We hypothesized that sequence features in the N terminus of DspE would affect the binding of DspF, with the caveat that additional sequences independent of DspF binding could also affect the translocation of DspE. In this study, we used a combination of secretion and translocation assays, virulence studies, and protein-protein interaction studies to characterize the minimal and optimal requirements for secretion, translocation, and chaperone binding in the N terminus of DspE. We demonstrate that the N terminus of DspE interacts with DspF and identify specific sequences that contribute to this interaction. Finally, we show that site-directed and deletion mutations of DspE that abolish DspF binding to the N terminus do not abolish the translocation of N-terminal DspE reporter fusions into host cells.

RESULTS

Creation and assessment of a *dspF* deletion mutant in *E. amylovora* Ea1189.

Gaudriault and associates (2002) reported that a *dspF* mutant of *E. amylovora* was weakly pathogenic on pear seedlings. To confirm these results and to determine whether a *dspF* mutant also caused symptoms on immature pear fruits, *E. amylovora* knockout strains Ea1189 Δ *dspE* and Ea1189 Δ *dspF* were constructed as described below. Ea1189 Δ *dspF* was complemented with the plasmid pLRT198, a construct containing the *dspF* gene and its promoter sequence. In a virulence assay on immature pear, Ea1189 Δ *dspE* caused no symptoms (data not shown), while Ea1189 Δ *dspF* caused symptoms that were dramatically reduced compared with that of the wild-type strain

(Fig. 1A). The virulence phenotype of Ea1189 Δ *dspF* was complemented by pLRT198. When infiltrated into leaves of *Nicotiana tabacum* cv. *Samsun*, wild-type Ea1189 caused complete hypersensitive response-like cell collapse within 24 h. Ea1189 Δ *dspE* and Ea1189 Δ *dspF* caused little to no necrosis, while Ea1189 Δ *dspF*(pLRT198) caused near wild-type levels of cell collapse (data not shown). Within 48 h, Ea1189 Δ *dspE* and Ea1189 Δ *dspF* caused a cell collapse, but this did not appear as severe as that caused by wild-type Ea1189 (Fig. 1B). Taken together, these results indicate that while DspE is required for *E. amylovora* pathogenicity on pear, its chaperone DspF is not. However, Ea1189 Δ *dspF* causes greatly diminished symptoms on pear and elicits a response on tobacco visually identical to that of Ea1189 Δ *dspE*.

Kinetics of DspE₍₁₋₂₀₃₎-CyaA (adenylate cyclase) translocation into tobacco leaves.

Bocsanczy and associates (2008) recently demonstrated that the N-terminal 737 amino acids of DspE confer translocation of the reporter CyaA into tobacco leaf cells from wild-type *E. amylovora* and that translocation levels increase steadily between 2 and 10 h postinfiltration. CyaA levels cannot be measured from tobacco infiltrated with wild-type *E. amylovora* after 12 h, due to rapid cell collapse. To determine the time required for maximum levels of DspE translocation into tobacco cells and to determine the kinetics of effector translocation into

Fig. 1. Pathogenicity and cell-death phenotypes of *Erwinia amylovora* Ea1189, Ea1189 Δ *dspF*, and Ea1189 Δ *dspF* (pLRT198). **A**, Symptom development on immature pears 5 days after inoculation with 3×10^3 CFU of three *E. amylovora* strains. **B**, Tobacco leaf cell death 48 h after infiltration with suspensions of Ea1189 (1), Ea1189 Δ *dspE* (2), Ea1189 Δ *dspF* (3), and Ea1189 Δ *dspF* (pLRT198) (4).

tobacco over a more extended period of time, we examined translocation levels of a DspE-CyaA fusion from Ea1189 Δ dspE. Ea1189 Δ dspE causes a delayed and greatly reduced cell collapse, allowing an extended period of time in which translocation assays can be performed. A fusion of DspE residues 1 through 203 to the CyaA reporter fragment was constructed and introduced into Ea1189 Δ dspE. The resulting strain was infiltrated into tobacco leaves, and cAMP accumulation in the leaves was measured at various timepoints. To control for the possibility that bacterial infiltration itself causes an increase in cAMP levels, we also measured cAMP accumulation elicited by Ea1189 Δ dspE carrying the parent vector pMJH20. cAMP accumulation of DspE(1-203)-CyaA expressed by Ea1189 Δ T3SS was measured to confirm that DspE translocation is dependent on the T3SS. Timepoints were optimized by preliminary studies indicating that a significant increase in cAMP levels was first detectable between 2 and 8 h postinfiltration with Ea1189 Δ expressing DspE(1-203)-CyaA (data not shown). cAMP levels increased significantly between 0 and 8 h, increased to a greater degree between 7 and 11 h, and remained steady between 11 and 24 (Fig. 2). cAMP levels did not increase in leaves infiltrated with the pMJH20 or type III secretion-deficient controls. These results show that, in the absence of cell collapse, *E. amylovora* requires up to 11 h to reach maximum levels of effector translocation. cAMP levels can fluctuate transiently in plant cells (Jiang et al. 2005; Kurosaki and Nishi 1993), so the persistence of high cAMP levels after 24 h suggests that *E. amylovora* maintains maximum effector expression and translocation levels for at least a day in a nonhost system. Based on these results, leaf samples in all subsequent studies were collected at 16 h postinfiltration.

Mapping of the secretion and translocation signals of DspE.

The minimal secretion signal of most model type III effector proteins is shorter than the minimal sequence required for translocation into host cells, and this translocation signal often coincides with the chaperone-binding domain (Cheng et al. 1997). The N-terminal 15, 33, 51, 77, 101, 150, 203, and 737 amino acids of DspE were fused to the CyaA reporter protein to map the minimal secretion and translocation signal of DspE. Fusion constructs were introduced into Ea1189 Δ dspE, and stable expression in Hrp-inducing minimal medium was confirmed by Western blot (Fig. 3A).

To determine the minimal signal for secretion of DspE, secretion of the shortest three fusion proteins from Ea1189 Δ dspE was assayed by Western blot of supernatants. While DspE₍₁₋₅₁₎-CyaA was strongly detected in the *E. amylovora* supernatant

fraction, secretion of DspE₍₁₋₁₅₎-CyaA and DspE₍₁₋₃₃₎-CyaA could only be detected very faintly (Fig. 3B). These results suggest that while the N-terminal 15 amino acids of DspE are sufficient to allow residual levels of secretion, 51 amino acids are required for substantial levels of secretion into culture medium. Fusions of longer DspE fragments were secreted at levels similar to that of DspE₍₁₋₅₁₎-CyaA (data not shown).

Translocation levels of the CyaA fusions were determined by assaying for cAMP accumulation in leaf disks. DspE₍₁₋₁₅₎-CyaA and DspE₍₁₋₃₃₎-CyaA did not cause an increase in cAMP levels in tobacco compared with the vector control, but fragments of 51 amino acids or longer caused an increase in cAMP levels that were significantly different from the pMJH20 control (Fig. 3C). These results indicate that the minimal signal for translocation is within the first 51 amino acids but not the first 33 amino acids of DspE. Fragments of 150 and 737 amino acids caused significantly greater levels of cAMP accumulation in plant cells than the first 51 amino acids, indicating that 51 amino acids are not sufficient for optimal translocation. Instead, optimal levels of translocation occurred with fragments of 151 amino acids or greater even though, for unknown reasons, the DspE₍₁₋₁₀₁₎-CyaA fragment was translocated at lower levels than DspE₍₁₋₅₁₎-CyaA. The experiments were repeated three additional times with consistent results.

Mutational analysis of conserved segments of the N-terminal 50 amino acids of DspE.

To identify features that might be important for translocation within the first 50 residues of DspE, the N-terminal fragment of DspE was aligned with that of homologs from related *Erwinia* species as well as *Pantoea agglomerans* pv. *gypsophylae*, *Pantoea stewartii*, and *Pseudomonas syringae* B728a (Fig. 4A). While the N-terminal 50 amino acids of these proteins did not share a high degree of conservation, there were two highly conserved stretches within residues 1 through 11 and 28 through 40. To determine whether specific residues within the translocation signal of *E. amylovora* might play an important role as part of the translocation signal, we used site-directed ligase independent mutagenesis (SLIM) to replace amino acids 2 through 10, 11 through 20, and 31 through 40 with stretches of consecutive alanine residues on the plasmid pLRT14, yielding plasmids pLRT113, pLRT114, and pLRT116. Alanine stretch replacement is a common strategy to assess the functional role of a sequence without imposing extreme steric constraints (Lefevre et al. 1997). Due to the nature of the SLIM technique, the number of amino acids varied by one or two; residues 2 through 10 and 11 through 20 were replaced with

Fig. 2. Kinetics of cAMP accumulation after infiltration into tobacco leaves of Ea1189 Δ dspE + DspE₍₁₋₂₀₃₎-CyaA (▲), Ea1189 Δ T3SS + DspE₍₁₋₂₀₃₎-CyaA (●), and Ea1189 Δ dspE + pMJH20 (■). Samples were collected at 0, 8, 11, and 24 h. Datapoints represent the mean and standard error of three replicate samples.

ten alanines, while 31 through 40 were replaced with eight alanine residues. Alanine-replacement mutants were subcloned into pLRT5, a full-length, high-copy clone of *dspE*, to create plasmids pLRT137, pLRT138, and pLRT140. These constructs were tested for complementation of the virulence phenotype of Eal189Δ*dspE* on immature pear. While *dspE*_(2-10A) did not complement Eal189Δ*dspE* on immature pear after four days, *dspE*_(11-20A) and *dspE*_(31-40A) complemented the mutant to levels similar to that of the parent vector (Fig. 4B), indicating that only residues 2 through 10 were required for translocation. To measure translocation levels of the site-directed mutants, DspE_(1-203; 2-10=10A)-CyaA, DspE_(1-203; 11-20=10A)-CyaA, and DspE_(1-203; 31-40=8A)-CyaA were amplified and recloned into the CyaA fusion vector pMJH20. Constructs were introduced into Eal189Δ*dspE*, expression was confirmed, and levels of translocation into tobacco were assayed. Translocation levels of DspE_(1-203; 11-20=10A)-CyaA were not significantly different than those of DspE₍₁₋₂₀₃₎-CyaA, but DspE_(1-203; 31-40=8A)-CyaA

was consistently translocated at levels significantly lower than the wild-type. DspE_(1-203; 2-10=10A)-CyaA was translocated at greatly reduced levels, although cAMP levels were significantly higher than the vector control (Fig. 4C). We did not obtain an alanine-replacement mutant of the conserved sequences between residues 41 and 50, but deletion of residues 35 through 125 from DspE₍₁₋₂₀₃₎-CyaA did not have a significant impact on translocation levels (data not shown), indicating that these residues are not important for protein translocation. Together, these results suggest that the first 10 amino-acid residues of DspE are required for translocation into host cells and pathogenicity and that other portions of the translocation signal, while tolerant to mutagenesis, could have a small but additive effect on translocation levels. The fact that decreases in translocation levels did not correspond to a decrease in virulence suggests that small reductions in DspE translocation do not adversely affect virulence levels.

DspF binds to DspE between amino acids 51 through 100.

Affinity-blot experiments have previously detected a direct interaction between DspE and DspF (Gaudriault et al. 2002). To date, almost all reported T3SS chaperones have interacted with cognate effector proteins via a single chaperone-binding domain near the N terminus (Parsot et al. 2003). However, recent studies showed that some chaperones of *Pseudomonas* effectors may bind to the middle third of an effector protein (Guo et al. 2005). Here, a yeast two-hybrid (Y2H) approach was used to identify the portion of the N terminus of DspE that interacts with the secretion chaperone DspF. N-terminal DspE fragments of varying lengths were fused to the LexA DNA binding domain and tested for interaction with a B42- hemagglutinin (HA)-DspF fusion in yeast as described below. The N-terminal 800, 600, 400, 200, 150, and 100 amino acids of DspE all interacted strongly with B42-HA-DspF, but the N-terminal 50 amino acids did not (Fig. 5A). Further analyses showed that residues 51 through 100 of DspE were sufficient for strong interaction with DspF, while deletion of residues 51 through 100 from LexA-DspE₍₁₋₁₅₀₎ abolished the interaction with DspF (Fig. 5A). Constructs expressing LexA-DspE₍₂₅₋₇₅₎ and LexA-DspE₍₇₅₋₁₂₅₎ interacted very weakly with B42-HA-DspF (data not shown), suggesting that the chaperone-binding domain is located squarely within residues 51 through 100 of DspE. Deletion of residues 51 through 100 from LexA - DspE₍₁₋₈₀₀₎ reduced the strength of the interaction with DspF but did not abolish the interaction. Further analysis mapped additional DspF Y2H interaction sites to residues 151 through 200, 401 through 450, 651 through 700, and 1,000 through 1,200 of DspE (data not shown). Because these secondary candidate chaperone-binding sites interacted weakly with DspF and because the N-terminal binding site in residues 51 through 100 corresponds to the chaperone-binding sites of the majority of characterized type III effector proteins, this study is focused on characterization of DspE residues 51 through 100 as a probable requirement for DspF chaperone function.

To confirm the DspE-DspF interaction in vitro, a glutathione S-transferase (GST) pull-down assay was performed. A GST-DspF fusion immobilized on glutathione agarose was tested for the ability to pull down DspE₍₁₋₁₅₀₎-CyaA and DspE_(1-150Δ51-100)-CyaA. DspE₍₁₋₁₅₀₎-CyaA was pulled down by GST-DspF in vitro but DspE_(1-150Δ51-100)-CyaA was not (Fig. 5B). This supports the Y2H evidence that residues 51 to 100 are required for interaction of the DspE N terminus with DspF.

DspE residues 51 through 60 and 71 through 85 affect the Y2H interaction between DspE₍₁₋₁₅₀₎ and DspF.

Crystallization studies and analyses of amino-acid sequences have identified secondary structures and specific residues

Fig. 3. Mapping of the secretion and translocation domain of DspE. **A**, The first 15, 33, 51, 77, 101, 150, and 203 amino acids of DspE were expressed in fusion with amino acids 2 through 402 of CyaA, and stable fusion expression in Eal189Δ*dspE* was confirmed by Western blot. **B**, Western blot of CyaA fusion proteins in supernatants (SN) and pellets (P) of Eal189Δ*dspE* expressing the first 15, 33, and 51 amino acids of DspE fused to CyaA. Samples were reprobbed with anti-NptII antibody as a cell lysis control. **C**, Translocation levels of N-terminal fragments of DspE, as measured by cAMP accumulation in tobacco leaves 16 h after infiltration with a 10^8 CFU/ml concentration of Eal189Δ*dspE* cells expressing DspE-CyaA fusions. Bars represent mean cAMP levels of three leaves on separate plants, plus or minus standard error. Asterisks above bars denote values significantly different from that of the pMJH20 control ($P < 0.05$). Asterisks above brackets denote values significantly different from one another ($P < 0.05$).

thought to be involved in chaperone-effector binding. Lilic and Stebbins (2006) showed that deletions of a single three-residue motif, the β -motif, in the chaperone-binding domains of several effector proteins led to a loss of chaperone-effector binding. CocrySTALLIZATION studies have indicated that hydrophobic grooves in class I chaperones interact with one or more α -helices on their cognate effector protein, but few functional analyses have been performed of specific chaperone-binding features on the effector (Ehrbar et al. 2006; Lilic et al. 2006). To identify DspE sequences within residues 51 through 100 that were important for DspF binding and to determine whether single or multiple sequences were involved, an alanine replacement strategy was employed. SLIM mutagenesis was used to replace DspE residues 51 through 60, 61 through 70, 71 through 85, and 86 through 100 with stretches of eight to ten alanines. Residues 71 through 85 were mutated at once because they correspond to a putative α helix-rich region predicted by the secondary structure prediction program PsiPred. In addition, we identified a potential β -motif consisting of residues L79, L83, and K86 that would be functionally disrupted by the 71 through 85 mutant (Fig. 6A). Alanine replacement constructs are described in Table 1. DspE residues 1 through 150 containing site-directed mutants in the 51 through 100 region were fused to the LexA protein and were tested for interaction with B42-HA-DspF in a Y2H assay. Replacement of residues 61 through 70 and 86 through 100 had no apparent effect on the interaction between LexA-DspE₍₁₋₁₅₀₎ and B42-HA-DspF (Fig. 6B). However, replacement of residues 51 through 60 appeared to cause a slight decrease in yeast growth and color development, while replacement of residues 71 through 85 caused a drastic reduction in growth and color development (Fig. 6B). Faint color development was still visible when the 71 through 85 alanine replacement strain was plated in high concentra-

tions on a less-selective medium amended with leucine, although this color development was not seen in a double mutant in which residues 51 through 60 and 73 through 82 were replaced with seven and ten alanines, respectively (data not shown). These results demonstrate that residues 71 through 85, which contain a putative α -helix and chaperone-binding motif, contribute strongly to the DspE-DspF interaction and that residues 51 through 60 may also contribute to this interaction.

The DspF-binding domain is not required for translocation of the N terminus of DspE.

The N-terminal chaperone-binding domain is required for translocation of many type III-secreted effector proteins (Higashide and Zhou 2006; Lee and Galan 2004). To determine whether the chaperone-binding domain in residues 51 through 100 of DspE affects protein translocation levels, a plasmid expressing DspE₍₁₋₁₅₀₎-CyaA lacking residues 51 through 100 was constructed. CyaA expression levels in Ea1189 Δ dspE were confirmed by Western blot to be equivalent to the wild type, and translocation levels into tobacco cells were tested. Deletion of amino acids 51 through 100 from residues 1 through 150 did not cause a change in translocation levels (Fig. 7A). To control for the possibility that residual chaperone-binding sites outside the 51 through 100 range were not affecting translocation levels, we also tested constructs with residues 35 to 125 deleted and found that these were not reduced in translocation (data not shown). We also tested constructs of DspE₍₁₋₇₃₇₎-CyaA with residues 51 through 100 deleted and found no effect of residues 51 through 100 on the longer fragment (data not shown). Although we hypothesized that translocation of the N terminus of DspE would be DspF-dependent, DspE₍₁₋₁₅₀₎-CyaA was translocated from Ea1189 Δ dspE and Ea1189 Δ dspF at similar levels (Fig 7B). Polymer-

Fig. 4. Analysis of alanine replacement mutants of amino acids 2 through 40 of DspE. **A**, Sequence alignment of amino acids 1 through 50 of DspE and homologs from other phytopathogenic bacteria. Bacterial species and Genbank accession numbers are as follows: *E. amylovora* (AAC04850), *E. pyrifoliae* (AY530755), *E. tasmaniensis* (YP_001906490), *Pantoea agglomerans* (AAF76343), *Pantoea stewartii* (AAG01467), and *Pseudomonas syringae* B728A (YP_234280). **B**, Lesion diameters of immature pears infected with Ea1189 Δ dspE and Ea1189 Δ dspE carrying pLRT5, a full-length clone expressing DspE, or pLRT137, pLRT138, and pLRT140, pLRT5 variants expressing DspE with amino acids 2 through 10, 11 through 20, or 31 through 40, respectively, replaced with stretches of alanine. **C**, cAMP levels of tobacco leaves infiltrated with Ea1189 Δ dspE carrying the pMJH20 parent vector, pLRT14(DspE₍₁₋₂₀₃₎-CyaA) or alanine replacement variants pLRT113, pLRT114, and pLRT116. Bars represent means plus or minus standard error. Letters above bars denote groups with significant differences ($P < 0.05$).

ase chain reaction (PCR) and pear virulence assays were used to confirm that the bacterial samples were not contaminated with any DspF-expressing cells. These results suggest that the accumulation and translocation of plasmid-expressed N-terminal fragments of DspE is not dependent on DspF binding and that the chaperone-binding domain itself does not inhibit or destabilize the N terminus of DspE in the absence of DspF.

DISCUSSION

This work reports a multifaceted analysis of the N-terminal 200 amino acids of the *E. amylovora*-secreted effector protein DspE. Three areas were examined with regards to functionality, i.e., requirements for secretion, requirements for translocation, and requirements for binding to the chaperone DspF. Our results demonstrated that, while DspF is required for virulence and binds to a specific site on the N terminus of DspE, the N-terminal minimal chaperone-binding domain is not required for translocation of the N terminus of DspE.

We report a timecourse analysis of DspE₍₁₋₂₀₃₎-CyaA translocation into tobacco leaves. cAMP accumulation in tobacco cells was detected at moderate levels after 8 h but had reached maximum levels within 11 h. This supports the recent finding of Boczanscy and associates (2008) that, after a short initial lag period, DspE translocation levels increase continuously up to 10 h postinoculation. *hrp* gene promoters require about 6 h for expression during induction in tobacco (Wei et al. 1992), which could explain the delay between leaf-disk infiltration and cAMP accumulation. In repeated studies, DspE-CyaA-induced cAMP levels remained at maximum levels until at least 24 h postinoculation. Work by Schechter and associates (2004) demonstrated that *Xanthomonas* protein translocation (cAMP

accumulation) levels into nonhost tobacco cells drop precipitously after about 8 h postinfiltration, and Wei and associates (1992) showed a decrease in *hrp* gene transcription in *E. amylovora* during the same timeframe. Our findings indicate that, in the absence of DspE-induced cell death, maximum translocation levels can be maintained for extended periods of time.

Studies on a variety of type III-secreted effectors in animal and plant pathogens have led to a model of two functional T3SS signals, a short N-terminal fragment sufficient for secretion into culture media and a longer fragment required for translocation into host cells (Mudgett et al. 2000; Sory et al. 1995). Here, we found that the N-terminal 15 and 33 amino acids of DspE were sufficient to allow very low levels of secretion of the CyaA reporter gene into Hrp-inducing minimal medium, while DspE₍₁₋₅₁₎-CyaA was strongly secreted. These results suggest that DspE does have a minimal secretion signal separate from the translocation signal but that optimal levels of secretion require a functional translocation domain. Previous work in plant pathogens indicated that, while the *X. oryzae* effector AvrBs2 has separate secretion and translocation signals (Casper-Lindley et al. 2002; Mudgett et al. 2000), the secretion and translocation requirements of the *P. syringae* effector AvrPto are not separate (Schechter et al. 2004).

This study found that translocation of the CyaA reporter protein required greater than 33 N-terminal amino acids of DspE and that translocation levels increased with greater than 51 amino acids. This finding echoes previous work that reported minimal and optimal translocation signals of the secreted effectors AvrPto, AvrBs2, SspH2, and YopE, among others (Casper-Lindley et al. 2002; Miao and Miller 2000; Schechter et al. 2004; Schesser et al. 1996). These findings suggest that the translocation signal itself could have multiple components.

Fig. 5. Mapping of the DspF-interacting regions in the N-terminal half of DspE. **A**, Yeast two-hybrid analysis of the N-terminal 800 amino acids of DspE. LexA-DspE fusions were coexpressed with fusions of DspF with B42-hemagglutinin. + and – indicate a weak and delayed color development. **B**, Pull-down analysis of DspE₍₁₋₁₅₀₎-CyaA and DspE_(1-150 Δ51-100)-CyaA with DspF-glutathione *S*-transferase (GST). Lysates of Ea1189Δ*dspE* carrying plasmids pLRT13 DspE₍₁₋₁₅₀₎-CyaA or pLRT109DspE_(1-150 Δ51-100)-CyaA were incubated overnight with lysates of *Escherichia coli* overexpressing GST-DspF and glutathione beads together or with glutathione beads alone.

The basis for increasing secretion competence of longer fragments is not known. In some cases, the structural conformation of effector or effector-reporter hybrids may be more secretion compatible in longer fragments. In some cases, longer gene fragments may allow greater levels of chaperone binding.

Mutational studies on the minimal translocation domain demonstrated that the sequence in residues 2 through 10 is required for all but the smallest detectable levels of translocation, a finding supported by previous studies on type III secretion signaling (Boyd et al. 1995; Schesser et al. 1996). Alanine replacement of residues 11 through 20 caused a minor but not significant decrease in translocation levels. Mutation of residues 31 through 40, a stretch rich in serine, caused a 40 to 60% reduction in translocation levels of DspE₍₁₋₂₀₃₎-CyaA in multiple studies. Residues 31 through 40 contain a stretch of five highly conserved serines. A high serine content (>10%) is a defining feature of the N terminus of T3SS effectors in plant pathogens (Guttman et al. 2002), although the N terminus of DspE does not contain any serines outside of residues 31 through 40. While at least one serine residue in the N terminus of the *P. syringae* effector protein AvrRpt2 was involved in protein stability, a fragment containing the serine-rich motif SSASS increased secretion or translocation efficiency without affecting levels of protein accumulation (Vinatzer et al. 2005). Mutagenesis of residues 31 through 40 did not affect accumulation of DspE₍₁₋₂₀₃₎-CyaA in Western blot experiments. Further studies are needed to determine whether the serine-rich stretch of DspE itself contributes to translocation efficiency.

Y2H and GST pulldown analyses in this study identified a DspF-binding domain within the first 150 residues of DspE, specifically within residues 51 through 100. A 50-amino acid domain is comparable to the sizes of other characterized chaperone-binding domains (Birtalan et al. 2002). Disruption of residues 51 through 60 and 71 through 86 strongly interfered with this interaction. Y2H studies also pointed to the possibility of several downstream sites of chaperone interaction, but these interactions have not been confirmed in vitro. To our knowledge, there are no previous reports of multiple distinct chaperone-binding sites on an effector. Bogdanove and associates (1998) reported that a deletion in the C-terminal half of

DspE is complemented with a cosmid expressing the overlapping C-terminal fragment, suggesting that DspE forms two independently folding domains. The mechanism of secretion of the C-terminal fragment is not known but, given the large size of DspE and the possibility of multiple secreted domains, multiple DspF interaction sites could be important for stabilization and secretion targeting of downstream portions of the protein. Although this study focused on the N terminus of DspE, it will be interesting in future studies to determine whether DspF binds to multiple downstream sites of DspE in a functionally significant manner.

Deletion of amino acids 51 through 100 of DspE or deletion of *dspF* did not result in a decrease in translocation levels of DspE₍₁₋₁₅₀₎-CyaA. Furthermore, DspE₍₁₋₁₅₀₎-CyaA showed no signs of destabilization in the absence of the chaperone-binding domain or DspF. These results would support a model in which, uncoupled from its stability role, the chaperone-binding domain does not have an effect on translocation of a stably expressed N terminus of DspE. This was surprising, since the N-terminal chaperone-binding domain is required for the T3SS-dependent translocation of the N terminus of many effectors (Parsot et al., 2003). One possibility for this difference is that a primary role of the chaperone-binding domain is to protect DspE from destabilization targeted to the C-terminal portions of the protein, as does the chaperone-binding domain of HopPtoM (Losada and Hutcheson 2005). If this were the case, deletion of the chaperone-binding domain would have little effect on translocation of N-terminal DspE fragments. Alternatively, expression of the N-terminal CyaA fusion of DspE from the medium-copy vector pMJH20 could overcome the need for the chaperone-binding domain, as multiple copies of the gene could potentially saturate proteolytic degradation or help DspE compete against other effectors in a hypothetical secretion hierarchy. However, it would be very unlikely for ectopic expression of the CyaA fusion to abolish the importance of chaperone binding, as effectors such as SspA and YscP require the chaperone for translocation even when expressed as a CyaA fusion from pMJH20-derived vectors (Bronstein et al. 2000; Matsumoto and Young 2009). This would suggest that the role of the N-terminal chaperone-binding domain of DspE differs from that of YscP and SspA, and our cur-

Fig. 6. Alanine-replacement mutagenesis of the N-terminal chaperone-binding domain of DspE. **A**, Amino-acid sequence of residues 51 to 100. Cylinders represent predicted α -helices. Underlined residues denote residues of a potential β -motif. **B**, Yeast two-hybrid interaction between alanine-replacement variants of LexA-DspE₍₁₋₁₅₀₎ and B42-hemagglutinin (HA)-DspF. The negative control strain carries the empty pGilda bait vector and B42-HA-DspF; the positive control strain carries pLexA-53 and pB42AD-T.

rent work is directed at determining whether alternate chaperones or chaperone-binding sites contribute to DspE secretion. Together, our results indicate that the N-terminal 150 amino acids of DspE are secretion- and translocation-competent even in the absence of DspF binding and that the chaperone-binding domain within residues 51 through 100 of DspE is not inhibitory to translocation in the absence of DspF. While chaperone-independent translocation of some effectors has been documented (Trulzsch et al. 2003), the virulence phenotype and secretion profile of *E. amylovora* *dspF* mutants demonstrates a strong role for the chaperone in translocation of wild-type DspE (Gaudriault et al. 2002). Studies involving full-length DspE with targeted deletions will be helpful in determining the true functional significance of the N-terminal chaperone-binding domain and its interaction with DspF.

In summary, our results provide evidence that the DspE chaperone-binding domain and the DspE-DspF interaction have the

same basic configuration as other characterized chaperone-effector pairs, including a minimal and optimal translocation signal and an N-terminal chaperone-binding domain. However, we have shown that the N terminus of DspE has some characteristics unlike that of many smaller secreted effectors, in that the N terminus of DspE required a functional translocation signal for substantial levels of secretion *in vitro* and did not require interaction with DspF for translocation competence.

MATERIALS AND METHODS

Genetic manipulations and analyses.

The bacterial strains and plasmids utilized in this study are listed in Table 1. Primers are listed in Supplementary Table S1. Genetic manipulations were performed using standard techniques and protocols (Ausubel et al. 1997). The inserts of all plasmid constructs were sequenced at the Michigan State Uni-

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics ^a	Reference or source
<i>Escherichia coli</i> strain		
DH5α	F ⁻ 80dlacZ ΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(r _K ⁻ m _K ⁺) deoR thi-1 supE44 gyrA96 relA1 λ-	Invitrogen, Carlsbad, CA, U.S.A.
<i>Erwinia amylovora</i> strains		
Ea1189	Wild type	Burse et al. 2004
Ea1189ΔdspE	dspE deletion mutant, Km ^r	This study
Ea1189ΔdspF	dspF deletion mutant, Km ^r	This study
Ea1189ΔT3SS	T3SS deletion mutant, Km ^r	Zhao et al. 2009
pBRR1-MCS5	broad host-range cloning vector, Gm ^r	Kovach et al. 1995
pLRT198	pBRR1-MCS5 expressing DspF	This study
pMJH20	pWSK29 containing codons 2 to 406 of CyaA, Amp ^r	Miao and Miller, 1999
pLRT8	pMJH20 expressing DspE ₍₁₋₁₅₎ -CyaA	This study
pLRT9	pMJH20 expressing DspE ₍₁₋₃₃₎ -CyaA	This study
pLRT10	pMJH20 expressing DspE ₍₁₋₅₁₎ -CyaA	This study
pLRT11	pMJH20 expressing DspE ₍₁₋₇₇₎ -CyaA	This study
pLRT12	pMJH20 expressing DspE ₍₁₋₁₀₁₎ -CyaA	This study
pLRT13	pMJH20 expressing DspE ₍₁₋₁₅₀₎ -CyaA	This study
pLRT14	pMJH20 expressing DspE ₍₁₋₂₀₃₎ -CyaA	This study
pLRT201	pMJH20 expressing DspE ₍₁₋₇₃₇₎ -CyaA	This study
pLRT109	pMJH20 expressing DspE _(1-150d. 51-100) -CyaA	This study
pLRT113	pMJH20 expressing DspE _(1-203; 2-10=10A) -CyaA	This study
pLRT114	pMJH20 expressing DspE _(1-203; 11-20=10A) -CyaA	This study
pLRT116	pMJH20 expressing DspE _(1-203 31-40=8A) -CyaA	This study
pGilda	HIS3 LexA BD bait vector, Amp ^r	Clontech, Mountain View, CA, U.S.A.
pB42AD	TRP1 B42 AD prey vector, Amp ^r	Clontech
pLexA-53	positive control bait vector, Amp ^r	Clontech
pB42-HA-T	positive control prey vector, Amp ^r	Clontech
pLRT192	LexA-DspE ₍₁₋₈₀₀₎	This study
pLRT216	LexA-DspE ₍₁₋₄₀₀₎	This study
pLRT217	LexA-DspE ₍₁₋₂₀₀₎	This study
pLRT31	LexA-DspE ₍₁₋₁₅₀₎	This study
pLRT30	LexA-DspE ₍₁₋₁₀₀₎	This study
pLRT29	LexA-DspE ₍₁₋₅₀₎	This study
pLRT32	LexA-DspE ₍₅₁₋₁₀₀₎	This study
pLRT35	LexA-DspE ₍₁₀₁₋₁₅₀₎	This study
pLRT37	LexA-DspE ₍₁₅₁₋₂₀₀₎	This study
pLRT155	LexA-DspE _(1-150 d. 51-100)	This study
pLRT149	LexA-DspE _(1-203; 51-60=7A)	This study
pLRT150	LexA-DspE _(1-203; 61-70=10A)	This study
pLRT151	LexA-DspE _(1-203; 71-86=8A)	This study
pLRT152	LexA-DspE _(1-203; 86-100=10A)	This study
pLRT194	LexA-DspE _(1-203; 51-60=7A, 73-82=10A)	This study
pLRT215	B42-HA-DspF	This study
pGEM T-Easy	high-copy TA cloning vector	Promega
pLRT5	5.6 kb dspEF locus in pGem T-Easy	This study
pLRT137	pLRT5 derivative expressing DspE _(1-203; 2-10=10A)	This study
pLRT138	pLRT5 derivative expressing DspE _(1-203; 11-20=10A)	This study
pLRT140	pLRT5 derivative expressing DspE _(1-203; 31-40=8A)	This study
pGEX 4T-1	Bacterial expression vector expressing a GST tag	GE Healthcare, Piscataway, NJ, U.S.A.
pLRT184	pGEX 4T-1 derivative expressing GST-DspF	This study
pKD4	Amp ^r , Km ^r , mutagenesis cassette template	Datsenko and Wanner 2006
pKD46	Amp ^r , expresses λ red recombinase	Datsenko and Wanner 2006

^a Km^r, Gm^r, Amp^r indicates resistant to kanamycin, gentamicin, and ampicillin, respectively

versity genomics facility. Sequence alignments were performed using T-Coffee (Notredame et al. 2000). Secondary structural prediction was performed by Psipred (Jones et al. 1999) and was supported by other secondary structure prediction programs.

Deletion mutagenesis of *dspE* and *dspF*.

E. amylovora nonpolar chromosomal deletion mutants were constructed according to the Red recombinase method (Datsenko and Wanner 2000). Briefly, the primers DspE_F1 and DspE_R1 as well as DspF_F1 and DspF_R1 were used to generate recombination fragments consisting of 50-nucleotide homology arms of the *dspE* or *dspF* genes flanking the neomycin phosphotransferase cassette from the plasmid pKD4. PCR products were purified, concentrated, and electroporated into Ea1189 expressing recombinase genes from the helper plasmid pKD46. Mutants were selected on Luria-Bertani (LB) agar media amended with kanamycin at a rate of 50 $\mu\text{g ml}^{-1}$. Single-gene disruption was confirmed by PCR, sequencing, and functional complementation. Virulence assays were conducted using immature pears as previously described (Zhao et al. 2005).

Cloning of *dspE* and derivative deletion mutants.

To create pLRT5, a 6.5-kb stretch encompassing the *dspE* and *dspF* open reading frames was amplified using primers DF1 and DspF-R and the Expand long template PCR system (Roche Diagnostics, Indianapolis, IN, U.S.A.), according to the manufacturer's instructions. The product was cloned into pGem T-Easy and complementation of Ea1189 Δ *dspE* and Ea1189 Δ *dspF* was confirmed. To create site-directed and deletion mutants of the full-length DspE, fragments were amplified from pLRT113, pLRT114, and pLRT116 using primers DF1-Apa and DR7 and were cloned into the *ApaI* and *BsmI* sites of pLRT5.

Site-directed alanine replacement and deletion mutagenesis.

Alanine replacement constructs of DspE (pLRT113, pLRT114, pLRT116, pLRT149, pLRT150, pLRT151, and

pLRT152) and deletions of the DspE N terminus (pLRT109) were created using the SLIM mutagenesis technique (Chiu et al. 2004). A set of two short primers were designed flanking each mutation site. In addition, two tailed primers were designed consisting of the short primer sequences with a 5' tail encoding a stretch of 10 alanines. Mutagenesis primer sequences are shown in Supplementary Figure S1. The four primers were combined in a PCR reaction to amplify the entire plasmid pLRT14 with complementary overhang ends. The PCR template was digested with *DpnI* to remove the template, the complementary ends were hybridized together, and the products were transformed into *Escherichia coli* DH5 α . Plasmids were extracted from the transformants and were screened by digestion to test for the addition of a *SacII* site incorporated into the alanine linker. Inserts were sequenced using the primers DF1 and CyaAR. The sequenced alanine replacement or deletion fragments were amplified, digested, and ligated into pMJH20 to create pLRT113, pLRT114, and pLRT116, into pGilda to create pLRT149, pLRT150, pLRT151, and pLRT152, and into pLRT5 to create pLRT137, pLRT138, and pLRT140.

The CyaA translocation assay.

N-terminal DspE fragments of increasing length, preceded by the native promoter, were amplified with primers DF1 and DR1 through DR8 and were cloned into the *XbaI* and *SmaI* sites of the plasmid pMJH20 in fusion with residues 2 through 402 of the CyaA. DspE-CyaA constructs were introduced into Ea1189 Δ *dspE* and Ea1189 Δ *dspF*. Stable fusion protein expression was confirmed via Western blot with anti-CyaA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and in vitro calmodulin-dependent adenylate cyclase activity of DspE₍₁₋₂₀₃₎-CyaA was confirmed using a published assay (Sory and Cornelis 1994). Overnight cultures were adjusted to 6×10^8 CFU/ml in phosphate-buffered saline and were infiltrated into the youngest three fully expanded leaves of 8-week-old *Nicotiana tabacum* cv. *Samsun* plants. Each treatment was

Fig. 7. Functional analysis of amino acids 51 through 100 of DspE. **A**, cAMP accumulation in tobacco leaves infiltrated with Ea1189 Δ *dspE* expressing DspE₍₁₋₁₅₀₎-CyaA and DspE_(1-150Δ51-100)-CyaA. **B**, cAMP accumulation in leaves infiltrated with Ea1189 Δ *dspE* and Ea1189 Δ *dspF* expressing DspE₍₁₋₁₅₀₎-CyaA. Results are the means and standard errors from three replicate leaves on separate plants.

infiltrated into three replicate leaves on different plants. Two 1-cm leaf disks were collected and flash-frozen at 20 h postinoculation. cAMP was extracted according to the method of Casper-Lindley and associates (2002); leaf disks were ground in liquid nitrogen and were resuspended in 325 μ l HClO₄ (1.1 M), and 280 μ l of the supernatants was neutralized with 37.5 μ l K₂CO₃ (6 M). cAMP levels in the resulting supernatants were measured using the cyclic AMP EIA kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) according to the manufacturer's instructions. Protein levels in leaf pellets were measured using the Bio-Rad (Hercules, CA, U.S.A.) protein assay. Statistical analyses were done using a one-way analysis of variance, and a mean separation was accomplished using Fisher's protected least significant difference test.

Y2H assays.

dspE fragments were cloned in fusion with the LexA DNA-binding domain in the *Bam*HI and *Xho*I sites of the bait vector pGilda (Clontech, Mountain View, CA, U.S.A.). *dspF* was cloned into the *Bam*HI and *Eco*RI sites of the prey vector pB42AD. Bait and prey constructs were cotransformed into the *Saccharomyces cerevisiae* EGY48 p80(pLacZ) using the Yeast transformation kit (Zymo Research Corporation, Orange, CA, U.S.A.). Transformants were selected on minimal SD agar amended with –Ura/–His/–Trp dropout supplement (Clontech). Reporter expression was visualized on minimal SD-galactose agar amended with –Ura/–His/–Trp/–Leu dropout supplement and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) at the rate of 80 μ g ml⁻¹. Stable expression of prey constructs was confirmed by Western blot using anti-LexA antibody (Millipore, Billerica, MA, U.S.A.).

Secretion assays.

Cultures (50 ml) of Ea1189 Δ *dspE* strains expressing DspE-CyaA fusions were grown overnight in LB broth, were pelleted by centrifugation, were washed with 40 ml of water, and were resuspended in 20 ml of Hrp-inducing minimal medium, pH 5.7 (Huynh et al. 1989). Cultures were induced for 9 h at room temperature. Induced cultures were pelleted for 20 min at 2,600 $\times$ g. The uppermost 35 ml of supernatant was removed, was amended with phenylmethylsulfonyl fluoride (PMSF) to a concentration of 0.5 mM, and was centrifuged again. The uppermost 30 ml of supernatant was removed and centrifuged a third time. The resulting supernatant was concentrated to 75 μ l (400 $\times$) using the Amicon 15-ml centrifugal filter unit (10 kDa molecular weight cut-off) and the Ultrafree 0.5-ml microcentrifugal filter unit (Millipore). Protein concentrations were measured by Bradford assay and were adjusted to 200 μ g/ml; 3 μ g of total supernatant protein was analyzed by Western blot using anti-CyaA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Anti-NPTII antibody (US Biological, Swampscott, MA, U.S.A.) was used as a lysis control.

In-vitro protein interaction assay.

dspF was cloned into the *Bam*HI and *Xho*I sites of the vector pGex 4T-1 (GE Healthcare; Piscataway, NJ, U.S.A.) in fusion with an N-terminal GST tag. *Escherichia coli* DH5 α cells carrying the fusions were induced in 1 mM isopropyl β -D-1-thiogalactopyranoside for 4 h at 37°C. To induce 15-ml overnight cultures of *E. amylovora*, Ea1189 Δ *dspF* cells harboring DspE-CyaA fusion vectors were washed twice with water, were resuspended in 30 ml of modified Hrp-inducing minimal medium, and were incubated at 25°C for 6 h. Induced *Escherichia coli* and *E. amylovora* cells were pelleted and lysed in 1 ml of B-Per reagent (Thermo-Fisher, Rockford, IL, U.S.A.). Lysates were diluted 1:1 in Tris-buffered saline (TBS);

20 mM Tris, 150 mM NaCl, pH 7.4), cleared by centrifugation, and amended with PMSF to a final concentration of 0.5 mM. Bait (DspF-GST) lysate (0.5 ml) was incubated with 0.5 ml of prey (DspE-CyaA) lysate and 50 μ l of TBS-equilibrated glutathione agarose (GE Healthcare) overnight at 4°C. The glutathione agarose fraction was washed eight times in 10-bed volumes of TBS and was boiled for 10 min in 100 μ l of sodium dodecyl sulfate (SDS) loading buffer (2% SDS, 4 mM EDTA, 5% B-mercaptoethanol, 0.02% bromophenol blue, and 10% glycerol). A 20- μ l sample of the resulting fraction was resolved on a 12% polyacrylamide gel, was transferred to nitrocellulose, and was probed with anti-CyaA or anti-GST antibody (Precision Antibody, Columbia, MD, U.S.A.).

ACKNOWLEDGMENTS

We thank S. Y. He for materials, technical advice, and critical reading of the manuscript, D. Dahlbeck for protocol advice, and Y. Zhao for construction of the *Erwinia amylovora* strains Ea1189 Δ *dspE* and Ea1189 Δ *dspF*. Funding for this work was provided by the United States Department of Agriculture-Cooperative State Research Education and Extension Service and the Michigan Agricultural Experiment Station.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Swiss Institute of Bioinformatics T-Coffee website:
tcffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi
 The PSIPRED protein structure prediction server:
bioinf.cs.ucl.ac.uk/psipred