***Pseudomonas syringae* CC1557: A Highly Virulent Strain With an Unusually Small Type III Effector Repertoire That Includes a Novel Effector**

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Both type III effector proteins and nonribosomal peptide toxins play important roles for *Pseudomonas syringae* pathogenicity in host plants, but whether and how these pathways interact to promote infection remains unclear. Genomic evidence from one clade of *P. syringae* suggests a tradeoff between the total number of type III effector proteins and presence of syringomycin, syringopeptin, and syringolin A toxins. Here, we report the complete genome sequence from *P. syringae* CC1557, which contains the lowest number of known type III effectors to date and has also acquired genes similar to sequences encoding syringomycin pathways from other strains. We demonstrate that this strain is pathogenic on *Nicotiana benthamiana* and that both the type III secretion system and a new type III effector, *hopBJ1*, contribute to pathogenicity. We further demonstrate that activity of HopBJ1 is dependent on residues structurally similar to the catalytic site of *Escherichia coli* CNF1 toxin. Taken together, our results provide additional support for a negative correlation between type III effector repertoires and the potential to produce syringomycin-like toxins while also highlighting how genomic synteny and bioinformatics can be used to identify and characterize novel virulence proteins.

Pseudomonas syringae, a bacterial phytopathogen of many crop species, utilizes a diverse arsenal of virulence factors to infect host plants (Hirano and Upper 2000; O'Brien et al. 2011). Much of the previous research on pathogenicity in *P. syringae* has focused on identifying and characterizing virulence factors within strains and on categorizing their presence across the species (Baltrus et al. 2011; Hwang et al. 2005; Lindeberg et al. 2012; O'Brien et al. 2011). Although this accumulation of knowledge has enabled tests of protein function during infection, many questions remain as to how virulence pathways interact and evolve at a systems level (Baltrus et al. 2011; Cunnac et al. 2011; Hwang et al. 2005). In the absence of direct tests, identification of similar genomic trends across independent

lineages provides strong support for the presence of such interactions over evolutionary time scales.

One of the main contributors to pathogenicity within *P. syringae* is a type III secretion system (T3SS) (O'Brien et al. 2011). The T3SS encodes a structure that translocates bacterial effector proteins (type III effectors [T3E]) into host cells to disrupt host physiological pathways and enable successful infection (Lindeberg et al. 2012). *P. syringae* genomes typically contain approximately 10 to 40 total T3E, the exact actions of which depend on the totality of effector repertoires as well as host genotype (Baltrus et al. 2011; Lindeberg et al. 2012; O'Brien et al. 2011). Another main contributor to pathogenicity within many *P. syringae* strains is nonribosomal peptide (NRP) toxin pathways. NRP pathways encode proteins that act as an assembly line, elongating and decorating peptide chains involved in pathogenicity (Bender et al. 1999). NRP pathways are often not conserved between closely related strains but, even if present, can differ in their outputs based on nucleotide polymorphisms (Baltrus et al. 2011; Bender et al. 1999; Hwang et al. 2005).

Multiple reports have suggested functional overlap or phenotypic interactions between T3E and NRP pathways. For instance, virulence functions of the toxin coronatine can be partially restored by the toxin syringolin A as well as T3E HopZ1 and AvrB1 (Cui et al. 2010; Jiang et al. 2013; Melotto et al. 2006; Schellenberg et al. 2010). Furthermore, within multilocus sequence analysis (MLSA) group II *P. syringae* strains as defined by Sarkar and Guttman (2004), there exists a negative correlation between presence of conserved toxin pathways (syringolin A, syringopeptin, and syringomycin) and size of the T3E repertoire compared with other phylogenetic clades (Baltrus et al. 2011) (Supplementary Fig. S1). In fact, MLSA group II strains possess the lowest number of T3E among analyzed genomes across the species. This correlation is strengthened by the observation that strains from pv. *pisi* possess some of the largest T3E repertoires within MLSA group II and have lost all three toxin pathways (Baltrus et al. 2013). Whether this negative correlation between NRP and T3E reflects an unrecognized difference in disease ecology is yet to be determined, although strains from MLSA group II are thought to survive as epiphytes much better than other clades within *P. syringae* (Feil et al. 2005).

The hypothesis of a phenotypic tradeoff between T3E and NRP pathways would be bolstered by discovery of an independent clade of *P. syringae* that has gained toxins similar to those of the MLSA group II strains but which also possesses a reduced effector repertoire. We have analyzed the genomes of a small clade of *P. syringae* isolated from environmental

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sources (Baltrus et al. 2014; Morris et al. 2008) and have found independent evidence supporting this genomic trend. Specifically, compared with a closely related outgroup, genomes for two strains appear to have lost T3E while gaining a path-

Fig. 1. *Pseudomonas syringae* CC1557 virulence in *Nicotiana benthamiana* is type III-secretion dependent. Growth of wild-type *P. syringae* CC1557 as well as an *hrcC* mutant was measured in planta 3 days post-syringe inoculation. Measurements are based on three independent experiments with at least four replicates in each. Bacterial population sizes are significantly different (Tukey's honestly significant difference, $P < 0.05$) between strains. Error bars represent two standard errors. Dashed lines indicate approximate population sizes at day 0, which were not significantly different.

way which potentially encodes proteins for the production of a syringomycin-like toxin. We demonstrate that one of these strains, CC1557, can infect *Nicotiana benthamiana* and cause disease in a T3SS-dependent manner. We further show that virulence of this strain is significantly increased by the presence of one new T3E effector, HopBJ1, which shares similar structure and catalytic residues with the CNF1 from *Escherichia coli*. Therefore, independent evidence suggests that acquisition of NRP pathways correlates strongly with the loss of T3E families and further strengthens the idea of an ecological or evolutionary tradeoff between these virulence factors.

RESULTS

A complete genome sequence for *P. syringae* CC1557.

2008). This genome consists of a 5,728,024-bp chromosome and a 53,629-bp plasmid (GenBank accession number AVEH02000000). According to PGAAP annotation (Angiuoli et al. 2008), this chromosome contains 5,001 open reading frames (ORF) while the single plasmid contains an additional 67 ORF.

P. syringae CC1557 can infect and cause disease in *N. benthamiana*.

P. syringae CC1557 was originally isolated from snow, whereas the closely related strain CC1466 was originally isolated from asymptomatic *Dodecantheon pulchellum*, a perennial herb (Morris et al. 2008). Using syringe inoculation under standard conditions, we have demonstrated that *P. syringae*

Fig. 2. Diversity of the CEL locus across *Pseudomonas syringae* sensu lato. **A**, Bayesian phylogenies for AvrE (left), HrpW (middle), and concatenated multilocus sequence analysis (MLSA) loci (right), for a subset of *P. syringae* strains. Posterior probabilities for all nodes are >0.95 . Scale bars indicate number of amino acid changes. Phylogenetic patterns for both of these loci approximate relationships based on core genomes and MLSA loci, except that HrpW from *P. syringae* CC1583 clusters more closely with *P. syringae* pv. *tomato* DC3000. This suggests a recombination event at this locus within *P. syringae* CC1583. **B**, Genomic context of the CEL across *P. syringae* sensu lato. In most strains, *hopM1* is bordered by *avrE* and *hrpW*. Some *P. viridiflava* strains lack *hopM1* (Bartoli et al. 2014), while CC1557 and CC1466 have replaced this region with *hopBJ1*. Arrows in represent open reading frames while the gap and triangle represent a loss of this region within strains CC1524 and CC1417.

CC1557 can grow to high population sizes in the apoplast of *N. benthamiana* after 3 days of infection (Fig. 1). This rate of growth is similar to the well-established pathogenic strain for *N. benthamiana*, *P. syringae* pv. *syringae* B728a (Supplementary Fig. S2). Furthermore, these large bacterial population sizes cause disease symptoms as shown by the visibility of tissue collapse (Fig. 2). High levels of growth and tissue collapse are both eliminated by deleting the *hrcC* gene (Figs. 1 and 2), which encodes a main structural protein for the type III secretion pilus. Therefore, CC1557 virulence in *N. benthamiana* under laboratory conditions is T3SS dependent.

The genomes of *P. syringae* CC1557 and CC1466 encode a reduced T3E repertoire.

We used bioinformatic methods as per Baltrus and associates (2011) to search the complete and draft genomes of *P. syringae* strains CC1557 and CC1466, and the closely related strain CC1583, for known T3E families (Baltrus et al. 2014). We found a total of four T3E shared across these three genomes, with eight T3E found only within CC1583 (Table 1). Therefore, together with HopBJ1 (see below), the genomes of CC1466 and CC1557 appear to encode a total of five potential T3E from known protein families. This stands in contrast to the immediate outgroup strain CC1583, which encodes a total of 12 potential T3E from known protein families.

P. syringae CC1466 and CC1557 have horizontally acquired pathways for production of NRP that resemble syringopeptin and syringomycin.

We used tBLASTn searches of the complete genome sequence of CC1557 in order to identify potential NRP toxin pathways, as per Baltrus and associates (2011). This genome contains highly similar full-length tBLASTn matches to a variety of proteins involved in syringomycin synthesis: SyrB (93% protein sequence identity), SyrC (99%), and SyrP (93%) (data not shown). Further investigation of all complete genomes for *P. syringae* demonstrates that the region containing putative NRP-related loci in CC1557 is found in approximately the same genomic context on the chromosome as the syringomycin-syringopeptin pathways in *P. syringae* pv. *syringae* B728a (Supplementary Fig. S3). Moreover, the genomic context surrounding these toxin pathways is conserved throughout *P. syringae*. Although regions of high similarity to the syringopeptin pathway do not appear to be present within CC1557, analysis of gene annotations suggests that a second NRP pathway occurs immediately downstream of the syringomycin-like region on the chromosome of CC1557 (data not shown). Although it is difficult to identify the NRP product of these CC1557 pathways by sequence alone, draft genomes sequences

suggest that this region is present in CC1466 but not CC1583. The most parsimonious explanation for this phylogenetic signal is that an immediate ancestor of CC1466 and CC1557 acquired this NRP region by horizontal transfer after divergence from the CC1583 lineage.

A new T3E protein family is present in the genomes of both *P. syringae* CC1466 and CC1557.

Similarity searches of the *P. syringae* genomes demonstrated that *hopM1*, which encodes an effector protein largely conserved throughout *P. syringae*, had likely been lost within strains CC1466 and CC1557. Further investigation demonstrated that an ORF encoding a hypothetical protein had replaced *hopM1* within these genomes (Fig. 2). In order to test whether this unknown protein is translocated into plant cells, we cloned the region encoding the predicted ORF into the translocation test vector pBAV178 and tested for delivery the C-terminal fragment of AvrRpt2 as a fusion protein. We found that the resulting HopBJ1-AvrRpt2 fusion protein triggered cell death in both *Arabidopsis thaliana* Col-0 wild type and *rps2* lines, indicating that HopBJ1 itself was capable of triggering cell death in the absence of recognition of AvrRpt2 (Table 2; Fig. 3B). In order to verify that HopBJ1 triggered cell death without AvrRpt2, we delivered HopBJ1:HA into a variety of plant genotypes using *P. syringae* pv. *tomato* DC3000 D28E (Cunnac et al. 2011). Delivery of HopBJ1:HA can cause tissue collapse in a variety of *Arabidopsis* accessions (Table 2). All genotypes tested displayed strong cell death by either 24 or 48 h post-inoculation.

HopBJ1-induced cell death is not dependent on the known resistance (*R*)-gene pathways because tissue collapse still occurs in *eds1*, *pad4*, or *rar1* mutants (Table 2). HopBJ1-induced cell death also occurs in plants expressing the salicylic acid-degrading enzyme NahG (Table 2). However, this rapid tissue collapse fails to occur out of a *hrcC*- background of *P. syringae* pv. *tomato* DC3000, indicating that HopBJ1 secretion is dependent on a functioning T3SS (data not shown). The genomic region immediately upstream of *hopBJ1* possesses a

Table 1. Type III effector family distribution^a

Effector name	CC1557	CC1466	CC1583
<i>avrE</i>	P	P	P
<i>hopF2-1</i>	–	–	P
<i>hopF2-2</i>	–	–	P
<i>hopM1</i>	–	–	P
<i>hopY1</i>	P	P	P
<i>hopAA1</i>	–	–	P
<i>hopAG1</i>	–	–	P
<i>hopAH1</i>	–	–	P
<i>hopAH2</i>	P	P	P
<i>hopAII</i>	–	–	P
<i>hopAS1</i>	–	–	P
<i>hopBF1</i>	P	P	P
<i>hopBJ1</i>	P	P	–

^a The letter P denotes presence of this particular effector family, whereas the symbol – denotes absence.

Table 2. Responses of *Arabidopsis* accessions to HopBJ1^a

<i>Arabidopsis</i> accession	Reaction
Col	++
Col <i>rps2</i>	++
Ws	++
Ws <i>eds1</i>	++
Ws <i>pad4</i>	++
Ws <i>rar1</i>	++
Ws <i>NahG</i>	++
Ag-0	++
Bur-0	+
Can-0	+
Ct-1	++
Edi-0	++
Hi-0	+
Kn-0	+
Ler-0	++
Mt-0	++
No-0	++
Oy-0	++
Po-0	++
Rsch	++
Sf-0	+
Tsu-0	+
Wil-2	+
Ws-0	+
Wu-0	+
Zu-0	+

^a Gene names next to accession indicate mutant lines; ++ and + indicate hypersensitive response in 24 and 48 h, respectively.

Fig. 3. Protein motifs and catalytic residues within HopBJ1 are similar to the *Escherichia coli* CNF1 toxin. **A**, Despite no sequence similarity between HopBJ1 and CNF1 by BLASTp searches, modeling of tertiary protein structure demonstrates limited similarity in protein structure between the two. Importantly, the catalytic dyad of *E. coli* CNF1 (Cys866-His881) perfectly lines up with a putative catalytic dyad within HopBJ1 (Cys174 and His192). **B**, All constructs of HopBJ1 can be translocated by *P. syringae* pv. *tomato* DC3000 D28E (Cunnac et al. 2011) into *Arabidopsis*. Top: translocation tests into accession Col-0. Bottom: translocation tests into *rps2-101C* mutant plants. Translocation of AvrB is shown as a positive control and causes hypersensitive response in both plant backgrounds, while the DC3000 D28E mutant causes no cell death. HopBJ1 causes rapid cell death across both plant backgrounds when translationally fused to the C-terminus of AvrRpt2, whereas putative catalytic mutants only show cell death in Col-0. Picture is representative of 9 to 12 independent leaves per strain-plant combination. **C**, Putative catalytic mutants of HopBJ1 cannot complement the growth defect of a CC1557 *hopBJ1* deletion strain. **D**, Translocation of HopBJ1 but not either of the two putative catalytic mutants lowers growth of DC3000 on *Arabidopsis* Col-0. **E**, Wild-type and the complemented Δ hopBJ1 deletion of strain CC1557 cause tissue collapse in *Nicotiana benthamiana*. Neither of the putative catalytic mutants complements the loss of virulence of the Δ hopBJ1 deletion strain. Picture is representative of multiple assays. **F**, Transient expression of HopBJ1 but not the putative catalytic mutants by *Agrobacterium* spp. in *N. benthamiana* causes cell death. Western blots and Ponceau staining for each of these *Agrobacterium* infiltrations are displayed below. Picture is representative of multiple assays. Each growth curve experiment consisted of at least two independent trials but representative data from only one trial is shown in each case. Letters indicate significant differences between bacterial population sizes between strains ($P < 0.05$, Tukey's honestly significant difference). Dashed lines indicate approximate population sizes at day 0, which were not significantly different.

canonical *hrp*-box sequence (Supplementary Fig. S4) and, therefore, regulation of this gene likely takes place through the action of the σ factor HrpL (Mucyn et al. 2014). Therefore, we conclude that this novel ORF encodes a new putative T3E family, HopBJ, and that this family possesses broad cytotoxicity. Further studies in diverse plant hosts will be required to determine the full extent of HopBJ cytotoxic activity.

Deletion of HopBJ1 from *P. syringae* CC1557 causes loss of disease symptoms and lowers bacterial populations in planta.

We have created a deletion mutant of *hopBJ1* in *P. syringae* strain CC1557 and have performed growth curves in planta using syringe infiltration. Deletion of *hopBJ1* causes a repeatable loss of growth in planta after 2 days of infection (Fig. 4A). We further find that deletion of *hopBJ1* leads to a loss of the tissue collapse phenotype after 3 days (Fig. 4B). We have been able to complement both of these phenotypes in planta through expression of *hopBJ1* using its native promoter in trans (Fig. 4).

HopBJ1 resembles cytotoxic genes present within other species.

Currently, the closest BLASTp hits for HopBJ1 are hypothetical proteins from *Serratia marcescens* (34% identity, YP_007346200.1) and *Hahella chejuensis* (30% identity, YP_435189.1). However, modeling of protein structure using the Phyre2 web server demonstrates that HopBJ1 displays limited similarity to the *E. coli* CNF1 toxin (Fig. 3A). Surprisingly, amino acids critical for the deamidase functions of CNF1 appear to be conserved in HopBJ1 (Fig. 3A) as well as in similar proteins from *S. marcescens* and *H. chejuensis* (Buetow et al. 2001) (data not shown). This modeling of protein structure further suggests that C174 and H192 are a functional catalytic dyad within HopBJ1.

Predicted catalytic residues of HopBJ1 are required for effector function.

To test for functionality of predicted catalytic residues for HopBJ1, we measured the effect of putative catalytic dead mu-

tants (C174S and H192A) in planta. Mutant versions of HopBJ1 are successfully translocated by strain DC3000 D28E into *Arabidopsis*, triggering RPS2-dependant cell death in Col-0. The wild-type HopBJ1:AvrRpt2 fusion also causes tissue collapse in *rps2-101c* plants, consistent with the ability of HopBJ1:HA to trigger cell death. (Fig. 3B). Moreover, both catalytic dead mutants are unable to complement virulence defects of a *hopBJ1* deletion in CC1557 in *N. benthamiana* (Fig. 3C and E). Both mutants, unlike the wild-type version, also fail to lower the growth of DC3000 in *Arabidopsis* (Fig. 3D) and fail to cause tissue collapse when transiently expressed in *N. benthamiana* (Fig. 3F). Therefore, as with similar residues in *E. coli* CNF1 (Buetow et al. 2001), we conclude that both C174 and H192 are required for the function of HopBJ1.

DISCUSSION

Recent advances in genome sequencing have spurred great interest in conservation and diversification of virulence pathways across *P. syringae* (O'Brien et al. 2011; Studholme 2011). Although genome gazing often leads to the identification of trends across a species, single observations must be treated with skepticism in the absence of additional data. One particularly interesting yet relatively little understood pattern across genomes involves a negative correlation in MLSA group II between size of T3E repertoire and presence of NRP pathways (Baltrus et al. 2011). Importantly, this trend does not appear to be a result of sampling bias or failure to discover novel T3E because strains within this clade have been thoroughly screened at both the genetic and transcriptome level (Baltrus et al. 2011; Mucyn et al. 2014). Indeed, the entire *hrpL* regulon, the major transcriptional regulator for all *P. syringae* T3E, is reduced within MLSA group II compared with other clades (Mucyn et al. 2014).

Strains CC1466 and CC1557 diverge early in the phylogeny of *P. syringae* sensu lato, and have not been isolated from diseased plants in nature (Morris et al. 2008). We have shown that CC1557 can act as a pathogen of *N. benthamiana* and, as with all other phytopathogenic *P. syringae* strains, that virulence

Fig. 4. HopBJ1 is a virulence factor for *Pseudomonas syringae* CC1557 in *Nicotiana benthamiana*. **A**, We created a deletion mutant of *hopBJ1* in *P. syringae* CC1557, and complemented this strain by expressing *hopBJ1* from a plasmid using the native promoter. Deletion of *hopBJ1* lowers growth of *P. syringae* CC1557 3 days postinoculation. Both of these phenotypes are complemented by expression of wild-type *hopBJ1* in trans. Three independent experiments were performed with at least four replicates in each treatment. Letters represent statistical differences within an analysis of variance (Tukey's honestly significant difference, $P < 0.05$). Error bars represent two standard errors. Dashed lines indicate approximate population sizes at day 0, which were not significantly different. **B**, Leaves from 2-week-old *N. benthamiana* plants were syringe inoculated with various strains derived from *P. syringae* CC1557. Three days after inoculation, plants showed evidence of tissue collapse dependent on a functioning type III secretion system (compare wild-type versus *hrcC* mutant) as well as HopBJ1 (compare *hopBJ1* deletion strain versus *hopBJ1* complement). Picture is representative of multiple trials.

requires a functional T3SS (Fig. 1). We have found that both of these genomes contain few T3E, with only five loci known to be present and shared by both (Table 1). Genomic comparisons between these two strains have further demonstrated that both contain an NRP toxin pathway very similar to those encoding syringomycin. As is the nature of NRP toxins, even a single amino acid change can have dramatic consequences on the final toxin product chemistry (Bender et al. 1999); however, it is important to note that many of the predicted ORF in these regions are highly similar (>90% protein identification) to syringomycin ORF present within MLSA group II strains. Comparison with closely related outgroup strains demonstrates that acquisition of this NRP pathway is evolutionarily correlated with loss of T3E families. Indeed, the most closely related sequenced outgroup strain, CC1583, lacks this NRP pathway and contains twice the number of known T3E loci (Table 1). As such, we interpret these patterns as independent evidence suggesting an evolutionary or ecological tradeoff between presence of syringomycin and number of T3E within the strain. We also note that there appears to be an additional, uncharacterized NRP pathway downstream of the syringomycin-like region within CC1557 (data not shown). Directed experiments will need to be performed to assess the contribution of these NRP pathways to virulence.

What selective pressure could underlie such a negative correlation between T3E and NRP toxins? It is possible that the functions of these NRP toxins are redundant with a specific suite of T3E. In this case, T3E loss can take place because there is no longer positive selection to counter selection against evolutionary (such as recognition by the plant immune system) or physiological costs. For MLSA clade II and CC1557, acquisition of syringomycin and, potentially, additional NRP pathways could render the functions of some T3E obsolete. If this is the case, we further predict that the potential second NRP toxin pathway (downstream of the syringomycin-like pathway in CC1557) could have functions similar to those of syringopeptin. It is also possible that disease ecologies of most MLSA group II strains, as well as CC1466 and CC1557, differ compared with other focal *P. syringae* strains. We have a rudimentary understanding of differences in disease ecology throughout *P. syringae* pathogens, although it is recognized that differences do exist across the phylogeny (Clarke et al. 2010). Specifically, one clade of strains from MLSA group II has exchanged the canonical *P. syringae* T3SS with a second divergent T3SS at an independent genomic location while still maintaining toxin pathways. Although these strains can grow in planta, it is unknown whether they can cause disease (Clarke et al. 2010). Furthermore, infection of woody hosts has arisen independent times on multiple hosts yet such strains are still dependent on a functioning T3SS (O'Brien et al. 2011; Ramos et al. 2012). Strains that contain syringopeptin and syringomycin potentially cause disease on a wider host range than other groups, which may be enabled by the generality of NRP toxins (Baltrus et al. 2011; Hwang et al. 2005; Quigley and Gross 1994). Alternatively, these strains may survive and persist across a variety of environments and host plants but only rarely reach sufficiently high population densities to cause disease. MLSA group II strains appear to survive better as epiphytes compared with other groups, and the presence of NRP toxins may tie into this strategy (Feil et al. 2005). Moreover, such differences in disease ecology may be difficult to precisely measure because they may only be visible under natural conditions of infection and dispersal.

It is possible that there are a number of undescribed T3E families within CC1466 and CC1557 and, to this point, we have successfully used comparisons of genome synteny to identify HopBJ1. *hopBJ1* is present within the conserved

effector locus within these strains and, as such, is proximate to both the structural genes for the T3SS and the T3E *avrE*. Although it is difficult to precisely map out the evolutionary history of this region, it does appear that *hopBJ1* has been recombined into this locus in place of *hopM1* (Fig. 4). In some respects, recombination of *hopM1* from this locus is analogous to a situation witnessed in MLSA group I *P. syringae* strains, where *hopM1* alleles have been cleanly swapped through recombination (Baltrus et al. 2011). HopM1 is an effector whose presence (although not necessarily in functional form) is conserved throughout *P. syringae* sensu stricto, and has been shown to act redundantly with AvrE during infection of plant hosts (Badel et al. 2006; Kvitko et al. 2009). Moreover, in contrast to frameshift or nonsense mutations disrupting the coding sequence of *hopM1* in *P. syringae*, presence of this gene is polymorphic within *P. viridiflava*. Although some strains maintain an intact version, others lack *hopM1* completely (Araki et al. 2006; Bartoli et al. 2014). It is currently unknown whether HopBJ1 can act redundantly with AvrE on specific hosts, although the fact that our single deletion mutant shows a virulence phenotype on *N. benthamiana* speaks against this possibility. Moreover, because HopBJ1 appears to be responsible for a significant portion of the growth of CC1557 in planta, it will be interesting to see how this effector behaves within different strain backgrounds on different hosts.

HopBJ1 itself displays protein structure similarity to CNF1 toxin found within pathogenic *E. coli* strains. CNF1 functions by causing deamidation of a glutamine residue, crucial for GTP hydrolysis for small GTPases of the Rho family, thereby leading to constitutive activation and actin disruption (Lemmonier et al. 2007). Furthermore, changing either of these amino acids within HopBJ1 eliminates virulence activity of CC1557 in planta, as well as during transient expression by *Agrobacterium* spp., and eliminates avirulence activity when delivered from DC3000 in *Arabidopsis* (Fig. 3). Potential functional parallels between CNF1 and HopBJ1 are particularly interesting because Rho GTPases are known to regulate cytoskeletal dynamics in plants (Mucha et al. 2011), actin has a demonstrated role in basal defense (Henty-Ridilla et al. 2013), and the *P. syringae* effector HopZ1a has been shown to target tubulin in order to promote virulence (Lee et al. 2012). If HopBJ1 does, indeed, target Rho family GTPases to disrupt the cytoskeleton, it would represent a striking example of molecular convergence across plant and animal pathogens.

The pathology of HopBJ1 on *Arabidopsis*, when delivered from strain DC3000, suggests that HopBJ1 could be acting as a general toxin because cell death appears regardless of accession and is not dependent on typical *R*-gene-related host defense pathways (Table 2). Moreover, HopBJ1 does act as an avirulence factor when delivered from DC3000 because cell death limits the growth of this strain in *Arabidopsis* (Fig. 3D). In some ways, this may be similar to the functions of AvrE, which was identified as causing cell death in soybean (Kobayashi et al. 1989). It is tempting to think that both AvrE and HopBJ1 ultimately lead to the same physiological outcomes during infection but, thus far, little is known about processes targeted by AvrE.

What advantages HopBJ1 provides to CC1557 in nature remains unknown, because it is currently unclear whether this strain is capable of causing disease in any organism outside of the lab environment or syringe infiltration. Indeed, both CC1557 (snow) and CC1466 (asymptomatic plants) were originally isolated in order to characterize environmental diversity of *P. syringae* strains outside of crop disease. It is certainly possible that CC1557 and related strains persist at low levels across a range of plant hosts and habitats, using a T3SS and toxins to survive epiphytically but never reaching sufficiently large

population sizes to cause disease. It is also possible that CC1557 is naturally pathogenic within a suite of understudied plant hosts or only under specific environmental conditions. However, given the disease symptoms measured under laboratory conditions, it is clear that CC1557 has the potential to cause rapid cell death in plant leaves relative to other *P. syringae* strains and that a significant amount of this activity is dependent on HopBJ1. These results suggest an intriguing possibility

that the disease ecology of CC1557 is fundamentally different (for instance, more necrotrophic) than other commonly studied *P. syringae* strains.

Herein, we have analyzed the genomes of two *P. syringae* strains isolated from environmental sources. These genomes contain a paucity of known T3E, even though virulence of CC1557 on *N. benthamiana* is dependent on a functioning T3SS. That these strains have also acquired an NRP pathway

Table 3. Strains and plasmids^a

Name	Description	Antibiotics	Reference
Strain			
CC1466	<i>Pseudomonas syringae</i> CC1466	...	Morris et al. 2008
CC1557	<i>P. syringae</i> CC1557	...	Morris et al. 2008
CC1583	<i>P. syringae</i> CC1583	...	Morris et al. 2008
DBL627	rif ^R version of <i>P. syringae</i> CC1557	...	This study
DBL704	DBL627 with pDAB334, CC1557 rif ^R mutant with EV	...	This study
DBL690	DBL627 with pDBL048 integrated, <i>hopBJ1</i> deletion construct integrated into CC1557 rif ^R	...	This study
DBL692	DBL627 with pDBL051 integrated, <i>hrcC</i> deletion construct integrated into CC1557 rif ^R	...	This study
DBL698	DBL627 <i>hrcC</i> mutant	...	This study
DBL696	DBL627 <i>hopBJ1</i> mutant	...	This study
DBL704	DBL627 with EV pDAB334, CC1557 rif ^R with EV	...	This study
DBL706	DBL696 del with pDAB334, <i>hopBJ1</i> deletion with EV	...	This study
DBL705	DBL696 del with pDBL052, <i>hopBJ1</i> deletion with <i>hopBJ1</i>	...	This study
DBL708	DBL698 with pDAB334, CC1557 rif ^R <i>hrcC</i> – with EV	...	This study
DBL707	DBL698 with pDBL052, CC1557 rif ^R <i>hrcC</i> – with <i>hopBJ1</i>	...	This study
DBL604	<i>P. syringae</i> pv. <i>tomato</i> DC3000 with pDBL061	...	This study
DBL888	DC3000 <i>hrcC</i> mutant with <i>hopBJ1</i> (no promoter) pDBL061	...	This study
MTN3101	GV3101 with pMTN3100; 35S HopBJ1:YFP	...	This study
MTN3131	GV3101 with pMTN3100; 35S HopBJ1:YFP	...	This study
MTN3132	GV3101 with pMTN3100; 35S HopBJ1:YFP	...	This study
MTN2890	DC3000 with pDBL052; HopBJ1 in pJC531	...	This study
MTN3151	DC3000 with pMTN3149; HopBJ1 C174S in pJC531	...	This study
MTN3152	DC3000 with pMTN3150; HopBJ1 H192A in pJC531	...	This study
MTN3155	DBL627 with pMTN3149; HopBJ1 C174S in pJC531	...	This study
MTN3156	DBL627 with pMTN3150; HopBJ1 H192A in pJC531	...	This study
MTN3240	DC3000-D28E with pMTN3224; HSP18-2 UTR in pJC532	...	This study
MTN3241	DC3000-D28E with pMTN3225; HopBJ1 in pJC532	...	This study
MTN3242	DC3000-D28E with pMTN3226; HopBJ1 C174S in pJC532	...	This study
MTN3243	DC3000-D28E with pMTN3227; HopBJ1 H192A in pJC532	...	This study
Plasmids			
pMTN1907	Gateway destination vector for making clean deletions in <i>P. syringae</i>	Tet ^R , Kan ^R , Cam ^R , Suc ^S	Baltrus et al. 2012
pDONR207	Gateway entry vector from Invitrogen	Gent ^R , Cam ^R	Invitrogen
pMTN1970	3' UTR of <i>Arabidopsis</i> HSP18.2 in pDONR207	Gent ^R	Baltrus et al. 2012
pJC531	Gateway destination vector based off of pBBRMCS1 no promoter; C-terminal HA tag	Kan ^R , Cam ^R	Chang et al. 2005
pJC532	Gateway destination vector based off of pBBRMCS1 no promoter; C-terminal ΔAvrRpt2	Kan ^R , Cam ^R	Chang et al. 2005
pDAB334	MTN1970-2 EV construct in pJC531	Kan ^R	Baltrus et al. 2012
pDBL040	<i>hopBJ1</i> cloned with promoter, no stop codon in pDONR207	Gent ^R	This study
pMTN3111	<i>hopBJ1</i> C174S with promoter, no stop codon in pDONR207	Gent ^R	This study
pMTN3112	<i>hopBJ1</i> H192A with promoter, no stop codon in pDONR207	Gent ^R	This study
pDBL052	<i>hopBJ1</i> cloned with promoter, no stop codon in pJC531	Kan ^R	This study
pMTN3149	<i>hopBJ1</i> C174S cloned with promoter, no stop codon in pJC531	Kan ^R	This study
pMTN3150	<i>hopBJ1</i> H192A cloned with promoter, no stop codon in pJC531	Kan ^R	This study
pDBL042	<i>hopBJ1</i> deletion construct in pDONR207	Gent ^R	This study
pDBL048	<i>hopBJ1</i> deletion construct in MTN1907	Tet ^R	This study
pDBL012	<i>hopBJ1</i> cloned without promoter into pDONR207, no stop	Gent ^R	This study
pMTN3113	<i>hopBJ1</i> C174S ORF, no stop codon in pDONR207	Gent ^R	This study
pMTN3114	<i>hopBJ1</i> H192A ORF, no stop codon in pDONR207	Gent ^R	This study
pDBL061	<i>hopBJ1</i> cloned without promoter into pBAV187	Tet ^R	This study
pMTN3224	HSP18.2 UTR in pJC532	Kan ^R	This study
pMTN3225	<i>hopBJ1</i> cloned with promoter, no stop codon in pJC532	Kan ^R	This study
pMTN3226	<i>hopBJ1</i> C174S cloned with promoter, no stop codon in pJC532	Kan ^R	This study
pMTN3227	<i>hopBJ1</i> H192A cloned with promoter, no stop codon in pJC532	Kan ^R	This study
pDBL045	CC1557 <i>hrcC</i> deletion construct in pDONR207	Gent ^R	This study
pDBL051	CC1557 <i>hrcC</i> deletion construct in MTN1907	Tet ^R	This study
pGWB641	Gateway destination vector; 35S promoter, C-terminal YFP	Spec ^R , Cam ^R	Nakamura et al. 2010
pMTN3100	<i>hopBJ1</i> ORF in pGWB641	Spec ^R	This study
pMTN3119	<i>hopBJ1</i> C174S ORF in pGWB641	Spec ^R	This study
pMTN3120	<i>hopBJ1</i> H192A ORF in pGWB641	Spec ^R	This study

^a rif^R, Tet^R, Kan^R, Cam^R, Gent^R, and Spec^R indicate resistant to rifampicin tetracycline, kanamycin, chloramphenicol, gentamycin, and spectinomycin; Suc^S indicates sensitive to sucrose; EV = empty vector, UTR = untranslated region, and ORF = open reading frame.

similar to syringomycin represents an independent evolutionary example that supports a negative correlation between NRP pathways and T3E repertoires. Furthermore, this data set highlights how genomic scans can lead to insights into pathogenesis while also demonstrating the power of genetic diversity to uncover genomewide changes in virulence gene architecture.

MATERIALS AND METHODS

Plasmids, bacterial isolates, and growth conditions.

All bacterial strains and plasmids used or created are listed in Table 3. Typically, *P. syringae* and *Agrobacterium* isolates were grown at 27°C on King's B (KB) media using 50 µg/ml rifampicin at 50 µg/ml. When necessary, cultures of *P. syringae*, *Agrobacterium* spp., and *E. coli* were supplemented with antibiotics or sugars in the following concentrations: tetracycline at 10 µg/ml, kanamycin at 50 µg/ml, spectinomycin at 50 µg/ml, gentamycin at 25 µg/ml (50 µg/ml for *Agrobacterium* spp.), and 5% sucrose.

All clones were created by first amplifying target sequences using *Pfx* polymerase (Invitrogen, Waltham, MA, U.S.A.), followed by recombination of these fragments into the entry vector pDONR207 using BP clonase (Invitrogen). Site-directed mutagenesis was performed using SLIM amplification (Chiu et al. 2004). All ORF (without an *hrp*-box promoter) and gene (including the promoter) sequences were confirmed by Sanger sequencing of these pDONR207 clones. Clones in entry vectors were recombined into destination vectors of interest using LR clonase (Invitrogen).

Genome sequences and searches.

Draft genome assemblies for CC1466, CC1557, and CC1583 are publicly available through GenBank (accession numbers AVE000000000, AVEH000000000, and AVEG000000000, respectively) (Baltrus et al. 2014). One PacBio SMRT cell yielded 37,509 reads for a total of 268,122,626 nucleotides after filtering for quality. To piece together the complete genome sequence for CC1557, PacBio reads were first assembled using the HGAP software (Chin et al. 2013), yielding two contigs total with no scaffolding gaps. Contigs from the previous assembly (using only 100-bp Illumina paired-end reads) (Baltrus et al. 2014) were then overlaid onto this HGAP assembly. When there was coverage by contigs from the Illumina assembly, the previous assembly sequence was chosen as the final sequence but, when there was no coverage, the PacBio assembly was chosen. In cases where multiple divergent Illumina contigs assembled to the same region in the HGAP assembly, the Illumina sequence which matched the PacBio assembly was chosen. Because the second contig from the HGAP assembly possesses numerous plasmid-related genes, we are confident that this does actually represent a plasmid present within this strain. Both the chromosome and plasmid were annotated using PGAAP (Angiuoli et al. 2008).

T3E and NRP pathways were identified as per Baltrus and associates (2011). Briefly, draft genome assemblies were queried using tBLASTn, with queries consisting of protein sequences for known T3E families or for key loci within toxin pathways. Each BLAST hit was validated by hand for copy number, identity, and completeness. All BLAST results were visually inspected to make sure that each sequence displayed high similarity to only one region in the assembly, that this similar region was not part of a larger ORF, and that the length of this region was greater than 40% of the length of the original query sequence.

The Phyre2 server was used to carry out protein structure similarity searches (Kelley and Sternberg 2009). In brief, this server performs an automated search of both the protein se-

quence and predicted folds for proteins of interest (in this case, HopBJ1) across multiple databases. Within this pipeline, iterative searches such as PSI-Blast enable identification of distantly similar sequence matches. Once a profile is constructed for the sequence of interest, it is compared against a database of known protein structures and returns the best matches as a prediction of protein folds within the query.

Generation of bacterial mutants.

Bacterial mutants were generated as per Baltrus and associates (2012). Regions (>500 bp) upstream and downstream of the target genes were polymerase chain reaction (PCR) amplified separately and then combined into one fragment by overlap extension PCR. The bridge PCR amplicon was then cloned into pDONR207, and further moved into pMTN1907 using LR clonase. Once mated into *P. syringae*, single recombination of a homologous region upstream or downstream of the target region and subsequent selection on sucrose allowed for screening of clean deletions. Mutants were confirmed by phenotyping for sucrose resistance, tetracycline sensitivity, PCR amplification of the deletion, and failure of PCR to amplify regions within the deletion.

Negative correlation between syringomycin-like toxin pathways and T3E.

We calculated statistical significance for negative correlation between toxin pathways and effectors using data on the number of full-length T3E per strain from Baltrus and associates (2011) as well as data from Table 1. To minimize bias due to phylogenetic relationships, we chose more than five diverse strains from MLSA groups 1, 2, and 3 as well as CC1557 and CC1583. We then performed a Wilcoxon rank sum test to compare the number of full-length effectors between genomes which either contain or lack the genetic capacity for production of a syringomycin-like toxin.

In planta growth curves, cell-death assays, and translocation tests.

N. benthamiana plants were grown for 4 to 6 weeks on a long-day cycle (16 h of light and 8 h of darkness). Plants were removed from the growth chamber and allowed to acclimate to ambient laboratory conditions for a period of 3 to 5 days prior to infiltration (for experiments taking place at the University of Arizona). Bacteria were cultured overnight on KB amended with proper antibiotics. Bacterial cells were washed 1× and resuspended to an optical density at 600 nm (OD₆₀₀) of 0.002 or 0.0002 in sterile 10 mM MgCl₂, yielding a final inoculum density of approximately 1 × 10⁵ (for experiments taking place at the University of North Carolina) or 1 × 10⁶ CFU/ml (for experiments taking place at the University of Arizona). Cell suspensions were then syringe infiltrated into the abaxial side of *N. benthamiana* leaves. Populations were recovered after two (for experiments taking place at the University of North Carolina) or three (for experiments taking place at the University of Arizona) days of growth using a cork borer, unless otherwise noted. Leaf disks were disrupted using glass beads and a bead-beater device and populations were enumerated by dilution plating onto KB amended with appropriate antibiotics. *Arabidopsis* growth assays were performed similarly, with the exception that plants were grown in walk-in rooms maintained on a short-day cycle (9 h of light at 21°C and 15 h of darkness at 18°C).

Cell death induced by HopBJ1 was assayed after transient expression of 35S-driven HopBJ1:YFP in *N. benthamiana*. Bacteria were grown to saturation overnight in 2× yeast tryptone media and then diluted to an OD₆₀₀ of 0.1. Cultures were syringe injected into fully expanded leaves of 5- to 6-week-old

plants and cell death was visualized 24 h postinoculation. Accumulation of wild-type and mutant HopBJ1 was verified with standard Western blotting of lysates from injected leaf cores using anti-GFP (Roche, Penzberg, Germany).

HopBJ1 was tested for the ability to secrete the active C-terminal fragment of AvrRpt2 to cause a hypersensitive response (HR) in *Arabidopsis* accession Col-0 (Guttman et al. 2002). For this test, promoterless *HopBJ1* was cloned into plasmid pBAV178 (Vinatzer et al. 2006) so that expression in strain DC3000 D28E was dependent on a *tet* promoter. This construct was also placed into an *hrcC*– mutant of DC3000 (Baltrus et al. 2012), in order to test the requirement of a functioning T3SS for tissue collapse. All HR tests were performed on approximately 5-week-old plants and utilized a bacterial density of OD₆₀₀ = 0.05. Tissue collapse was measured at either 24 or 48 h postinfection and confirmed by at least four independent tests. For translocation of HopBJ1 and HopBJ1 site-directed mutants, similar assays were done with native promoter clones in pJC532 delivered from the D28E effector-deleted strain of DC3000. (Chang et al. 2005).

Phylogenetic methods.

Phylogenetic analyses were performed on concatenated MLSA loci as described previously using concatenated fragments from seven MLSA genes across a diverse array of isolates from *P. syringae* (Baltrus et al. 2011) as well as AvrE and HrpW protein sequences. MrBayes was used to perform Bayesian phylogenetic analyses with flat priors, a burn-in period of 250,000 generations, and convergence after 1,000,000 total generations (Ronquist et al. 2012).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Phyre2 server, <http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>