The Type III Effector AvrBsT Enhances *Xanthomonas perforans* Fitness in Field-Grown Tomato

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Accepted for publication 11 June 2018.

ABSTRACT

Type III secretion system effectors contribute to pathogenicity through various mechanisms. Recent surveys showed an increasing prevalence of the type III secretion effector *avrBsT* among *Xanthomonas perforans* strains. We hypothesized that the acquisition of *avrBsT* has a fitness advantage for the pathogen. The contribution of *avrBsT* to fitness on tomato was evaluated based on disease severity, in planta growth, competition, and recovery rates of wild-type (WT) and *avrBsT* mutant strains in greenhouse and field plants. GEV872 and GEV1001, representative strains of two phylogenomic groups of *X. perforans*, were selected for generating *avrBsT* mutants. Disease severity was higher for WT strains compared with the *avrBsT* mutant

strains. *X. perforans* WT and *avrBsT* mutant strains did not differ following leaf infiltration of greenhouse plants in direct competition and in planta growth assays. The effect of *avrBsT* on pathogen fitness was noticeable under field conditions. Differences in strain recovery were significant, with WT being recovered two to eight times more than *avrBsT* mutant strains in the case of both strains GEV872 and GEV1001. WT strains were capable of spreading longer distances across field plots compared with *avrBsT* mutant strains. Findings suggest that the functional AvrBsT affects the fitness of *X. perforans* under field conditions, making it an ideal candidate for bacterial spot resistance breeding efforts in tomato.

Plant-pathogenic bacteria have evolved diverse strategies to suppress plant defense responses (Galán and Collmer 1999). Most gram-negative plant-pathogenic bacteria rely on the type III secretion system (T3SS) to suppress plant defenses (Gürlebeck et al. 2006). The T3SS is encoded by a large conserved gene cluster termed *hrp* (Alfano and Collmer 2004). The *hrp* gene cluster is essential for maintaining pathogenicity in several gram-negative plant-pathogenic bacteria (Alfano and Collmer 2004). The T3SS secretes effector proteins into plant cells that are responsible for modulating cellular processes, mimicking host proteins, and contributing to virulence (Jacques et al. 2016)). Plant-pathogenic bacteria can acquire novel effectors mainly through horizontal gene transfer (Alfano and Collmer 2004; Barak et al. 2016).

Bacterial spot of tomato (BST) in Florida is caused primarily by *Xanthomonas perforans* (Potnis et al. 2015). BST leads to high yield losses in the field due to defoliation (Pohronezny et al. 1986). *X. euvesicatoria* was the only causal organism of BST in Florida through the early 1990s but was gradually replaced by the introduction of *X. perforans* race T3 strains (Jones et al. 1998). *X. perforans* race T4 is the prevalent cause of BST, and other BST-causing *Xanthomonas* spp. such as *X. euvesicatoria*, *X. gardneri*, and *X. vesicatoria* were not recovered from tomato fields in Florida (Horvath et al. 2012; Schwartz et al. 2015; Stall et al. 2009). Phylogenomic analysis of a number of strains from recent surveys revealed the presence of two groups (1 and 2) within the T4 race

structure (Schwartz et al. 2015; Timilsina et al. 2015). Based on a comparative genomic study, the majority of the strains of both groups acquired a new effector, *avrBsT* (Schwartz et al. 2015; Timilsina et al. 2016). *avrBsT* was initially characterized from *X. euvesicatoria* in Early Cal Wonder (ECW) pepper plants (Minsavage et al. 1990). These *X. euvesicatoria* strains were pathogenic on tomato only and elicited a hypersensitive response (HR) on ECW pepper plants carrying the *Bst* resistance locus (Minsavage et al. 1990). *avrBsT* was initially identified in the first race T4 strain isolated in 1998 from Florida (Timilsina et al. 2016). Race T3 strains collected prior to 1998 did not carry *avrBsT* but T3 strains isolated later did (Timilsina et al. 2016). The presence of *avrBsT* in *X. perforans* limits its host range to tomato only (Minsavage et al. 1990). Furthermore, *avrBsT* is present in *X. vesicatoria* strains but not in *X. gardneri* (Kim et al. 2010; Schwartz et al. 2015).

AvrBsT (syn. XopJ2) is a plasmid-borne type III effector belonging to the XopJ clade within the YopJ superfamily of effectors that are present in human and plant bacterial pathogens (Ma and Ma 2016). The hallmark feature of this effector group is the presence of a conserved cysteine protease catalytic triad domain which is essential for cellular activity (Lewis et al. 2011; Orth et al. 2000). The XopJ effector group contains several characterized effectors such as XopJ1, AvrBsT (XopJ2), AvrRxv (XopJ3), AvrXv4 (XopJ4), and AvrXccB (XopJ5) (Büttner and Bonas 2010; Lewis et al. 2011; Ma and Ma 2016; Orth et al. 2000; White et al. 2009). Effectors of this family have diverse and unique cellular functions in planta even within the same species (Lewis et al. 2011; Szczesny et al. 2010). AvrBsT is not a core effector but has a conserved sequence and is commonly found in *X. perforans* and some strains of *X. vesicatoria* and *X. euvesicatoria* (Minsavage et al. 1990; Potnis et al. 2011; Schwartz et al. 2015; Stall et al. 2009; Timilsina et al. 2016). *X. euvesicatoria*, *X. vesicatoria*, or *X. perforans* carrying *avrBsT* can cause an HR in nonhosts such as *Arabidopsis thaliana* Pi-0 and *Nicotiana benthamiana* (Cunnac et al. 2007; Escobar et al. 2001; Kim et al. 2010; Orth et al. 2000; Schwartz

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Funding: This research is based upon work that is supported by the United States Department of Agriculture National Institute of Food and Agriculture under award number 2015-51181-24312.

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and four supplementary tables are published online.

et al. 2015). Deletion mutants of *avrBsT* in *X. vesicatoria* (strain BV5-4a) produced mild symptoms in pepper when infiltrated at 5×10^4 CFU/ml (Kim et al. 2010). *X. perforans* race T4, group 2 strains carrying nonfunctional *avrBsT* were virulent on pepper when inoculated with a nonfunctional *avrBsT* (Schwartz et al. 2015). However, *avrBsT* deletion mutants in *X. perforans* group 1 strains did not cause disease in pepper, indicating additional factors restricting host-range expansion in these strains (Schwartz et al. 2015). Furthermore, site-directed mutational analysis of the catalytic triad (His, Glu, and Cys) in the *avrBsT* gene abolished cell death but not effector delivery into plant cells (Cunnac et al. 2007; Orth et al. 2000). The catalytic triad in *AvrBsT* is essential for HR and cellular function and a single amino acid mutation in the catalytic triad abolishes HR ability in *N. benthamiana* and *A. thaliana* Pi-0 (Orth et al. 2000). Kim et al. (2010) demonstrated the requirement of a full-length copy of *avrBsT* for inducing HR in pepper.

AvrBsT interacts with a number of effectors and host proteins in planta (Han and Hwang 2017; Szczesny et al. 2010). Unlike other XopJ-like effectors, *AvrBsT* is capable of interfering with *AvrBs1*-induced HR, indicating a direct interference with effector-triggered immunity (ETI) through a possible interaction with cell signaling factors such as SNF1-related kinase 1 (Szczesny et al. 2010). *AvrBsT* has acetyltransferase activity and acetylates the acetylated interacting protein 1 protein which is involved in ETI and pathogen-associated molecular pattern-triggered immunity responses (Cheong et al. 2014). *AvrBsT* downregulates defense-related genes (*Senu4*, *Cevi16*, and *Tgas118*) in tomato (Kim et al. 2010), whereas the HR in pepper is accompanied with induction of defense-related genes (*CaBPR1*, *CaPO2*, *CaSAR82A*, and *CaDEF1*), electrolyte leakage, and hydrogen peroxide accumulation (Kim et al. 2010). *AvrXccB*, an *AvrBsT*-homolog, suppressed immunity and interacted with cellular proteins in *Brassica* plants (Liu et al. 2017).

Pathogen fitness is ideally measured as the ability of a pathogen to survive and reproduce on a particular host (Laine and Barrès 2013; Leach et al. 2001). According to Vanderplank (1968), pathogens carrying unnecessary virulence genes have a fitness cost or penalty. Furthermore, mutations in avirulence genes occur to increase fitness in the presence of a resistance (*R*) gene but can incur a fitness cost if an *R* gene is not present (Vanderplank 1968). Fitness in plant-pathogenic bacteria can be measured by examining several

factors such as the rate of aggressiveness, infection efficiency, population growth, and survival under adverse environmental conditions (Leach et al. 2001). Several avirulence genes have been studied for their contribution to pathogen fitness in *Xanthomonas* spp. causing bacterial spot (Kearney and Staskawicz 1990; Leach et al. 2001). The highly conserved avirulence gene *avrBs2* contributes to pathogenicity and its deletion negatively affects fitness by reducing in planta growth of *X. euvesicatoria* (Kearney and Staskawicz 1990). In contrast, *avrBs3* does not reduce bacterial populations and does not reduce aggressiveness but still contributes to pathogen fitness in the field (Wichmann and Bergelson 2004). Deletion of more than one effector gene in an *avrBs2* mutant strain resulted in an additive fitness cost (Wichmann and Bergelson 2004).

Most studies focus on evaluating the effect of type III effectors on pathogen virulence under controlled environmental conditions. Therefore, a knowledge gap exists in corroborating greenhouse findings with actual field conditions. Furthermore, fitness studies examine pathogen fitness within the context of gene-by-gene interaction, yet the rate of discovering novel effector genes resulted in a shortage of discovering corresponding *R* genes. As a result, using such *R*-gene-based approaches is inadequate or unavailable for evaluating specific and recently introduced effectors into a population. Nevertheless, the significance of possessing such effectors for pathogen virulence under field conditions remains crucial for disease management purposes. Therefore, in this study, we evaluated the effect of a type III effector, *AvrBsT*, under field conditions. We hypothesized that the ubiquitous presence of *AvrBsT* confers a selective advantage in the Florida *X. perforans* population and the loss of *avrBsT* should lead to a reduced pathogen fitness. For this purpose, we generated *avrBsT* deletion mutants in *X. perforans* strains representative of phylogenomic groups 1 and 2 and studied the effect of the *avrBsT* mutant strains on in planta growth, competition, movement, and epiphytic survival in comparison with the wild-type (WT) strain in tomato under field conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and plants. *X. perforans* strains GEV872 and GEV1001 were collected during a survey in 2012 from commercial grower fields (Schwartz et al. 2015). Strains GEV872 and GEV1001 were selected as representative strains of *X. perforans* phylogenomic groups 1 and 2 (Schwartz et al. 2015). *X. perforans* GEV872 and GEV1001 were induced for rifampicin resistance by plating a 10^8 CFU/ml bacterial culture of each strain on tryptone soy agar media amended with rifampicin at 200 mg/ml. All GEV872 and GEV1001 strains referred to hereafter are resistant to rifampicin. Plates were monitored over a period of 10 days and resistant colonies were selected and verified for rifampicin resistance. Antibiotics were added to media at the following concentrations: ampicillin at 100 µg/ml, rifampicin at 100 µg/ml, kanamycin at 100 µg/ml, spectinomycin at 100 µg/ml, and cycloheximide at 100 mg/ml. Bacterial strains, vectors, and plasmids used and constructed in this study are described in Table 1. *X. perforans* strains were cultured on nutrient agar (NA) or yeast glycerol agar at 28°C with or without amended antibiotics. Bacterial strains were cultured on NA at 28 and 37°C for *X. perforans* and *Escherichia coli*, respectively. Bacterial cultures used for inoculating plants were suspended in sterile tap water and the concentration was adjusted to 10^8 CFU/ml (optical density at 600 nm = 0.3) using a spectrophotometer.

Tomato cultivar HM1823 (HM Clause, Davis, CA) was used in field trials conducted at the Gulf Coast Research and Education Center. Tomato cultivars HM1823 and FL47 (Seminis, St. Louis) were used for in planta growth curve and coinfiltration assays. Both tomato cultivars do not have any tolerance to bacterial spot. Pepper (*Capsicum annuum* L.) cultivar ECW, carrying the *Bst* resistance locus, was used for determining HR.

Mutant construction. *X. perforans* strain GEV872 was used as a template for amplifying the full-length *avrBsT* with primers 872F

Table 1. List of bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source or reference ^b
<i>Xanthomonas perforans</i>		
GEV872	Wild type; Rif ^R	Schwartz et al. 2015; this study
GEV872 <i>avrBsT</i> del ₅₇₂₋₆₁₅ fs	<i>avrBsT</i> with an internal deletion and frameshift mutant; Rif ^R	This study
GEV1001	Wild type; Rif ^R	Schwartz et al. 2015; this study
GEV1001 <i>avrBsT</i> del ₅₇₂₋₆₁₅ fs	<i>avrBsT</i> with an internal deletion and frameshift mutant; Rif ^R	This study
<i>Escherichia coli</i>		
DH5α	F ⁻ <i>recA</i>	BRL
λpir	Host for pOK1; Sp ^f	UB
Plasmids		
pGEM-T	Amp ^R	Promega Inc.
pOK1	Suicide vector; SacB	Huguet et al. 1998
pRK2013	Helper plasmid; Kan ^R	Figurski and Helinski 1979

^a Rif^R, Sp^f, Amp^R, and Kan^R indicate resistant to rifampicin, spectinomycin, ampicillin, and kanamycin, respectively.

^b BRL = Bethesda Research Laboratories and UB = University of Berkley.

(CAAACATGCCTTCGGTGA) and 872R (ATGTGCATAGTGC GGTGCAT). An amplicon of 1,858 bp, including 0.5-kb flanking regions, was cloned into pGEM-T Easy vector and sequenced using F20 and R24 primers for sequence confirmation. A 44-nucleotide internal gene deletion (*avrBsT*₅₇₂₋₆₁₅) was introduced into p-GEM-T:*avrBsT* using internal primers with a *Hind*III unique restriction site (FBstDel: tatataagcttGGAGACATGGCGGTCATCCAAC and RBstDel: tatataagcttAGCCGGCCATAACCTTAATTTTCG) to produce p-GEM-T:*avrBsTdel*₅₇₂₋₆₁₅fs, resulting in a 12-amino-acid deletion and frameshift in the *avrBsT* open reading frame (ORF). The insert was sequenced, excised from the clone by digestion using restriction enzymes (*Apal*/*SpeI*), and subcloned into the suicide vector pOK1 restricted with *Apal*/*XbaI* to produce pOK1:*avrBsTdel*₅₇₂₋₆₁₅fs. Transformants were made in *E. coli* λ pir. pOK1:*avrBsTdel*₅₇₂₋₆₁₅fs was introduced into *X. perforans* by triparental mating using the helper plasmid pRK2013. Transformants carrying the mutated *avrBsT* were negatively selected using the *sacB* gene on NA plates amended with 5% sucrose and replicated on rifampicin-amended NA plates. Transformants with a complete double crossover that resulted in loss of pOK1 and, subsequently, *sacB* gene activity were selected from rifampicin-amended plates. The *avrBsT* deletion was introduced into *X. perforans* GEV872 and GEV1001 by two-step homologous recombination and confirmation of mutants was made by polymerase chain reaction (PCR), sequencing, and restriction digestion of the *avrBsT* locus (Huguet et al. 1998). All strains were stored as a 20% glycerol stock at -80°C .

In planta growth and competition assays. Strains were grown in nutrient broth (Difco Laboratories, Detroit) for 18 h, harvested by centrifugation, and resuspended in sterile tap water or 0.01 M MgSO_4 . WT strains, mutant strains, and water were infiltrated into ECW pepper leaves at a concentration of 5×10^6 and 5×10^8 CFU/ml. Four pepper leaves were infiltrated with each strain and water control at both concentrations and symptoms of HR were visually evaluated 1 to 3 days postinoculation (dpi). Pepper infiltration was conducted twice.

Bacterial suspensions of each strain were infiltrated separately in 4- or 6-week-old tomato FL47 or HM1823 seedlings at a concentration of 5×10^5 CFU/ml. Three leaves in multiple plants were infiltrated with each *X. perforans* strain (GEV872:WT, GEV872:*avrBsTdel*₅₇₂₋₆₁₅fs, GEV1001:WT, and GEV1001:*avrBsTdel*₅₇₂₋₆₁₅fs). Following inoculation, plants were incubated at 24 to 28°C . Three tomato leaflets were collected at a 24-h interval starting at 0 to 96 h after inoculation. Three leaf discs from inoculated leaves were collected for each infiltrated strain at each time point. Leaf discs (1 cm^2) were individually macerated in 1 ml of sterile tap water and a 10-fold serial dilution was plated on NA media amended with rifampicin. Plates were incubated at 28°C and colonies were counted after 48 to 72 h. CFU values were log-transformed and calculated as CFU/ cm^2 . In planta growth assays were repeated twice.

Furthermore, a comixture of *X. perforans* GEV872:WT + GEV872:*avrBsTdel*₅₇₂₋₆₁₅fs or *X. perforans* GEV1001:WT + GEV1001:*avrBsTdel*₅₇₂₋₆₁₅fs was infiltrated into 4-week-old tomato HM1823 seedlings at a 1:1 ratio and adjusted to 5×10^5 CFU/ml. Leaf discs (1 cm^2) were collected at 1, 3, and 5 or 1, 3, 5, and 7 dpi for the first and second assay, respectively. Two and three leaf discs were collected in the first and second assay, respectively. Leaf discs were processed as described above and diluted as necessary. Plates were incubated for 48 h at 28°C . Eight single colonies from each leaf disc representative of the comixture were isolated, sub streaked, and stored for further testing (see colony testing section).

Disease severity. *X. perforans* GEV872:WT, GEV872:*avrBsTdel*₅₇₂₋₆₁₅fs, GEV1001:WT, and GEV1001:*avrBsTdel*₅₇₂₋₆₁₅fs strains were individually spray inoculated until runoff on 4-week-old tomato seedlings at a concentration of 5×10^5 and 5×10^3 CFU/ml. Three plants were inoculated per strain. Plants were bagged for 48 h after inoculation. Plants were monitored daily for disease development. At 12 dpi, three leaflets per strain were detached and

evaluated for the area of disease using Assess 2.0 (American Phytopathological Society, St. Paul, MN).

Field trials. Three field trials were conducted during the 2015 (fall), 2016 (spring), and 2017 (spring) growing seasons at the Gulf Coast Research and Education Center in Balm, FL. Beds were fumigated, fertilized, sprayed with preplant herbicide, and covered with a white plastic mulch according to standard grower practices (Freeman et al. 2017). Gloves were changed between plots whenever touching plants was required to reduce cross contamination between plots. Plots were on 7.6-m-long beds with at least 15- to 21-m buffer zones between plots. Plants were obtained from a commercial grower. Tomato cultivars FL47 and HM1823 were used in the fall and springs seasons, respectively. Seedlings were transplanted at a 0.6-m distance within plots. Each plot contained 15 plants and treatments were set up in a randomized complete block design. The center plant of each plot was inoculated at a concentration of 5×10^6 CFU/ml. Each plot was inoculated with either *X. perforans* GEV872 or GEV1001. During fall 2015, four leaves of the center plant in each plot were individually infiltrated with *X. perforans* GEV872:WT and GEV872:*avrBsTdel*₅₇₂₋₆₁₅fs, or with GEV1001:WT and GEV1001:*avrBsTdel*₅₇₂₋₆₁₅fs. In the spring 2016 and 2017 trials, center plants were dip inoculated into a suspension containing a mixture of GEV872:WT + GEV872:*avrBsTdel*₅₇₂₋₆₁₅fs or GEV1001:WT + GEV1001:*avrBsTdel*₅₇₂₋₆₁₅fs, each adjusted to 5×10^6 CFU/ml. The suspension also contained 0.05% (vol/vol) Silwett L77 (Helena, Collierville, TN). All trials included noninoculated control plots.

Disease severity was evaluated weekly and simultaneously with sample collection according to the Horsfall-Barratt scale. In fall 2015, only symptomatic leaves were sampled every 7 days starting at 7 to 49 dpi. In spring 2016 and 2017, all leaves were sampled every 7 days starting at 7 to 70 dpi. Furthermore, noninoculated control plants were also sampled randomly throughout the trials. Four leaflets (approximately 2 g) were sampled from the new growth to avoid redundant sampling. Leaves were washed in an extraction buffer (0.01 M MgSO_4 + 0.2% Tween 20) (Sigma-Aldrich, St. Louis), and a 1-ml aliquot was sampled and diluted 10- to 200-fold. Only during the fall 2015 trial, the suspension was streaked using a sterile loop on unamended NA and rifampicin-amended NA. During the spring 2016 and 2017 trials, a 50- μl aliquot was plated using the exponential slow deposition mode in an Autoplate 4000 Spiroplater (Spiral Biotech, Norwood, MA) on unamended NA and rifampicin-amended NA. Plates were incubated for 2 days at 28°C . Bacterial concentrations were determined by counting colonies in the outer plate sectors in a grid, dividing the number of colonies by the volume deposited in the sector, and multiplying by the dilution factor for conversion into CFU/ml. The minimum detection threshold in our field trials was at 50 CFU/ml (approximately log 1.6). Plants with no CFU recovered were assigned the detection threshold for statistical analyses. Up to four single colonies, representative of each plant, were picked, sub streaked, and stored for further colony testing.

Cleaved amplified polymorphic sequence PCR on rifampicin-resistant colonies. Colonies collected from rifampicin-amended plates of leaf washings were tested using cleaved amplified polymorphic sequence (CAPS)-PCR for the presence of the full-length or truncated *avrBsT* in WT or mutant strains, respectively. The maximum number of colonies tested for each plot was 60, based on 4 colonies per 15 plants. DNA was extracted using the boiling method. Briefly, bacterial stocks of each single colony (total of approximately 6,000) were freshly streaked on NA amended with rifampicin. Using the tip of a loop, a small amount of pure bacterial growth was transferred into a 1.5-ml microcentrifuge tube and resuspended in 400 μl of sterile water. Tubes were boiled at 100°C for 15 min, then immediately placed on ice for 5 min, followed by centrifugation at 10,000 rpm for 5 min. A 2- μl aliquot of the supernatant was used in PCR. Primers 872F and 872R were used for amplifying the whole gene with flanking

regions (1,858 bp). PCR products were analyzed using agarose gel electrophoresis to confirm amplicon presence. Amplicons were then digested overnight using *Hind*III restriction enzyme and analyzed by electrophoresis on a 1% agarose gel. *X. perforans* colonies with a disrupted *avrBsT* ORF yielded two equal-sized bands (approximately 900 bp).

Statistical analyses. All statistical analysis were performed in SAS (v. 9.2; SAS Institute, Gary, NC). Generalized linear mixed models were employed using PROC GLIMMIX in SAS to determine the effect of treatments on response variables. Data were log transformed into a normal distribution prior to statistical analysis. Data were analyzed using a repeated measure model including a SPLICE function to compare treatments at individual intervals and to identify any treatment, time or time–treatment effect. Data were back-transformed and standard errors of the means were determined.

RESULTS

Loss of HR in pepper. To characterize the contribution of *avrBsT* to HR activity, pepper (ECW) carrying the BsT cognate resistance gene was infiltrated with four different *X. perforans* strains. *X. perforans* *avrBsT* mutants were generated in the background of two *X. perforans* strains, GEV872 and GEV1001. Leaves of pepper plants were infiltrated with WT strains of GEV872

and GEV1001. An HR was visible at 36 to 48 h after inoculation (Fig. 1A). Infiltrated areas were completely necrotic by 72 h. On the same leaf, *X. perforans* strains GEV872:*avrBsTdel*_{572-615fs} and GEV1001:*avrBsTdel*_{572-615fs} carrying an internal deletion of *avrBsT* did not elicit an HR response in ECW pepper plants carrying the cognate *Bst* resistance gene. The absence of HR activity in pepper indicated loss of *AvrBsT* function in the mutant strains of GEV872 and GEV1001.

In planta growth in tomato leaf infiltration assays. The effect of *avrBsT* deletion was compared with the full-length *avrBsT* in different strains using bacterial growth curves. The growth curves for the WT and *avrBsT* mutant *X. perforans* strains were determined by infiltrating each strain separately in tomato leaves. In leaf-infiltration assays, WT strains and mutant strains of GEV872 or GEV1001 did not show significant differences between populations (Fig. 1B). Populations initially started at approximately 3.4×10^4 and 2.7×10^4 CFU/cm² and then increased by 96 h to 1.7×10^8 and 3.2×10^8 CFU/cm² for GEV872:WT and GEV872:*avrBsTdel*_{572-615fs}, respectively. GEV1001:WT and GEV1001:*avrBsTdel*_{572-615fs} populations were initially at 2×10^4 and 5.5×10^4 CFU/cm² and reached 4.2×10^7 and 4.98×10^7 , respectively. Based on the growth curves herein, *avrBsT* does not affect bacterial populations sizes under these conditions.

In order to evaluate direct competition in planta, tomato leaves were coinfiltrated with rifampicin-resistant *X. perforans* strains

Fig. 1. In planta greenhouse assays of representative experiments. **A**, Infiltration of pepper Early Cal Wonder at 10^8 CFU/ml with *Xanthomonas perforans* wild-type (WT) and *avrBsTdel*_{572-615fs} of two representative genotypes (GEV872 and GEV1001) for evaluating hypersensitive response (HR) and loss of *avrBsT* function. Photos were taken 48 h after inoculation. Pepper infiltration experiments were repeated twice. **B**, Bacterial growth curve showing population dynamics after infiltrating tomato FL47 at 10^5 CFU/ml with representative isolates of *X. perforans* WT *avrBsT* and *avrBsTdel*_{572-615fs} strains in the greenhouse. GEV872: WT, GEV872:*avrBsTdel*_{572-615fs}, GEV1001:WT, and GEV1001:*avrBsTdel*_{572-615fs} were each infiltrated into separate leaves. Values at each point are the average of three replicates. In planta bacterial growth assays were repeated twice. Error bars refer to the standard error of the mean. **C**, Disease symptoms elicited by spray inoculation of tomato plants at 10^5 CFU/ml with two representative strains (GEV872 and GEV1001) of *X. perforans* WT and *avrBsTdel*_{572-615fs}. Photos were taken 12 days postinoculation (dpi). **D**, Measurement of diseased area on inoculated leaves at 12 dpi. Each treatment had three replicates. Asterisks above bars indicate significant difference at $P < 0.05$ between WT and mutants strains for GEV872 and GEV1001.

GEV872WT + GEV872:*avrBsTdel*_{572-615fs} or GEV1001WT + GEV1001:*avrBsTdel*_{572-615fs}. Eight randomly selected colonies were tested from each coinfiltrated leaf disc by PCR and restriction digestion. Three leaves from three different plants were tested for the presence of WT or mutant strains of *X. perforans* GEV872 or GEV1001. Coinfiltration assays showed no statistical differences between WT strains and mutant strains of GEV872 or GEV1001 in three and two trials, respectively (data not shown). Only in one trial, the frequency of GEV1001:WT was two- to threefold higher ($P = 0.0227$) than GEV1001:*avrBsTdel*_{572-615fs}. These results indicate the absence of direct competition between strains for survival.

Disease severity in greenhouse experiments. The disease severity of each strain was evaluated to assess whether disrupting *avrBsT* has an effect on symptom progression. Tomato seedlings were dip inoculated with *X. perforans* GEV872:WT, GEV872:*avrBsTdel*_{572-615fs}, GEV1001:WT, or GEV1001:*avrBsTdel*_{572-615fs} at 5×10^3 and 5×10^5 CFU/ml. Water-soaked lesions were observed starting at 4 and 10 dpi with plants inoculated with 10^5 and 10^3 CFU/ml, respectively (Fig. 1C). Necrotic lesions became well defined by 7 and 15 dpi, respectively. At 12 dpi, we found the necrotic area of the leaf caused by GEV872:WT and GEV1001:WT greater than that of the strains lacking *avrBsT* (Fig. 1D). The average diseased leaf area was 14.4 and 4.5% for plants inoculated with GEV872:WT and GEV872:*avrBsTdel*_{572-615fs}, respectively, at 5×10^5 CFU/ml. Similarly, the average diseased leaf area was 12.8 and 6.1% for strains GEV1001:WT and GEV1001:*avrBsTdel*_{572-615fs}, respectively. Similarly, plants infiltrated at a lower concentration of 10^3 CFU/ml showed a similar trend; however, with an overall lower number of lesions and diseased area for both WT and *avrBsTdel*_{572-615fs} strains (data not shown).

Disease severity and bacterial populations in field plants. Three field trials were conducted during fall 2015, spring 2016, and spring 2017 to evaluate the effect of *avrBsT* on several fitness factors. Center plants of each field plot were inoculated with a comixture of *X. perforans* GEV872:WT + GEV872:*avrBsTdel*_{572-615fs} or GEV1001:WT + GEV1001:*avrBsTdel*_{572-615fs}. A noninoculated control plot was also included to monitor bacterial spread into neighboring plots. None of the noninoculated plots showed any disease throughout the season. Initially and later during the season, disease severity was highest on the center inoculated plants. Disease severity and bacterial population levels across the noninoculated plants within each plot were determined to monitor *X. perforans* infection and spread in the field. Representative data for disease severity and bacterial populations are presented for spring 2017. In spring 2017, disease severity of field plants initially increased to approximately 10% for strains GEV872 and GEV1001 during the first 2 weeks and then dropped to 2 to 4% over the next 6 weeks of the field trial. At 63 dpi, weather conditions became more conducive and disease severity rapidly increased to 55%. Sharp increases in disease severity coincided with wet conditions. Disease severity and environmental conditions for all field trials are presented in Supplementary Figure S1.

Total bacterial populations of rifampicin-marked strains *X. perforans*. GEV872:WT + GEV872:*avrBsTdel*_{572-615fs} or GEV1001:WT + GEV1001:*avrBsTdel*_{572-615fs} were determined for each field plant to visualize bacterial spread across the field plots at a weekly interval. A subset of colonies representative of the bacterial population from each plant was stored and used for subsequent testing. Bacterial populations across trial plots initially exhibited a slightly bell-shaped distribution during the first few weeks, with the highest recovery of *Xanthomonas* levels occurring on plants within the center of the plot (Supplementary Fig. S2). Representative data from spring 2017 showed CFU levels of the center plants initially at 4.1×10^6 and 3.7×10^6 CFU/g of tissue but levels decreased within 14 days to 6.8×10^3 and 50 CFU/g of tissue for *X. perforans* GEV872 and GEV1001, respectively. Between 14 and 42 dpi, several plants had very low CFU levels and CFU values below the limit of detection except for the center inoculated plants.

At 21 and 28 dpi, GEV1001 was below the limit of detection and GEV872 was recovered at very low levels of 5×10^3 CFU/g of tissue. The recovery of GEV1001 and GEV872 increased by 49 dpi but was not significantly different from the levels during the first 2 weeks. At 56 dpi, CFU levels reached an average of 5.6×10^5 and 9.5×10^5 CFU/g of tissue for GEV1001 and GEV872, respectively. The recovery of both strains eventually plateaued after 56 dpi and levels were not significantly different from each other. There was no significant interaction for bacterial populations between plant distance from center inoculated plant and time in spring 2016 or 2017. Bacterial populations from spring 2016 showed similar trends.

Effect of *avrBsT* on *X. perforans* movement in field plants. A subset of bacterial colonies from each plant within each plot was further tested for identification of WT or mutant strains for *X. perforans* GEV872 and GEV1001. The movement of *X. perforans* WT and *avrBsTdel*_{572-615fs} strains was evaluated as a function of incidence over distance and time across the plot to either side of the center inoculated plant. GEV872 and GEV1001 showed similar movement trends across each plot in each trial (Fig. 2A and B). Slight variations occurred in the overall movement of strains in each trial; however, the overall trend across all three field trials consistently showed that WT strains of either GEV872 or GEV1001 moved greater distances than the mutants for each *X. perforans* genotypes. During fall 2015, GEV872:WT was initially recovered at distances not significantly different than GEV872:*avrBsTdel*_{572-615fs}. At 21 to 28 dpi, GEV872:*avrBsTdel*_{572-615fs} surpassed GEV872:WT in distance but was not statistically different (data not shown). At 43 dpi, GEV872:WT was detected at a distance 1.2 times greater than GEV872:*avrBsTdel*_{572-615fs}, whereas GEV1001:WT was capable of moving across plots at greater distances and was significantly ($P = 0.0004$) different than GEV1001:*avrBsTdel*_{572-615fs} across all time points (data not shown). Furthermore, between 7 and 28 dpi, GEV1001:*avrBsTdel*_{572-615fs} did not readily spread across the plots. By 36 dpi, GEV1001:*avrBsTdel*_{572-615fs} movement lagged behind that of GEV1001:WT. GEV1001:WT spread a distance of 1.7 times greater than the mutant strain along the plots (data not shown).

In spring 2016, *X. perforans* GEV1001:WT moved greater distances across the plots compared with *X. perforans* GEV1001:*avrBsTdel*_{572-615fs}, with significant differences at 21 and 42 dpi. Furthermore, GEV872:WT was detected at a further distance than GEV872:*avrBsTdel*_{572-615fs} at each time period except 21 dpi (Fig. 2A). Nevertheless, there were no statistical differences in movement between GEV872:WT and GEV872:*avrBsTdel*_{572-615fs} strains over time (Fig. 2A). In spring 2017, both GEV872:WT and GEV1001:WT spread significantly ($P = 0.0048$ and $P = 0.0085$, respectively) further than both *avrBsTdel*_{572-615fs} strains (Fig. 2B). At 7 dpi, GEV872:WT and GEV1001:WT spread a distance of 4 and 1.1 times more than GEV872:*avrBsTdel*_{572-615fs} and GEV1001:*avrBsTdel*_{572-615fs}, respectively. The *avrBsTdel*_{572-615fs} strains of either genotype were not able to surpass the spread of the WT strains at any time period in the spring 2016 and 2017 trials and only at one time period during the fall 2015 trial. Statistical analysis did not show an interaction between time and treatments in all trials (Supplementary Tables S1 and S2).

Effect of *avrBsT* on strain recovery in field plants. For comparing the frequencies of WT and mutants strains in field plants, rifampicin-resistant colonies were recovered from the leaf surface of symptomatic or nonsymptomatic leaves. A subset of the rifampicin-resistant colonies recovered from leaf washings from each field plant was tested for the full-length or truncated *avrBsT* gene using CAPS-PCR. This subset included four colonies of rifampicin-marked *X. perforans* for each plant in the plot at a weekly interval. The number of colonies recovered from each plot varied due to fluctuations in disease severity and epiphytic populations. In total, more than 6,000 rifampicin-resistant colonies were tested from all three field trials. In all three trials, WT strains were recovered at a higher frequency compared with *avrBsTdel*_{572-615fs} strains. In fall

2015, plots coinoculated with both WT and mutant strains showed that *X. perforans* GEV872:WT was recovered at a frequency significantly higher than *X. perforans* GEV872:*avrBsTdel*_{572-615fs} at 42 dpi ($P = 0.0052$) and 49 dpi ($P = 0.0219$) but not at 14 to 35 dpi (data not shown). On the other hand, GEV1001:WT strains were recovered at a higher frequency than GEV1001:*avrBsTdel*_{572-615fs} ($P = 0.0004$) (data not shown).

In spring 2016, there was a significant difference ($P = 0.0001$) between the frequency of WT and *avrBsTdel*_{572-615fs} strains of *X. perforans* GEV872 and GEV1001. At 21 dpi, frequency of GEV872:WT colonies were 1.1-fold higher compared with GEV872:*avrBsTdel*_{572-615fs} (Fig. 2C). However, after 28 dpi, the frequency of GEV872 WT colonies ranged between 2- and 4.4-fold higher than mutant strains. On the other hand, at 21 dpi, the frequency of GEV1001:WT colonies was 2.8-fold higher than GEV1001:*avrBsTdel*_{572-615fs}. GEV1001:WT decreased slightly to a 1.5-fold difference by 35 dpi and then increased to 3.1-fold difference after 56 dpi (Fig. 2C). A low number of colonies was tested at 42 dpi, coinciding with low total bacterial CFU (Fig. 2C).

In spring 2017, during the first 7 weeks, there was a low recovery rate of bacterial colonies (Fig. 2D), resulting in a low number of tested colonies for either WT or mutant strains. At 7 dpi, there was no significant difference between the frequency of WT or mutant strains. However, at 14 dpi, the frequency of *X. perforans* GEV1001:

WT strains was approximately twofold higher than the corresponding *X. perforans* GEV1001:*avrBsTdel*_{572-615fs}. At 21 and 28 dpi, GEV1001 was not recovered whereas GEV872 colony recovery was low (Fig. 2D). By 42 and 49 dpi, recovery of WT strains of each genotype was higher but not statistically different than the corresponding *avrBsTdel*_{572-615fs} strains. Recovery of *X. perforans* WT strains of GEV872 and GEV1001 significantly peaked at 56 dpi to approximately five- and sevenfold, respectively, compared with *avrBsTdel*_{572-615fs} strains. A slight decrease followed at 63 and 70 dpi in the frequency of WT colonies; however, the difference with *avrBsTdel*_{572-615fs} remained significant. Furthermore, only in the third trial (spring 2017), we recovered a very low number ($n = 39$) of rifampicin-resistant strains from the five noninoculated control plots at 70 dpi (data not shown). The recovered colonies from non-inoculated plots were dominantly carrying the full-length *avrBsT* gene ($n = 37$) whereas the remaining colonies carried a nonfunctional *avrBsT*. There was no interaction between time and treatments in all trials except for GEV1001 in spring 2017. The type III tests for fixed effects are included in Supplementary Tables S3 and S4).

DISCUSSION

Effector genes variably contribute to pathogen fitness and virulence levels. Effector genes are translocated into host cells by the T3SS

Fig. 2. Effect of *avrBsT* under field conditions. **A**, Recovery of *Xanthomonas perforans* GEV872 and GEV1001 wild-type (WT) and mutant (*avrBsTdel*_{572-615fs}) strains across field plots during 2016. **B**, Recovery of *X. perforans* GEV872 and GEV1001 WT and mutant (*avrBsTdel*_{572-615fs}) strains across field plots during 2017. **C**, Average number of colonies recovered for each WT and *avrBsTdel*_{572-615fs} strain during spring 2016. **D**, Average number of colonies recovered for each WT and *avrBsTdel*_{572-615fs} strain during spring 2017. Each data point is an average of five replicates. Each replicate contained 15 experimental units. Field trials were repeated three times and only results from two trials are presented in this figure. Error bars refer to the standard error of the mean.

which is regulated by *hrp* genes. It is well documented that that deletion of *hrp* genes of the T3SS in most plant-pathogenic gram-negative bacteria leads to a dramatic decrease or abolishment of infection (Gürlebeck et al. 2006). Furthermore, several effectors were shown to contribute to pathogen fitness in planta or epiphytically under greenhouse conditions and very few under field conditions (Hirano et al. 1999; Leach et al. 2001; Vera Cruz et al. 2000; Wichmann and Bergelson 2004). In this study, we examined for the first time the effect of *avrBsT*, an increasingly prevalent type III effector in the *X. perforans* population, on pathogen fitness at a large scale.

X. perforans mutant strains in this study were disrupted at the acetyltransferase catalytic domain of the gene, resulting in the abolishment of two essential amino acids responsible for cell death and auto- and transacetylation (Han and Hwang 2017). Therefore, based on our infiltration assays in pepper plants carrying the *BsT* resistance gene, our mutant strains did not elicit an HR, indicating gene inactivation and disruption of cellular catalytic activity. Comparison of population growth curves between strains carrying the full-length *avrBsT* gene and strains with a nonfunctional *avrBsT* gene did not affect bacterial growth in planta in the greenhouse. Therefore, *avrBsT* appears to be a nonessential virulence factor and does not have a fitness effect on in planta population under the conditions tested. Likewise, other studies showed that knocking out *avrBs1* and *avrBs3* in *X. euvesicatoria* does not alter in planta population sizes but does have a fitness cost in the field (Wichmann and Bergelson 2004). However, this is in contrast to another study in which *avrBsT* deletion mutant strains of *X. vesicatoria* showed a lower population than the WT strain (Kim et al. 2010). The effect of *AvrBsT* on fitness might be species or strain specific but further studies are needed for confirmation. However, our results are in line with those described elsewhere for other XopJ-like effectors (Ciesiolka et al. 1999; Jiang et al. 2009; Noel et al. 2003). Deletion mutants of *avrRxv* (Ciesiolka et al. 1999) and *avrXccb* (Jiang et al. 2009) in *X. euvesicatoria* and *X. campestris* pv. *campestris* and a frameshift deletion mutant of *XopJ* in *X. euvesicatoria* (Noel et al. 2003; Üstün et al. 2013) showed slight or no difference in bacterial populations compared with WT strains. Furthermore, a coinfiltration assay confirmed that there is no in planta interference between WT and *avrBsT* mutant strains. Evidence based on previous studies and within this study suggests that members of the XopJ family do not appear to have a significant effect on bacterial in planta growth.

Environmental conditions play a role in influencing pathogen population dynamics in the field. *Xanthomonas* spp. can spread between plant beds and throughout production fields by wind-driven rain, irrigation water that is contaminated with the bacteria, and handling of plants (Pohronezny et al. 1990). Throughout the three trials, prolonged dry periods reduced rates of pathogen movement throughout the field; however, after periods of wetness, disease severity increased. Because plants were coinoculated simultaneously, disease severity was not distinguishable between WT and mutant strains. Disease severity levels were also similar across different time periods between the group 1 and group 2 strains, indicating a uniform level of disease development in the field. Although disease severity was low at specific time periods, bacterial population levels on plant leaves remained high. When epiphytic populations were examined in the field plants, we observed a strong trend in which mutant strains of either genotype had lower recovery levels than WT strains. The reduction in epiphytic populations of strains lacking certain effectors is not unusual. For example, *X. campestris* pv. *malvacearum* mutant strains with a nonfunctional *avrB6* did not reduce pathogen population in planta; however, pathogen egress to the leaf surface was reduced (Yang et al. 1994). Similarly, *avrBsT* mutant strains appear to be significantly less prevalent as an epiphyte and, thus, disperse shorter distances than WT strains. As a result of higher epiphytic WT populations and conducive environmental conditions, WT strains were more readily disseminated across plants for both genotypes compared with strains lacking *avrBsT*.

Currently, there are no commercially deployed *R* genes against *avrBsT* and, therefore, the presence of *avrBsT* should incur a fitness cost with no selective advantage in *X. perforans*. Nevertheless, under the conditions explored in these experiments, *avrBsT* provides a benefit to *X. perforans* with no associated cost. We showed that deploying *avrBsT* mutant strains in the field incurred a fitness cost apart from the presence of an *R* gene. In this study, we used two genotypes representative of two phylogenomic groups and observed that *avrBsT* disruption in both genotypes is detrimental to fitness. Our data suggest that *avrBsT* might have a strain-dependent fitness cost; however, this needs to be confirmed in more strains of the same group. Strain-dependent virulence has been shown for *avrPto*, an effector in *Pseudomonas syringae* pv. *tomato* (Shan et al. 2000). Furthermore, it is worth noting that *X. perforans* group 2 strains might be more virulent compared with group 1, which might explain the higher prevalence of group 2 strains in Florida (P. Abrahamian, unpublished data). *avrBsT* is highly conserved and has no polymorphism based on recent comparative genomic studies in more than 100 strains (P. Abrahamian, unpublished data) (Schwartz et al. 2015; Timilsina et al. 2016). From a fitness cost perspective, the presence *avrBsT* should be dispensable for *X. perforans* but this is not the case for *avrBsT* based on our results in this work. The presence of this effector for almost 20 years in the *X. perforans* population makes this locus a good target in resistance breeding efforts. Sources of resistance to *avrBsT* were found in almost all pepper lines and in *Solanum lycopersicoides* and a line of *S. lycopersicum* (Wang 1992). Future work should focus on mapping the *Bst* locus in tomato and introgress the *R* gene into commercially grown tomato cultivars. Pyramiding resistance genes against other conserved bacterial loci such as *avrBs2* and *avrXv4* is essential to reduce resistance breakdown based on single-locus resistance.

ACKNOWLEDGMENTS

We thank R. Willis, S. Hughes, S. Kalb, K. Triner, C. Land, and A. Riniker for excellent technical assistance.

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