The Contributions to Virulence of the Effectors Eop1 and DspE Differ Between Two Clades of *Erwinia tracheiphila* Strains

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Strains of *Erwinia tracheiphila*, causal agent of bacterial wilt of cucurbits, are divided into distinct clades. *Et-melo* clade strains wilt *Cucumis* spp. but not *Cucurbita* spp., thus exhibiting host specificity, whereas *Et-C1* clade strains wilt *Cucurbita* spp. more rapidly than *Cucumis melo*, thus exhibiting a host preference. This study investigated the contribution of the effector proteins Eop1 and DspE to *E. tracheiphila* pathogenicity and host adaptation. Loss of *eop1* did not enable *Et-melo* strains to infect squash (*Cucurbita pepo*) or an *Et-C1* strain to induce a more rapid wilt of muskmelon (*Cucumis melo*), indicating that Eop1 did not function in host specificity or preference as in the related pathogen *E. amylovora*. However, overexpression of *eop1* from *Et-melo* strain MDCuke but not from *Et-C1* strain BHKY increased the virulence of a BHKY *eop1* deletion mutant on muskmelon, demonstrating that the Eop1 variants in the two clades are distinct in their virulence functions. Loss of *dspE* from *Et-melo* strains reduced but did not eliminate virulence on hosts muskmelon and cucumber, whereas loss of *dspE* from an *Et-C1* strain eliminated pathogenicity on hosts squash, muskmelon, and cucumber. Thus, the centrality of DspE to virulence differs in the two clades. *Et-melo* mutants lacking the chaperone DspF exhibited similar virulence to mutants lacking DspE, indicating that DspF is the sole chaperone for DspE in *E. tracheiphila*, unlike in *E. amylovora*. Collectively, these results provide the first functional evaluation of effectors in *E. tracheiphila* and demonstrate clade-specific differences in the roles of Eop1 and DspE.

Keywords: bacterial pathogenesis, chaperone, cucurbit, effector, host-specificity, muskmelon, phytopathogenic bacteria, squash, type III secretion system, wilt, xylem

Erwinia tracheiphila is a xylem pathogen that causes bacterial wilt of cucurbits in the *Cucurbita* (squash and pumpkin) and *Cucumis* (muskmelon and cucumber) genera (Smith 1911). This pathogen is transmitted by striped and spotted cucumber beetles (*Acalymma vittatum* and *Diabrotica undecimpunctata*, respectively). When these beetles feed on leaves or flowers of cucurbits, they can deposit *E. tracheiphila*-infested frass onto fresh feeding wounds, allowing bacterial entry into the xylem (Rojas et al. 2015). *E. tracheiphila* cells multiply in the xylem, producing exopolysaccharides that likely help obstruct the vascular system; this can lead to wilt, collapse, and eventual plant death. Cucurbits are grown throughout the United States but bacterial wilt is restricted to the Northeastern and Midwestern regions; these regions account for approximately 68% of the nation's total cultivated cucurbit acreage (Rojas et al. 2015). Bacterial wilt is managed primarily by frequent insecticide applications to reduce the cucumber beetle populations, although alternative management strategies include the deployment of row tunnels to prevent beetle feeding (Sánchez et al. 2015) and perimeter trap crops to lure beetle vectors to an alternate crop (Cavanagh et al. 2009; Weber 2018).

E. tracheiphila strains fall into distinct clades based on their genetics. In all, 69 strains were initially separated into two groups based on repetitive element sequence-based (rep)-PCR fingerprints (Rojas et al. 2013), with a subsequent study separating 88 strains into three phylogenetic clades based on comparative genomics of whole-genome sequences (Shapiro et al. 2018). The rep-PCR-based groups generally correspond to the phylogenetic clades designated *Et-melo* and *Et-C1* and exhibit host-specific virulence patterns. *Et-melo* strains induce rapid wilt on the *Cucumis* spp. muskmelon (*Cucumis melo*) and cucumber (*C. sativa*) but do not induce wilt on *Cucurbita* spp. (Nazareno and Dumenyo 2015; Vrisman et al. 2016), although a study in the greenhouse reported induction of non-lethal, localized wilt symptoms on squash (*Cucurbita pepo*) (Shapiro et al. 2018). In contrast, *Et-C1* strains induce wilt on *Cucumis* and *Cucurbita* spp. but induce a more rapid wilt on squash than on muskmelon (Nazareno and Dumenyo 2015; Rojas et al. 2013; Shapiro et al. 2018; Vrisman et al. 2016). Thus, *Et-C1* strains demonstrate a greater aggressiveness to, or a host preference for, squash over muskmelon. A third clade, *Et-C2*, contains recently isolated strains that exhibit the same host preference as the *Et-C1* strains but originated from a more localized geographic range (Shapiro et al. 2018). The host adaptation of these clades may be even more marked in the field, because all of the *Et-melo* strains in current collections were isolated from muskmelon or cucumber, and all of the *Et-C1* and *Et-C2* strains were isolated from squash or cucumber (Rojas et al. 2013; Shapiro et al. 2018).

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We are interested in understanding the factors contributing to the pathogenicity and host adaptation of *E. tracheiphila* strains. Like many gram-negative phytopathogens, *E. tracheiphila* has a type III secretion system (T3SS), which is a membrane-spanning structure that enables the secretion and delivery of bacterial effector proteins into host cells. These effector proteins are often key factors in pathogenicity and host specificity. *E. tracheiphila* strains share several effectors with the well-characterized and related pathogen, *E. amylovora*. *E. amylovora* secretes only a limited repertoire of effectors (Yuan et al. 2020), of which two, Eop1 and DspE, have been particularly well studied. Like *E. tracheiphila*, *E. amylovora* strains fall into groups that differ in their host specificity. *Erwinia* outer protein 1 (Eop1) prevents *Rubus*-specific strains from infecting rosaceous plants outside of the *Rubus* genus (Asselin et al. 2011) and, thus, functions as a typical avirulence gene in *Rubus* strains on these non*Rubus* hosts (Asselin et al. 2011; Wöhner et al. 2018); Eop1 was not shown to function in virulence (Asselin et al. 2011). In contrast, the effector disease-specific protein E (DspE) is essential to the pathogenicity of *E. amylovora* (Gaudriault et al. 1997) and has variable roles in the pathogenicity of other phytopathogens (Badel et al. 2006; Bogdanove et al. 1998; Coplin et al. 2008; Guy et al. 2013; Ham et al. 2006; Jacobs et al. 2013; Kim et al. 2011). Here, we investigated the roles of Eop1 and DspE in *E. tracheiphila* pathogenicity and host adaptation.

Eop1 belongs to the YopJ family of T3SS effectors (T3SEs). This is a widely distributed family of T3SEs that has been divided into five major clusters (Ma and Ma 2016). Of the 18 distinct YopJ family effectors in plant pathogens, the best studied are the HopZ alleles of *Pseudomonas syringae*, XopJ and AvrBsT of *Xanthomonas* spp., and RipP2 (previously PopP2) (Peeters et al. 2013) of *Ralstonia solanacearum*. Eop1, which was previously called EopB and OrfB (Oh et al. 2005), is phylogenetically clustered with the *P. syringae* HopZ1 and HopZ3 effectors (Ma and Ma 2016). All YopJ family effectors share a conserved triad of amino acids that initially suggested cysteine protease activity (Orth et al. 2000) but is now known to contribute to acetyltransferase activity within eukaryotic cells (Ma and Ma 2016; Mittal et al. 2010). Although the host protein targets for acetylation generally have kinase activity in animal cells (Orth et al. 2000), they have diverse functions in plant cells, which leads to multiple pathways by which YopJ effectors influence plant pathogenesis and resistance. For example, HopZ1a suppresses pathogen-associated molecular pattern (PAMP)-triggered immunity, activates jasmonic acid signaling that suppresses salicylic acid (SA)-mediated defenses (Jiang et al. 2013), and potentially interferes with trafficking of plant defense molecules by acetylating tubulin (Cheong et al. 2014; Lee et al. 2012). Other YopJ effectors interfere with resistance by suppressing the immune response to other effectors (Lee et al. 2015; Le Roux et al. 2015; Sarris et al. 2015). Strain variation in the sequence of Eop1 in *E. psidii*, a close relative of *E. tracheiphila*, correlated with strain aggressiveness on eucalyptus (Pereira et al. 2021), suggesting that even minor variation among YopJ effectors may affect their impact on plant hosts.

DspE belongs to the widely distributed AvrE family of T3SEs. AvrE family effectors are required for the pathogenicity of some pathogens such as *E. amylovora*, *Pantoea agglomerans* pv. *gypsophylae*, and *Pectobacterium carotovorum* (Bogdanove et al. 1998; Gaudriault et al. 1997; Kim et al. 2011; Mor et al. 2001; Siamer et al. 2013), which each secrete multiple T3SEs, and *Pantoea stewartii*, which secretes only the DspE homolog WtsE (Ham et al. 2006). In contrast, the loss of AvrE homologs reduced but did not eliminate virulence in *Pseudomonas syringae* (Badel et al. 2006) and *R. solanacearum* (Jacobs et al. 2013). AvrE effectors are unusually large proteins (approximately 2,000 amino acids), possibly reflecting multiple mechanisms by which

they contribute to virulence (Degraeve et al. 2015), although their biochemical functions are not yet known. In addition to suppressing PAMP-triggered immunity and SA-mediated defenses, AvrE family proteins can elicit plant cell death in the absence of resistance proteins, consistent with their toxicity to yeast and cultured plant cells (DebRoy et al. 2004; Degraeve et al. 2015; Yuan et al. 2020). Moreover, *P. syringae* AvrE contributes to effector-triggered immunity based on its role as an avirulence factor on soybean (Kobayashi et al. 1989) and as a trigger for resistance when interacting with the *Arabidopsis* receptor protein CAR1 (Laflamme et al. 2020).

Here, we report the first functional studies of the T3SEs of *E. tracheiphila*. We targeted Eop1 and DspE based on their importance in the host specificity and pathogenesis of the related and well-studied pathogen *E. amylovora*. We tested four hypotheses. First, Eop1 contributes to the inability of *Et-melo* strains to infect squash or to the lower aggressiveness (i.e., slower wilt) of *Et-C1* strains on muskmelon than squash, as suggested by the avirulence function of Eop1 in *E. amylovora*. Any evidence for the involvement of effectors in resistance, even if weak, could be a useful first step toward identifying genetic resistance to bacterial wilt of cucurbits. Second, Eop1 contributes to the virulence of the *Et-melo* and *Et-C1* clades, consistent with the multifaceted roles in virulence of the phylogenetically related effectors HrpZ1 and HrpZ3. Third, differences in Eop1 sequence between the *Et-melo* and *Et-C1* clades correlate with differences in aggressiveness on muskmelon, as suggested by a similar correlation among *E. psidii* strains on eucalyptus. And fourth, DspE is required for the pathogenicity of *E. tracheiphila*, in general, consistent with the critical role of DspE and WtsE in all known phytopathogens of *Erwinia* and *Pantoea* spp.

RESULTS

Eop1 sequence differences correlate with *E. tracheiphila* clades.

The *E. tracheiphila* *eop1* and *dspE* genes occur in a locus that is generally conserved among *Erwinia* spp. (Fig. 1). Homologs of Eop1 fall into the group III clade of the five YopJ effector clades identified by Ma and Ma (2016). A maximum-likelihood phylogenetic tree of these group III YopJ homologs shows that the *E. tracheiphila* Eop1 effectors are more closely related to those in some *Pantoea* spp. (71 to 72% identity) than to the OrfB effectors in many *Erwinia* spp. (57 to 59% identity) (Fig. 2). These group III YopJ family effectors fall into at least three distinct subgroups; namely, those of *E. tracheiphila*/*E. psidii* and *Pantoea* spp., the other phytopathogenic *Erwinia* spp., and the *P. syringae* effectors, with the latter group potentially including HopZ2, the only effector previously assigned to group II (Ma and Ma 2016). Alignment of the Eop1 sequences from nine strains within *Et-melo* and nine strains within *Et-C1* indicated that Eop1 did not vary within a clade but differed between clades (Fig. 3, shown for only two strains per clade). These differences were not correlated with sequence differences among YopJ homologs in the other members of the *Erwiniaceae* family (Fig. 3).

Deletion of *eop1* did not alter the virulence of *E. tracheiphila* strains on muskmelon or squash.

To generate deletion mutants of *E. tracheiphila*, we first optimized the λ red recombineering system (LRRS) (Datsenko and Wanner 2000) for use with this pathogen. We introduced the λ red recombinase on a stable plasmid, pKD46, to enable λ red recombinase-mediated recombination into the chromosome; used 90-bp homologous flanking arms to generate linear fragments for the LRRS; and induced electrocompetency in *E. tracheiphila* cells at room temperature rather than on ice. In this manner, we

generated unmarked $\Delta eop1$ deletion mutants (Supplementary Fig. S1) of several *E. tracheiphila* strains (Supplementary Table S1).

The *Et-melo* strains MDCuke and SCR3 both wilt muskmelon more rapidly than the *Et-C1* strains BHKY and BuffGH and do not wilt squash (Supplementary Fig. S2A), and SCR3 and BHKY, as representatives of each clade, both lost all wilt activity upon loss of the T3SS (Supplementary Fig. S2B). Loss of *eop1* did not alter the virulence of the mutants MDCuke $\Delta eop1$ and SCR3 $\Delta eop1$ on muskmelon (Supplementary Fig. S3A and B), nor did it enable them to infect squash (data not shown). Similarly, loss of *eop1* from the *Et-C1* strain BHKY, designated BHKY $\Delta eop1$, did not alter its virulence on either muskmelon or squash (Supplementary Fig. S3C and D). These findings indicate that *E. tracheiphila* Eop1 does not function as an avirulence factor that prevents *Et-melo* strains from infecting squash, or as a host-limiting factor that reduces the aggressiveness of *Et-C1* strains on muskmelon. These results also do not support a role for Eop1 in virulence, although this contribution could have been masked by other effectors, as is common for T3SEs.

Overexpression of *eop1* from an *Et-melo* strain in an *Et-C1* strain increased virulence on muskmelon but not squash.

We constructed derivatives of MDCuke $\Delta eop1$ and BHKY- $\Delta eop1$ that expressed homologous and heterologous variants of *eop1* and evaluated their virulence. Expression of *eop1* from MDCuke or BHKY in MDCuke $\Delta eop1$ did not alter virulence on muskmelon (Supplementary Fig. S4A) or enable infection of squash (data not shown). Similarly, expression of these *eop1* variants in BHKY did not alter virulence on squash (Supplementary Fig. S4B) but expression of *eop1*_{MDCuke} in BHKY increased virulence on muskmelon (Fig. 4A). Expression of these *eop1* variants in BHKY in planta was confirmed using RNA isolated from the xylem sap of infected muskmelon plants (Fig. 4B). These findings demonstrate that the Eop1 variants in the two *E. tracheiphila* clades have distinct functions, with the Eop1 variant from the *Et-melo* strain increasing the virulence, or aggressiveness, of an *Et-C1* strain on muskmelon. Although BHKY $\Delta eop1$ (pN-*eop1*_{MDCuke}) was more virulent than the wild-type BHKY (Fig. 4A), it remained less virulent than the *Et-melo* strains on muskmelon (Supplementary Figs. S2 to S4), indicating that *eop1*_{MDCuke} contributed to but was not sufficient for rapid wilt of muskmelon.

DspE contributes to the virulence of *Et-melo* strains but is required for the pathogenicity of an *Et-C1* strain.

Similar to Eop1, the DspE effectors exhibit sequence differences between the two *E. tracheiphila* clades but not within the clades (Supplementary Fig. S5). Loss of DspE and its cognate chaperone DspF reduced the virulence of *Et-melo* strains MDCuke and SCR3 on muskmelon and cucumber, although these mutants retained the ability to induce wilt (Fig. 5). The large reduction in virulence suggests a central role for DspE in

the virulence of *Et-melo* strains, while the remaining virulence demonstrates that DspE is not absolutely required for the pathogenicity of this clade. Loss of *dspEF* did not enable MDCuke or SCR3 to wilt squash (Supplementary Fig. S6), indicating that DspE does not have a role in avirulence. In contrast, loss of *dspEF* completely eliminated the ability of *Et-C1* strain BHKY to cause disease on squash, muskmelon, and cucumber (Fig. 6).

These collective results highlight distinct roles for DspE in the two *E. tracheiphila* clades. Specifically, DspE is a major factor contributing to but is not required for virulence of the *Et-melo* clade, whereas it is absolutely required for the pathogenicity of the *Et-C1* clade. Prior studies have found that DspE is required for the pathogenicity of all *Erwiniaceae* family pathogens examined; thus, the *Et-melo* clade strains are exceptional within this family (Fig. 7). Among a taxonomically broader array of pathogens, only the *Pectobacterium* genus includes strains that differ in their requirement for DspE for pathogenicity. Differences in the impact of DspE loss likely reflect the presence of one or more additional effectors with compensatory functions in the strains exhibiting residual virulence.

Fig. 2. Phylogenetic relatedness of YopJ effectors within the group III clade, with representatives from other YopJ family groups for comparison. Protein sequences were aligned using MUSCLE (Edgar 2004) and the phylogenetic tree was produced using MEGA X (Kumar et al. 2018) using a maximum-likelihood approach and bootstrapping with 1,000 replicates to generate the consensus trees shown. Protein sequences for the effectors representing groups I, II, IV, and V, along with the HopZ1a and HopZ1b effectors of *Pseudomonas syringae*, are from Ma and Ma (2016), with RipP3 (Peeters et al. 2013) referring to PopP3 (Ma and Ma 2016). The GenBank accession numbers for the remaining YopJ family effectors were *Et-melo* Eop1 (*Erwinia tracheiphila* MDCuke, AXF77196.1) *Et-C1* Eop1 (*E. tracheiphila* BuffGH, WP_016191031.1), *Pantoea agglomerans* (WP_158150056.1), *P. vagans* (WP_061060943.1), *E. psidii* Eop1 (WP_124231866.1), *E. amylovora* OrfB (AAF63400), *E. tasmaniensis* OrfB (WP_012440293.1), and *E. pyrifoliae* OrfB (WP_012669297.1).

Fig. 1. Genetic organization of the *eop1-dspE* locus of several *Erwinia* spp.

The chaperone DspF is required for virulence of *Et-melo* strains.

Whereas DspE was required for the virulence of *E. amylovora* (Triplett et al. 2008) and *Pantoea stewartii* subsp. *stewartii*

(Ham et al. 2006), loss of the cognate chaperone DspF in these pathogens reduced but did not eliminate their virulence. These results indicate that DspF contributes to but is not absolutely required for stability and secretion of DspE in these pathogens.

<i>E. amylovora</i>	MNISGLRGYKSAQAQADNASSSS----TQGSAPTGR--RLQRQDA-LPANYRYHASQMPATPERARVAARYASQASSS----- 73
<i>E. pyrifoliae</i>	MNVSGLRAGQSPSQADHAPSSS----TQASPAQTGR--RLQRQDA-LPANNRYHASQTPATPDRARAAARYASGASSSAAAPAGP 80
<i>E. psidii</i>	MNVFGIKIGHRNPSQQTERTPSP----SPQASPLPAGRAGRLQRQNA-LSPTIRYNARSTPTPDRARASMHGAGSSSPACSSGA 82
<i>Et-melo</i> SCR3	MNVFGVIRGRKSSSQEEQTPSSSPLASQSSPLPAGRAGRLQRQATLSNNTRYNARSTPGTPDRARATSRHSGESSSSAYSTGP 87
<i>Et-melo</i> MDCuke	MNVFGVIRGRKSSSQEEQTPSSSPLASQSSPLPAGRAGRLQRQATLSNNTRYNARSTPGTPDRARATSRHSGESSSSAYSTGP 87
<i>Et-C1</i> BuffGH	MNVFGVIRGRKSSSQEEQTPSSSPLASQSSPLPAGRAGRLQRQATLSNNTRYNARSTPGTPDRARATSRHSGESSSSAYSTGP 87
<i>Et-C1</i> BHKY	MNVFGVIRGRKSSSQEEQTPSSSPLASQSSPLPAGRAGRLQRQATLSNNTRYNARSTPGTPDRARATSRHSGESSSSAYSTGP 87
<i>E. amylovora</i>	AGPSIL-LSRQSGHRENPSLVRFHETMQQSPKMSRGDPLPEKPEIIVPKRLQEKIDSVNLPRLNKLKDNLYEYKGMATELAKEGSSGS 159
<i>E. pyrifoliae</i>	AGPSMA-LSRQHANNRNPFTARFHDAMQQSPKMLRASVPEKEKIPERLQKADAIDLPGKKLKDLSLEYAKLATELVKEGAGPD 166
<i>E. psidii</i>	ASPSRG-LTRQAGRENAQLAQFHDTRASPMSRNDPIPETPETPKRLQKMDAIDLPLKSLDKDLYHYAPMATELVNENNSN 168
<i>Et-melo</i> SCR3	ASSSRVLRQGGNREHSQLAQFHEMMQVSPKISRNDPLPETPESIPRRLQEKMDTVNLPELEKLDGGLYAYAKMAIERVNEKKGAD 174
<i>Et-melo</i> MDCuke	ASSSRVLRQGGNREHSQLAQFHEMMQVSPKISRNDPLPETPESIPRRLQEKMDTVNLPELEKLDGGLYAYAKMAIERVNEKKGAD 174
<i>Et-C1</i> BuffGH	ASSSRVLRQGGNREHSQLAQFHEMMQVSPKISRNDPLPETPESIPRRLQEKMDTVNLPELEKLDGGLYAYAKMAIERVNEKKGAD 174
<i>Et-C1</i> BHKY	ASSSRVLRQGGNREHSQLAQFHEMMQVSPKISRNDPLPETPESIPRRLQEKMDTVNLPELEKLDGGLYAYAKMAIERVNEKKGAD 174
<i>E. amylovora</i>	VALMRMDKKVLPPLADAENARNPGLNLHVYKRGEECYQAIKEQHKKIVQSSGQPKTMRALYPPFFIGMPDHHIALDIHLRPGHPSIVG 246
<i>E. pyrifoliae</i>	NDLADMDBKLPLPLADAENARNPGLNLRTFKSSEECYRAIKDNQKSVQSSRQPMRMRVLYPPLKGARDHRVALDIQFRPGHRPSIVG 253
<i>E. psidii</i>	AVLMRMDKKMLPLIAEAENARHPGLNLHVFKNADECYKAIKMNQKQVWSSQPMNMRVLYSPFKGMPDHHVALDVQFRPGHRPSVVC 255
<i>Et-melo</i> SCR3	KQLSELDDKMLPLFAEAENARHPDLNLHVFRGPEACYKAIKEQNKKAWDSRQPMNMRVVFSPSRGIPDHHVALDVQLRPGHHPSVVC 261
<i>Et-melo</i> MDCuke	KQLSELDDKMLPLFAEAENARHPDLNLHVFRGPEACYKAIKEQNKKAWDSRQPMNMRVVFSPSRGIPDHHVALDVQLRPGHHPSVVC 261
<i>Et-C1</i> BuffGH	KQLSELDDKMLPLFAEAENARHPDLNLHVFRGPEACYKAIKEQNKKAWDSRQPMNMRVVFSPSRGIPDHHVALDVQLRPGHHPSVVC 261
<i>Et-C1</i> BHKY	KQLSELDDKMLPLFAEAENARHPDLNLHVFRGPEACYKAIKEQNKKAWDSRQPMNMRVVFSPSRGIPDHHVALDVQLRPGHHPSVVC 261
<i>E. amylovora</i>	FESALGHMVDPIRQGIQAQGLRGAKVHMVGNRIQNSEWDCIMYSLNNALKSFKHHDEYTLRLHKGEE--K-IPVPAEFFKHAQSKSMVE 330
<i>E. pyrifoliae</i>	FESAPGNLAELLQHELEHALRGAKVQVVENTIQNSLRGCSMFALNNALKSFKHHDEYTLRLHSGE--KQVPVPAEFFLKHAKSKALVE 338
<i>E. psidii</i>	MESALGHMMDTIQMGIEHGLKGAQKVMVGNITQASQWDCAMYALNNALKLFKHHDEYTLRVHGE--TDVSLPAEFFLKHAKQSRSHIR 340
<i>Et-melo</i> SCR3	FESALWGMNNEIRQDIEHGLKESKVKLIGNFVQASDWCAMFALSNAKLYKHHDEYTSRLHAGE--ENVRIPSEELIKHAQSKGHAE 346
<i>Et-melo</i> MDCuke	FESALWGMNNEIRQDIEHGLKESKVKLIGNFVQASDWCAMFALSNAKLYKHHDEYTSRLHAGE--ENVRIPSEELIKHAQSKGHAE 346
<i>Et-C1</i> BuffGH	FESALWGMNNEIRQDIEHGLKESKVKLIGNFVQASDWCAMFALSNAKLYKHHDEYTSRLHAGE--EENVRIPSEELIKHAQSKGHAE 348
<i>Et-C1</i> BHKY	FESALWGMNNEIRQDIEHGLKESKVKLIGNFVQASDWCAMFALSNAKLYKHHDEYTSRLHAGE--EENVRIPSEELIKHAQSKGHAE 348
<i>E. amylovora</i>	GLPHQDAIVTKDKGGLHAETLLHRNLAYRADRFDHACNTSIEGFRMQEIQRAGEFLSAQNRKS 393
<i>E. pyrifoliae</i>	GHRHQDAIVSKDKGGLHAETLLHRNLAYRADRINHSYSTSIEGFRQLQEIQRAGEFLAARKQRR 401
<i>E. psidii</i>	ESPNKNDIVSKDKGGLHAETLMHRNLAYRTQRFKAYSTSIEGFRFQEIQRAGDYLAQGRGLK 403
<i>Et-melo</i> SCR3	RQGRRNDIVTKDKGGLHAETLLHRNLAYRAQRFDKAYSTSIEGFRFQEIQRAGDYLAQGRGRK 409
<i>Et-melo</i> MDCuke	RQGRRNDIVTKDKGGLHAETLLHRNLAYRAQRFDKAYSTSIEGFRFQEIQRAGDYLAQGRGRK 409
<i>Et-C1</i> BuffGH	RQGRRNDIVTKDKGGLHAETLLHRNLAYRAQRFDKAYSTSIEGFRFQEIQRAGDYLAQGRGRK 411
<i>Et-C1</i> BHKY	RQGRRNDIVTKDKGGLHAETLLHRNLAYRAQRFDKAYSTSIEGFRFQEIQRAGDYLAQGRGRK 411

Fig. 3. Eop1 exhibits clade-specific sequence differences among *Erwinia tracheiphila* strains. Eop1 sequences of *Et-C1* and *Et-melo* strains exhibit three single-amino acid differences and one two-amino acid-insertion-deletion. Amino acid sequences were aligned using Clustal Omega (Madeira et al. 2019). The GenBank accession numbers for the proteins shown are *Et-melo* (AXF7196.1) *Et-C1* (WP_016191031.1), *E. psidii* (WP_124231866.1), *E. amylovora* (AAF63400), and *E. pyrifoliae* (WP_012669297.1). Eop1 sequences for additional *E. tracheiphila* strains are from Liu (2015).

Fig. 4. Expression of *eop1* from *Et-melo* in *Et-C1* increased virulence on muskmelon. **A**, Proportion of leaves wilted per plant (left panel) and area under disease progress curve (right panel) of the wild-type *Et-C1* strain BHKY, BHKYΔ*eop1*, and BHKYΔ*eop1* expressing homologous (*eop1*_{BHKY}) and heterologous (*eop1*_{MDCuke}) variants of *eop1* on muskmelon. The experiment was repeated four times with 5 to 10 replicates each; the results of one representative experiment are shown. **B**, In planta expression of *eop1* in the indicated cells was measured using real-time quantitative reverse-transcription PCR (RT-qPCR) and relative expression was calculated from the cycle threshold (C_T) values using expression of the *recA* gene for normalization; results are shown as 2^{-ΔΔC_T} values. The experiment was repeated twice with three technical replicates. Strains are indicated by the same color in all figures. Values shown are mean ± standard error of the mean, and were analyzed by analysis of variance (A, *P* = 0.0002; B, *P* = 0.03) followed by a Tukey's multiple comparison test.

In contrast, the loss of *dspF* reduced the virulence of SCR3 and MDCuke to a similar extent as the loss of *dspE* or *dspEF* (Fig. 8), suggesting that DspF is required for DspE secretion in the *E. tracheiphila* strains.

Fig. 5. Effector DspE contributes to the virulence of the *Et-melo* strains on **A** and **B**, muskmelon and **C** and **D**, cucumber. Plants were infected with wild-type strains and $\Delta dspEF$ mutants of SCR3 (**A** and **C**) and MDCuke (**B** and **D**). Comparisons were performed using Student's *t* tests ($P < 0.0001$).

DISCUSSION

This report presents the first study to explore the genetic basis of pathogenicity and host adaptation of *E. tracheiphila* and the contribution of individual *E. tracheiphila* T3SEs to virulence. Most gram-negative phytopathogens exploit T3SEs as part of a coevolutionary arms race in which the T3SEs influence pathogen virulence and plant recognition of the pathogen (Bastedo et al. 2020; Schreiber et al. 2021). Although the T3SS enables the introduction of T3SEs into living host cells, the conductive elements colonized by xylem pathogens are composed of dead cells. The T3SS of xylem pathogens that can invade nonvascular tissues may enable protein translocation into living cortical and mesophyll cells whereas, in the xylem, the T3SS may enable protein translocation at the pit membrane into the living parenchyma cells that surround the xylem vessels (Hilaire et al. 2001; Morris et al. 2018). *E. tracheiphila* has an unusual mix of traits among xylem pathogens, making it challenging to predict the role of the T3SEs. It is a xylem-restricted, obligately vector-transmitted pathogen that has a T3SS. Thus, it is distinct from *R. solanacearum* and *Xanthomonas campestris* pv. *campestris*, which are not xylem restricted; from *X. oryzae* pv. *oryzae* and *X. translucens* pv. *translucens*, which are xylem restricted but not vector transmitted; from *Xylella fastidiosa*, *Xanthomonas albilineans*, and gram-positive xylem pathogens, which all lack a T3SS; and from the closely related pathogens *P. stewartii* subsp. *stewartii* and *E. amylovora*, which cause wilt but are not

Fig. 7. Phylogeny of the AvrE effector family and their contributions to virulence. A maximum-likelihood tree of the AvrE sequences was generated. The DspE sequences of *Erwinia tracheiphila* strains SCR3 and BHKY (Supplementary Fig. S5) were obtained from their complete genome sequences, which we generated using the PacBio platform (unpublished data). GenBank accession numbers for the AvrE family effector proteins were: *E. tracheiphila* strain MDCuke DspE (AXF77194.1), *Pantoea agglomerans* pv. *gypsophillae* DspE (AAF76343.1), *P. stewartii* subsp. *stewartii* WtsE (AAG01467.2), *E. amylovora* DspE (CCO81193.1), *Pectobacterium carotovorum* DspE (CBI45053.1), *P. atrosepticum* DspE (WP_039292044.1), *Pseudomonas syringae* pv. *tomato* strain DC3000 DspE (AAF71499.1), *Ralstonia solanacearum* RipR (previously PopA) (WP_003262779.1), and *Xanthomonas vesicatoria* XopAM (EGD07146.1).

Fig. 6. Effector DspE and its chaperone DspF are required for pathogenicity of the *Et-C1* strain BHKY on **A**, squash, **B**, muskmelon and **C**, cucumber. A *BHKYΔdspE* mutant was similar to *BHKYΔdspEF* in its inability to wilt squash and muskmelon (data not shown); it was not examined on cucumber.

xylem restricted or obligately vectored (Bae et al. 2015; Eigenbrode et al. 2018; Yadeta and Thomma 2013). We used knowledge of the T3SEs in *E. amylovora* and *P. stewartii* subsp. *stewartii* to guide our studies based on both the phylogenetic relatedness of these pathogens to *E. tracheiphila* and the prior body of literature on the role of their T3SEs in host adaptation and virulence.

E. tracheiphila strains fall into phylogenetic clades that differ in their virulence patterns across cucurbit species (Nazareno and Dumenyo 2015; Rojas et al. 2013; Shapiro et al. 2018). We identified Eop1 as a candidate T3SE contributing to the host specificity of the *Et-melo* clade based on its role as a host-limiting factor in *E. amylovora* strains (Asselin et al. 2011). Here, we found that loss of Eop1 did not enable *Et-melo* clade strains to infect squash; thus, Eop1 did not have a detectable avirulence function in the *Et-melo* strains. This finding could reflect the absence of resistance genes in the squash cultivar

used, although we cannot discount that resistance resulted from an alternative T3SE. Our results are consistent with the absence of known genetic resistance to bacterial wilt in cucurbits.

Strains of the *E. tracheiphila* clade *Et-C1* exhibit a host preference indicated by their more rapid induction of wilt and, thus, greater aggressiveness on squash than on muskmelon. This type of host preference has been seen with other phytopathogens such as the cucurbit pathogen *Acidovorax avenae* subsp. *citrulli*, for which strains have similarly been divided into groups based on differential aggressiveness on distinct cucurbit genera (Ecksh-tain-Levi et al. 2014; Walcott et al. 2004). Loss of *eop1* did not influence the virulence of the *Et-melo* or *Et-C1* clade strains on any host, as is common in pathogens with many T3SEs due to the dispensability of individual T3SEs. In contrast, overexpression of *eop1* increased the virulence of an *Et-C1* strain on muskmelon, demonstrating a role for Eop1 in virulence. Interestingly, this contribution was specific to the Eop1 variant present in the *Et-melo* clade, because Eop1 from *Et-melo* but not from *Et-C1* increased the virulence of an *Et-C1*Δ*eop1* mutant. The *eop1* gene sequence was invariable within each clade, and the *eop1* gene from each clade was cloned into the same vector and expressed by the same overexpression promoter. The clade-specific Eop1 variants exhibited only four sequence polymorphisms, three single-amino-acid differences, and one two-amino-acid insertion-deletion, but none were in regions of known function. This result is concordant with a correlation among differences in Eop1 sequence and *E. psidii* aggressiveness on eucalyptus (Pereira et al. 2021), and suggests that minor sequence variations within YopJ-family effectors affect their impact on plant hosts. Collectively, our data demonstrate that Eop1 has distinct roles in the virulence of each clade on muskmelon, supporting the possibility that Eop1 is one factor contributing to adaptation of *E. tracheiphila* to this host.

We also demonstrated that the role of DspE in *E. tracheiphila* virulence differed in the two *E. tracheiphila* clades. A large reduction in *Et-melo* virulence upon the loss of DspE supports a central role for DspE in virulence; however, its residual virulence activity indicates that DspE is not absolutely required for the pathogenicity of the *Et-melo* clade, unlike the *Et-C1* clade and in all previous *Erwiniaceae* family phytopathogens examined. The *Et-melo* clade strains may have one or more other effectors that compensate for the loss of DspE, similar to a *Pseudomonas syringae* pv. *tomato* strain in which AvrE is functionally redundant with HopM1 (Badel et al. 2006; Xin et al. 2016), although *E. tracheiphila* strains lack an HopM1 homolog. The loss of DspE did not enable two *Et-melo* strains to wilt squash, which is consistent with the lack of evidence for a role for DspE homologs in effector-triggered immunity in *E. amylovora* and *Pantoea stewartii* subsp. *stewartii*. The difference in the requirement for DspE for virulence between the two *E. tracheiphila* clades identifies this as the only phytopathogenic species that includes both strains that do and strains that do not require an AvrE family member for disease induction. To date, a similar diversity of DspE functions has been detected only at the species level, namely, in *Pectobacterium carotovorum* and *P. atrosepticum* strains. Our data showing distinct roles of DspE in the *Et-melo* and *Et-C1* clades are consistent with the distinct T3SE repertoires in these clades, as highlighted in *E. tracheiphila* genomic sequence data (Shapiro et al. 2018).

Like many T3SEs (Ham et al. 2006), most T3SEs in the AvrE family are coexpressed with a chaperone that is required for efficient folding, stability, secretion, and translocation of the effector into host cells (Gaudriault et al. 2002). Studies with *E. amylovora* and *Pantoea stewartii* subsp. *stewartii* found that loss of DspE eliminated pathogenicity but loss of the coexpressed chaperone DspF did not (Ham et al. 2006; Triplett et al. 2008). More recently, three chaperones were shown to

Fig. 8. The chaperone DspF is required for the virulence function of DspE in the *Et-melo* strains MDCuke and SCR3 on **A** and **B**, muskmelon and **C** and **D**, cucumber. Plants were infected with wild-type strains and Δ*dspEF* mutants of SCR3 (**A** and **C**) and MDCuke (**B** and **D**). Comparisons were performed using one-way analysis of variance and a Tukey's multiple comparison test (**A**, $P < 0.0001$; **B**, $P < 0.0001$; **C**, $P = 0.001$; and **D**, $P < 0.0001$).

contribute additively to the secretion and translocation of DspE in *E. amylovora* (Castiblanco et al. 2018); these three chaperones include DspF, the Eop1 chaperone Esc1, and a chaperone to the T3SE Eop4. We found that two *Et-melo* strains exhibited a similar loss in virulence upon loss of DspF or DspE, indicating that DspF is sufficient for the secretion of DspE. Thus, even though these *Et-melo* strains encode an Esc1 chaperone, this chaperone did not measurably compensate for the loss of DspF as in *E. amylovora* (Castiblanco et al. 2018).

Due to the lack of known genetic resistance to bacterial wilt of cucurbits, this disease is managed in conventional agriculture primarily by insecticides that kill the cucumber beetle vectors (Rojas et al. 2015). Although Eop1 was shown to induce resistance in apple cultivars against the fire blight pathogen *E. amylovora*, we did not find evidence that Eop1-triggered immunity was involved in host specificity or host preference in the *E. tracheiphila* clades examined. Thus, these studies do not provide a foundation to support further search for such resistance. However, these studies have illustrated differences in the portfolio of effectors influencing the virulence of the two clades, with differences in both the function of homologous effectors and the functional redundancy within their effector repertoires. Knowledge of the molecular drivers of virulence in *E. tracheiphila* and their diversification among *E. tracheiphila* strains may help in identifying future management alternatives for bacterial wilt of cucurbits.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and inoculum preparation.

The bacterial strains and plasmids for this study are described in Supplementary Table S1. *E. tracheiphila* strains were grown in King's B (KB) medium (King et al. 1954) at 28°C, unless otherwise described. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. The following antibiotics were added, when needed, at 50 µg/ml: rifampin (Rif), kanamycin (Km), spectinomycin, and ampicillin (Amp). To prepare cells for plant inoculation assays, *Erwinia tracheiphila* strains were recovered from cryogenically preserved glycerol stocks (−70°C) on KB agar amended with Rif. The cells were incubated for 3 to 4 days, after which a single colony was transferred to a fresh plate to make a lawn. Cells from a 2-day-old lawn were suspended in 10 mM phosphate buffer (PB) and normalized to an optical density at 600 nm (OD₆₀₀) of 0.5 (approximately 2.5 × 10⁸ CFU/ml). A nonionic organosilicone surfactant (Silwet L-77) was added to each bacterial suspension at 0.02% (vol/vol).

Construction of deletion mutants and complementation constructs.

Deletion mutants were generated using the λ red recombinase system, which comprises recombinase components expressed under the control of an arabinose-inducible promoter on the plasmid pKD46 (Datsenko and Wanner 2000). PCR products were generated using primers (Supplementary Table S2) that target sequences that were conserved across the *Et-melo* and *Et-C1* clades. The PCR products used to generate the *dspEF* mutants included 1-kb regions flanking *dspEF* in the genomes, whereas the PCR products used to generate the *eop1*, *dspE*, and *dspF* mutants included 90-nucleotide (nt) regions flanking these genes in the genomes. The PCR products also contained a kanamycin cassette (*kan*) flanked by flippase recognition target (FRT) sites, generating chimeric PCR products that were subsequently introduced via electroporation into *E. tracheiphila* cells.

More specifically, the linear fragments used to generate the *ΔdspEF* mutants were constructed by using splice-overlap-extension PCR (Ho et al. 1989). PCR products containing the

1-kb regions upstream and downstream of *dspEF* were ligated to a FRT-*kan*-FRT cassette that was generated by PCR using pKD13 (Datsenko and Wanner 2000) as a template. The resulting fragment was cloned into pTOK2T (Chen et al. 2010). This plasmid was used as a template to generate a chimeric linear fragment, which was electroporated into cells of *E. tracheiphila* strains that had been transformed with pKD46 (Datsenko and Wanner 2000). The linear fragments used to generate the *Δeop1*, *ΔdspE*, and *ΔdspF* mutants contained the FRT-*kan*-FRT cassette that was amplified from plasmid pKD13 using primers that incorporated into the final product 90-bp sequences homologous to the regions immediately flanking the target genes in the genomes (Supplementary Table S2).

To generate mutants, *E. tracheiphila* cells containing pKD46 were grown at 28°C for 2 to 3 days in KB broth amended with Amp. When cells reached an OD₆₀₀ of 0.3, freshly made L-arabinose was added to a final concentration of 10 mM to induce expression of the λ phage red recombinase genes. Cells were harvested at an OD₆₀₀ of 0.5 and washed three times with room-temperature, sterile distilled water. The room-temperature wash was adopted because *E. tracheiphila* competency appeared to be reduced by washing in cold sterile water. After the washes, the cells were suspended in sterile distilled water and placed on ice for 15 to 20 min immediately prior to electroporation. The linear chimeric fragments that contained the flanked *kan* cassette were introduced into a high-density suspension (10¹⁰ CFU/ml) of ice-cold electrocompetent cells at a concentration of >5 µg per 100 µl of cells in a 0.2-cm cuvette using a gene pulser electroporator (Bio-Rad, Hercules, CA, U.S.A.). The high DNA density was achieved by pooling and concentrating products generated by multiple PCR reactions. Electroporated cells were transferred to 1 ml of ice-cold KB broth and incubated for 6 to 10 h, then plated on KB agar supplemented with Km. Mutants were confirmed by PCR using primers shown in Supplementary Table S2, and the *kan* cassette was removed by introduction of pFlp2Q (Wu et al. 2013).

To evaluate complementation of the *Δeop1* mutants, PCR fragments containing the *eop1* genes from MDCuke and BHKY were cloned into the *EcoRV* site of pN (Chen et al. 2010). These fragments included the regions upstream of the translation start sites to capture their native promoters; specifically, they included the complete 109-nt intergenic region along with 138 nt (MDCuke) or 131 nt (BHKY) of 3' terminus of the upstream gene. Plasmid pN is a derivative of the broad-host-range vector pME6041 (Heeb et al. 2000) that has a 733-bp *nptII* promoter inserted upstream of the multiple cloning site. The resulting constructs, pN-*eop1*_{MDCuke} and pN-*eop1*_{BHKY}, contained *eop1* under the control of the native promoter fused in tandem with the *nptII* promoter. These plasmids were introduced into the MDCuke*Δeop1* and BHKY*Δeop1* mutants by electroporation.

Plant growth conditions.

The cucurbit cultivars used in this study included muskmelon (*Cucumis melo* 'Athena'), zucchini squash (*Cucurbita pepo* 'Partenon F1'), and cucumber (*Cucumis sativus* 'Marketmore') (organically grown seed from Johnny's Selected Seeds, Winslow, ME, U.S.A.). Seeds were sown in 10-cm pots containing a matrix of peat moss, coarse perlite, and Metro-Mix 300 (Sun Gro Horticulture, Canada Ltd., Vancouver, BC, Canada). Seedlings were maintained in a growth chamber (Percival Scientific Inc.) at 28°C under a photoperiod of 12 h of light and 12 h of darkness and 70% relative humidity. Plants were watered every other day and fertilized with 15-5-15 NPK (Peters Excel; ICL Specialty Fertilizers) 2 days before inoculation.

Inoculation and disease assessment.

Plants were inoculated with *E. tracheiphila* when they were 2 weeks old. The site closest to the petiole of the adaxial surface

of the youngest fully expanded leaf was punctured with a florist's pin frog, as described previously (Liu et al. 2018), and 200 µl of cell suspension (approximately 2.5×10^8 CFU/ml) was applied to the punctured site. For negative controls, plants were inoculated with PB in the same manner. Inoculated plants were incubated as described for the seedlings and rated daily for wilt development based on the total number of leaves and the number of leaves wilted per inoculated plant. Once plants reached 100% wilt, newly emerging leaves were not counted and the plants were not monitored further. Data were collected daily for at least 2 weeks.

Evaluation of gene expression in cells recovered from the xylem.

To evaluate *eop1* expression in bacteria during growth in planta, 2-week-old seedlings of muskmelon were inoculated with BHKY, BHKYΔ*eop1*, BHKYΔ*eop1*(pN-*eop1*_{MDCuke}), and BHKYΔ*eop1*(pN-*eop1*_{BHKY}). At the first appearance of wilt symptoms, xylem sap was collected from infected plants following surface sterilization of an excised segment with 70% ethanol. The xylem sap was collected by manually squeezing the aerial portion of the cut stem and collecting the sap with a pipette. Immediately following xylem sap collection, the sap was combined with RNeasy Protect Cell Reagent (Qiagen) for 10 min at room temperature to preserve RNA integrity. The cells in this mixture were collected by centrifugation and the pellets were either used immediately for RNA extraction or frozen at -70°C for RNA extraction at a later time. RNA was extracted using an RNeasy Mini Kit (Qiagen) and the DNA was removed using an on-column DNase I digestion (Qiagen).

The *eop1* transcripts were measured using real-time quantitative reverse-transcription PCR (RT-qPCR) with the qScript One-Step SYBR Green RT-qPCR kit (Quantabio, Beverly, MA, U.S.A.). Relative expression was calculated from the cycle threshold (C_T) values, using expression of the *recA* gene for normalization. The primers 5'-GGGGTATGATGAATGAGATTCGGC-3' and 5'-ATTTCAGCGCATTACTCAGCGC-3' were used to evaluate expression of *eop1*, and 5'-CCATATCTACGGGTCTCTCTCGC-3' and 5'-AGCGATAACCTGCAAA GTCAGC-3' were used to evaluate expression of *recA*. In all, 100 µg of total RNA was used for each of three technical replicates, each in a total reaction of 25 µl. RT-qPCR was performed using the Mastercycler ep *realplex* thermocycler (Eppendorf) under the following conditions: 1 cycle at 95°C for 2.5 min, 40 cycles at 95°C for 15 s, and 1 cycle at 60°C for 30 s. The resulting C_T values were calculated using the relative standard curve method (Applied Biosystems 2016), in which the C_T value of each tested gene was normalized to the C_T values of *recA*.

Sequence alignment and generation of phylogenetic trees.

Protein sequences were aligned using Clustal Omega (Madeira et al. 2019) for visualization. Phylogenetic trees were generated by aligning sequences using MUSCLE (Edgar 2004) and applying the maximum-likelihood method using MEGA X (Kumar et al. 2018), with a Jones-Taylor-Thornton substitution model with uniform rates at all sites, a nearest-neighbor-interchange tree for inference, and bootstrapping with 1,000 replicates to generate consensus trees.

Statistical analyses.

Total and wilted numbers of leaves were used to calculate the proportion of wilted leaves per plant. Virulence was visually represented as disease progress curves (proportion of leaves wilted over time), which were quantified based on the area under the disease progress curve (AUDPC) as calculated in GraphPad (Prism 9 for Windows). Statistical comparisons were made using GraphPad. AUDPC values were evaluated for a normal

distribution with the Shapiro-Wilk test. For two-way comparisons, AUDPC values were subjected to a Student's *t* test (unpaired, two-tailed, equal variances) with a Welch's correction when the variances were not equal. For multiple comparisons, AUDPC values were subjected to an analysis of variance followed by a Tukey's multiple comparisons test.

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