Genome Context Influences Evolutionary Flexibility of Nearly Identical Type III Effectors in Two Phytopathogenic Pseudomonads

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Integrative Conjugative Elements (ICEs) are replicons that can insert and excise from chromosomal locations in a site-specific manner, can conjugate across strains, and which often carry a variety of genes useful for bacterial growth and survival under specific conditions. Although ICEs have been identified and vetted within certain clades of the agricultural pathogen Pseudomonas syringae, the impact of ICE carriage and transfer across the entire P. syringae species complex remains underexplored. Here we identify and vet an ICE (PmaICE-DQ) from P. syringae pv. maculicola ES4326, a strain commonly used for laboratory virulence experiments, demonstrate that this element can excise and conjugate across strains, and highlight that this element contains loci encoding multiple type III effector proteins. Moreover, genome context suggests that another ICE (PmaICE-AOAB) is highly similar in comparison with and found immediately adjacent to PmaICE-DQ within the chromosome of strain ES4326, and also contains multiple type III effectors. Lastly, we present passage data from in planta experiments that suggests that genomic plasticity associated with ICEs may enable strains to more rapidly lose type III effectors that trigger R-gene mediated resistance in comparison to strains where nearly isogenic effectors are not present in active ICEs. Taken together, our study sheds light on a set of ICE elements from P. syringae pv. maculicola ES4326 and suggests how genomic context may lead to different evolutionary dynamics for shared virulence genes between strains.

Keywords: Pseudomonas syringae, integrative conjugative element (ICE), type III effector, phytopathogen, Nicotiana benthamiana

INTRODUCTION

Genome fluidity is crucial for survival of bacterial phytopathogens, as selection pressures on the presence and function of specific virulence genes can dramatically change from host to host (Dillon et al., 2019a; de Vries et al., 2020). Much research characterizing gene composition of bacterial pathogens focuses on presence and absence of specific virulence genes, and therefore often extrapolates from lists of loci to predict virulence and evolutionary potential (Baltrus et al., 2011; Dillon et al., 2019b). However, even if virulence genes are conserved across strains of a particular pathogen, differences in genomic flexibility for shared virulence genes could potentiate different evolutionary outcomes between strains under conditions of strong selection. Here we characterize one instance where such differences in genomic context and fluidity for a set of type III effectors
affects evolutionary potential for *Pseudomonas syringae*, and we speculate about the ability of such systems to shift evolutionary dynamics for phytopathogens moving forward.

Integrative Conjugative Elements (ICEs) are mobile replicons that blend characteristics of both plasmids and prophage and are well known for their ability to harbor antibiotic resistance genes and other niche-association traits across bacteria (Johnson and Grossman, 2015). Like conjugative plasmids, ICEs contain all genes and pathways required for conjugation between bacterial cells. Like prophage, ICEs contain site specific recombinases that enable recombination into chromosomal locations and repress the genes responsible for conjugation and replication while in this quiescent state. Most importantly, ICEs also contain cargo regions that can house genes and pathways that directly contribute to dramatic changes in bacterial phenotypes (Lovell et al., 2009; Johnson and Grossman, 2015). Not only can these elements modify phenotypes in their current host cells, but the ability of ICEs to transfer throughout populations and across communities through horizontal gene transfer means that cargo genes present on ICEs can rapidly proliferate across strains if they are beneficial. The rise of sequencing technologies enabling closed bacterial genomes has reinforced the importance of mobile elements in genomes and increased awareness of the prevalence of ICEs throughout bacteria.

The phytopathogen *Pseudomonas syringae* (sensu lato) is a recognized agricultural pest for many crops throughout the world, with numerous strains well established as laboratory systems for understanding virulence of plant pathogens *in vitro* and *in planta* (Baltrus et al., 2017). The presence of a type III secretion system is critical for virulence *in planta* for many strains of this pathogen, and this system is used to translocate upwards of 40 effector proteins per strain from the bacterial cytoplasm into plant cells (Dillon et al., 2019a; Laflamme et al., 2020). Once inside the plant cells, effector proteins can disrupt plant immune responses in a variety of ways to promote bacterial growth and infection. The presence of effector proteins can also be monitored by plant immune responses through the action of R-genes, with recognition of effector protein functions leading to an overarching immune reaction termed effector triggered immunity (ETI) (Collmer et al., 2000; Jones and Dangl, 2006; Jayaraman et al., 2021). Triggering of the ETI response can quickly shut down nascent infections in resistant plant cultivars and has thus formed the basis of future plans to engineer durable crop resistance to infection through genetic modification and selective breeding (Dong and Ronald, 2019; Laflamme et al., 2020). Thus, type III effectors sit at an evolutionary infection point where they can be highly beneficial for bacterial growth in some host backgrounds and highly detrimental in others.

Throughout this manuscript, we use the phrase “genome context” as a catch-all term to represent both the placement and relative location of specific genes within a genome compared to orthologs and homologs of these specific genes. We intend this as an open-ended term to describe how sequence characteristics (gene order, location, etc.) can affect parameters that contribute to evolutionary potential. For instance, imagine two antibiotic resistance genes that are sequence identical and found within a single genome but where one copy is present on a chromosome and stably maintained whereas the other copy is found in a region that is much more easily lost (e.g., on a small mobilizable plasmid). While these two genes may play similar if not redundant phenotypic roles for cells containing this particular version of the genome, differences between copies in rates of loss and horizontal transfer could significantly influence the evolutionary potential of each copy across the population and microbial community by altering population genetic parameters like population size and deletion rates.

The presence of virulence genes, including type III effectors, on plasmids and consequent movement across strains can dramatically alter trajectories of virulence evolution for *P. syringae* on different host species (Cazorla et al., 2002; Schierstaedt et al., 2019). Plasmids can be lost or modified if effector proteins are recognized by a potential host, which could enable infections to proceed at a population level despite the ETI response (Grant et al., 2006; Bardaji et al., 2019). Plasmids are not the only element within *P. syringae* genomes displaying plasticity, though. For instance, the effector AvrPphB (aka HopA1) triggers HR in bean plants that contain the R-gene RPS5 (Qi et al., 2014). AvrPphB is found on an genomic island in certain strains of *P. syringae* pathovar phaseolicola, and under selective pressure from ETI responses, bacteria with the island excised from the chromosome and maintained independently as an episome rapidly dominate the population (Godfrey et al., 2011; Neale et al., 2018). Excision of the genomic island containing AvrPphB prevents ETI because this Avr gene is downregulated under episomal replication, but enables this gene to be maintained within a population for infection of host plants where it may be beneficial. ICE elements have also been identified and characterized in *P. syringae* pathovar actinidiae strains where their presence/absence is one of the most glaring differences between closely related strains and where they contribute to large scale differences in strain metabolism and resistance to antibacterial compounds (Colombi et al., 2017; McCann et al., 2017; Poulet et al., 2018).

Here we describe how genomic context in a locus encoding an effector protein, HopQ, differentially affects evolutionary flexibility for strains containing this effector. Recognition of HopQ by the plant R-gene Rq1 triggers ETI in the host plant *Nicotiana benthamiana*, and thus limits growth of strain *P. syringae* pv. *tomato* DC3000 (*Pto*) in this host (Wei et al., 2007; Schultink et al., 2017). We characterize the genomic context for hopQ and other linked virulence genes in both *PtoDC3000* and another pathogen, *Pseudomonas syringae* pv. maculicola ES4326 (*PmaES4326*). We demonstrate that this and other effectors are differentially present in an active ICE element in *PmaES4326* but not in *PtoDC3000*, and confirm that recognition of HopQ also limits growth of *PmaES4326* in *N. benthamiana*. These differences are somewhat surprising because at a broad scale these regions appear syntenic. We further show how this differential genomic context allows for differential genomic plasticity for hopQ between these two strains, by demonstrating that passage of strains in *Nicotiana benthamiana* leads hopQ to be more readily lost in *PmaES4326* than in *PtoDC3000*. Thus, we directly demonstrate how genomic context for homologous virulence
genes in two closely related pathogens can contribute to different evolutionary trajectories for these strains.

**MATERIALS AND METHODS**

**Bacterial Strains and Culturing Conditions**

*E. coli* was routinely cultured at 37°C in LB (Lysogeny broth; per 1 L = 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.5 + with 15 g agar for solidified media). *P. syringae* was routinely cultured at 25°C or 28°C in either LM (LB modified; per 1 L = 10.0 g tryptone, 6.0 g yeast extract, 2.0 g K2HPO4·3H2O, 0.6 g NaCl, 0.4 g MgSO4·7H2O, with 18 g agar for solidified media), KB (King’s B; per 1 L = 20.0 g proteose peptone 3, 0.4 g MgSO4·7H2O, glycerol 10 mL, 2.0 g K2HPO4·3H2O, with 18 g agar for solidified media) or KBC (KB amended with boric acid 1.5 g/L and cephalxin 80 mg/L). Liquid cultures were incubated with shaking at 200 rpm. Where appropriate, media was augmented at final concentration with rifampicin (Rf) 40–60 µg/mL, gentamicin (Gm) 10 µg/mL, kanamycin (Km) 50 µg/mL, spectinomycin (Sp) 50 µg/mL, and diaminopimelic acid (DAP) 200 µg/mL for liquid media, 400 µg/mL for solid media for the growth of DAP-auxotrophic *E. coli* strains.

**Genome Sequencing and Assembly**

For each strain with a genome sequence reported herein, a frozen stock was streaked to single colonies on King’s B (KB) agar plates, at which point a single colony was picked to 2 mL KB liquid and grown overnight on a shaker at 27°C or 28°C in either LM (LB modified; with 18 g agar for solidified plates, at which point a single colony was picked to 2 mL KB liquid and grown overnight on a shaker at 27°C or 28°C) in LB (Lysogeny broth; per 1 L = 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.5 + with 15 g agar for solidified media). *P. syringae* was routinely cultured at 25°C or 28°C in either LM (LB modified; per 1 L = 10.0 g tryptone, 6.0 g yeast extract, 2.0 g K2HPO4·3H2O, 0.6 g NaCl, 0.4 g MgSO4·7H2O, with 18 g agar for solidified media) or KBC (KB amended with boric acid 1.5 g/L and cephalxin 80 mg/L). Liquid cultures were incubated with shaking at 200 rpm. Where appropriate, media was augmented at final concentration with rifampicin (Rf) 40–60 µg/mL, gentamicin (Gm) 10 µg/mL, kanamycin (Km) 50 µg/mL, spectinomycin (Sp) 50 µg/mL, and diaminopimelic acid (DAP) 200 µg/mL for liquid media, 400 µg/mL for solid media for the growth of DAP-auxotrophic *E. coli* strains.

**DNA Manipulation**

Plasmid DNA was routinely purified using the GeneJet plasmid miniprep kit (Thermo Fisher Scientific). PCR was conducted with Phusion HiFi polymerase (Thermo Fisher Scientific). PCR/reaction cleanup and gel extraction were conducted with the Monarch PCR and DNA cleanup kit and Monarch DNA gel extraction kits (NEB). *E. coli* transformation was conducted by either by preparing competent cells using the Mix and Go! *E. coli* transformation kit (Zymo Research) or standard electro-transformation protocols. *Pma*ES4326 strains were transformed with pCPP5372hopQ (pHopQ) (Wei et al., 2018) plasmid DNA via electro-transformation after washing and concentration of overnight liquid cultures with 300 mM sucrose (Choi et al., 2006). Restriction enzymes, T4 ligase, and Gibson Assembly Mastermix were purchased from NEB. Oligonucleotide primers were synthesized by IDT. Commercial molecular biology reagents were used in accordance with their manufacturer’s recommendations.

To create the site-specific Tn7 3xmCherry labeling vector pURR25DK-3xmcherry, pURR25 (Teal et al., 2006) was first digested with *PstI* and recircularized with T4 ligase to remove the *nptII* KmR marker gene creating pURR25DK. To replace the *gfp* gene in pURR25DK, the 3xmCherry cassette was PCR amplified from pGGC026 (pGGC026 was a gift from Jan Lohmann (Addgene plasmid # 48831); RRID:Addgene_48831) using primers bko375 (’5’ACATCTAGATTAAGAGGAAATAAGCATGGTG AGCAAGGGCGAGGAGGATAACATG 3’) and bko375 (’5’CAGGAGTCCAGATCGACTAATATAGCCTTTGATC AACATCATTACCACCCTTTGTA 3’) to introduce 30 bp 5’ overlaps corresponding to the *gfp* flanking regions. Gibson assembly was used to join the 3xmCherry PCR amplicon with pURR25DK backbone digested with BseRI and partially digested with HindIII.

**Bacterial Conjugation and Creation of Mutant Strains**

All genetic manipulations of *Pma*ES4326 were conducted with the *Pma*ES4326-C strain. Conjugations were performed by mixing 15 µL of fivefold concentrated, washed, overnight LM liquid cultures of each parent strain and co-culturing at 28°C changes that occurred in the passage strain *Pma*ES4326 LA-P5-20-1 (hereafter *Pma*ES4326 LAP5-20). Default parameters were used for all software unless otherwise specified.

**Integrative Conjugative Element Identification**

Potential ICE elements were identified by manual searches of gene annotations for loci that could code for proteins critical for processing of conjugative elements. These include a site specific recombinase, a TraG-like NTPase, an ATPase, and numerous proteins involved in creation of the conjugation pilus. To be present within an ICE, these elements must all be present in a contiguous segment of the genome which is bordered by a potential attachment site (often tRNA loci).

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1http://n2t.net/addgene:48831
each lineage were directly scraped from the dilution plate with E. coli RH03 pTNS3 Tn7 transposase helper strain as a third parent. For all conjugation experiments of each parent strain were included separately as controls. PmaES4326 merodiploid exconjugants of pCPP5729 (pK18msGmΔhopQ1) were recovered on LM Km (Kvitko et al., 2009). Resolved Pma ES4326 pCPP5729 merodiploids were recovered via counter-selection on LM Rf + 10% sucrose and sucrose resistant clones were screened for kanamycin sensitivity by patch plating indicating the loss of the pK18ms plasmid backbone (Kvitko and Collmer, 2011). Derivative attTn7-3xmCherry transposant strains were recovered on LM Sp and pink clones were selected after 4 days incubation at 4°C and restreaked to isolation. To test the native mobility of ICE-DQ, conjugation was conducted as described above with the PmaES4326 pCPP5729 merodiploid as the donor parent and attTn7-3xmCherry derivatives of PtoDC3000 and PmaES4326 ΔICE-DQ strains (Pma DQ3 and Pma LAP5-20) as recipients. ICE-DQ exconjugants were recovered on LM Rf Sp Km. For all conjugation experiments cultures of each parent strain were included separately as controls. Conjugation frequency was calculated as the number of SpKm colonies recovered per recipient CFU as determined by dilution plating.

*Nicotiana benthamiana* Growth, Inoculation and Bacterial Passage Assays

*Nicotiana benthamiana*, WT LAB accession, and *roq1-1* (Qi et al., 2018) were grown in a Conviron Adaptis growth chamber with 12 h light (125 μmol/m²/s) at 26°C and 12 h dark at 23°C. Plants were used at 5-7 weeks post germination. To prepare inoculum, *P. syringae* cultures were recovered from fresh KB plate cultures, resuspended in 0.25 mM MgCl₂, standardized to OD₆₀₀ 0.2, and serially diluted 10,000X to ~3 × 10⁴ CFU/mL. Cell suspensions were infiltrated with a blunt syringe into either the third, 4th, or 5th leaves. Infiltrated spots were allowed to dry fully and then leaves were covered with a humidity dome and kept at 100% humidity for 6–8 days to allow symptoms to develop. At end point, leaves were photographed to document symptoms and four 4 mm diameter leaf punches (~0.65 cm² total) were collected with a 4 mm diameter biopsy punch from each infiltrated area. Discs were macerated in 0.1 mL of 0.25 mM MgCl₂ using an Analytik Jena SpeedMill Plus homogenizer and the bacterial CFU/cm² leaf tissue was determined by serial dilution spot plating from 10 μL volumes on LM Rf.

For *P. syringae* pasaging in *N. benthamiana*, single colonies of Pto DC3000 WT and PmaES4326 were inoculated into LM Rf liquid cultures. Samples of the initial cultures (P0) were cryo-preserved at ~80°C in 15% final strength sterile glycerol. The liquid cultures were diluted 1000X in 0.25 mM MgCl₂ to approximately 5 × 10⁵ CFU/mL prior to syringe infiltration into three leaf areas establishing three lineages (A, B, C) each for PtoDC3000 and PmaES4326. Tissue samples were collected 6–7 days post inoculation and processed as described above. Bacteria cultured from the 10⁻¹ tissue macerate dilutions of each lineage were directly scraped from the dilution plate with an inoculation loop and suspended in 1 mL 0.25 mM MgCl₂. These suspensions were then sub-cultured 5 μL into 5 mL LM Rf and diluted as described above to create inoculum for the next passage. This passaging scheme was repeated five times and samples of both the post-passage recovered bacteria (P1) and the corresponding sub-cultured bacteria (P1c) were cryo-preserved for each lineage and each passage. To screen for changes in *N. benthamiana* disease compatibility with passaged strains in a medium-throughput format, bacteria from the P5 cryo-preserved samples were streaked to isolation on KBC plates and isolated “P5” colonies were cultured in 200 μL of LM Rf in sterile 96 well microtiter plates along with Pto DC3000, Pto DC3000ΔhopQ, Pma ES4326 and PmaES4326ΔDQ3 control strains. Cultures were serially diluted 5000X in 0.25 mM MgCl₂ to ~3 × 10⁴ CFU/mL and inoculated into *N. benthamiana* leaves as described. Inoculum concentration was verified via serial dilution spot plating. Inoculated areas were monitored visually for qualitative changes to disease symptoms compared to their respective WT and targeted hopQ deletion control strains over the course to 6–8 days. The CFU/cm² leaf tissue bacterial load of select clones was determined for each strain as described above. Isolated colonies of strains that displayed bacterial load and symptoms comparable to their respective ΔhopQ backgrounds were subcultured from the dilution spot count plates and retained.

We estimate that Nₑ of these passage populations to be on the order of 2.5 × 10⁴ over the course of five passages. This number was calculated by taking the inoculum of each passage (5 × 10⁵ CFU/mL in 50 μL) and estimating that approximately 10 μL were inoculated per cm² for a total of 2.5 × 10⁴ for the area inoculated (~5 cm²). We further estimate that this is the smallest bottleneck over the course of the passages, and that populations undergo ~10 generations of division, as these populations grow from 10⁴ to 10⁷ cm² in planta). Given these assumptions, Nₑ can be estimated by taking the harmonic mean of fluctuating population sizes (Sjödin et al., 2005) and will be highly skewed by smaller numbers. Since 1 cm² is harvested at the end of each cycle, a conservative estimate for Nₑ over five passages is therefore approximately 2.5 × 10⁴ CFU.

**RESULTS**

Complete Genome Sequences for Multiple Isolates of *PmaES4326*

We previously reported a draft genome sequence for an isolate of PmaES4326 acquired from the lab of Jeff Dangi (Baltrus et al., 2011), and our first goal was to generate a complete genome sequence for this strain (referred to herein as *PmaES4326-D*, Table 1). We used MiGS (Pittsburgh, PA) to generate Illumina reads for PmaES4326-D, and their workflow generated a total of 3,284,990 paired reads and 418 Mb (~64× coverage) of sequence. We also independently isolated genomic DNA and sequenced using an Oxford Nanopore MinION to generate 32,291 reads for a total of 465 Mb (~71× coverage) of sequence with a read N50 of 30,656 bp. Assembly of these reads resulted in a complete circular chromosome and four separate plasmids.
TABLE 1 | Sequencing of PmaES4326 strains.

| Strain       | Reads (Illumina/Nanopore) | Total bp sequenced Mb (Illumina/Nanopore) | Read N50 Nanopore bp | Total sequencing depth (Illumina/Nanopore) | SRA accession (Illumina/Nanopore) |
|--------------|---------------------------|------------------------------------------|----------------------|------------------------------------------|----------------------------------|
| PmaES4326-D  | 3,284,990/32,291          | 418,372/465.212                         | 30,656               | 64×/71×                                   | SRR1598672/ SRR15986724         |
| PmaES4326-C  | 3,757,284/55,845          | 485.469/700.483                         | 23,669               | 74×/107×                                  | SRR1598671/ SRR15988568         |
| PmaES4326-DQ3| 2,479,212/15,275          | 351.276/178.118                         | 31,491               | 51×/27×                                   | SRR15988570/ SRR15988567       |
| PmaES4326-C-LA-P5-20-1 | 1,372,720/9,934        | 189.971/146.897                         | 31,020               | 29×/22×                                   | SRR17005742/ SRR15988569       |

TABLE 2 | Assembly of PmaES4326 strains.

| Strain       | Complete genome | Number of contigs in assembly | Replicons present | Assembly accession |
|--------------|-----------------|------------------------------|-------------------|--------------------|
| PmaES4326-D  | Yes             | 5                            | Chromosome (circular), pPmaES4326ABCF | GCA_000145845.2   |
| PmaES4326-C  | Yes             | 7                            | Chromosome (circular), pPmaES4326ABCEF | GCA_020309905.1   |
| PmaES4326-DQ3| Yes             | 6                            | Chromosome (circular), pPmaES4326ABCF | doi:10.6084/m9.figshare.17064080 |
| PmaES4326-C-LA-P5-20-1 | No | 7 | Chromosome (not circular), pPmaES4326ABCEF | doi:10.6084/m9.figshare.17064080 |

Notably, our isolate of PmaES4326-D contains three previously reported plasmids (pPma4326A, pPma4326B, pPma4326E) but also appears to have lost two different plasmids (pPma4326C and pPma4326D) first reported as present in this strain by Stavrinides and Guttman (2004). The assembly for this strain appears to contain an additional ~350 kb plasmid that was not reported by Stavrinides and Guttman (2004) and which we name pPma4326F following earlier naming conventions.

Given the absence of two plasmids from PmaES4326-D, we sought to generate a genome assembly from a different lab isolate, acquired from the lab of Alan Collmer referred to here as PmaES4326-C (Table 1). We used MiGS (Pittsburgh, PA) to generate Illumina reads for the Collmer lab version of PmaES4326, and their workflow generated a total of 3,757,284 paired reads and 485 Mb (~74× coverage) of sequence. We also independently isolated genomic DNA and sequenced using an Oxford Nanopore MinION to generate 55,845 reads for a total of 700 Mb (~70× coverage) of sequence with a read N50 of 24,669 bp. Hybrid assembly of all read types resulted in a single chromosome that appears nearly complete but was not circularized by Unicycler. However, this assembly does contain all predicted circular plasmids as well as pPmaES4326F.

Sequencing and assembly characteristics for all strains can be found in Tables 1, 2, respectfully.

An Integrative Conjugative Element Hotspot in PmaES4326

Genomic inspection of the region containing type III effectors hopQ and hopD in strains PmaES4326-C and PmaES4326-D indicate that this area is a potential hotspot for genomic plasticity. Specifically, in a region bordered by clpB and the type III effector hopR, gene content characterization strongly suggests the presence of two independent integrative conjugative elements (ICEs, Figure 1). Both ICEs are approximately 70 kb in length, and are found adjacent to rRNA loci encoding a proline anticodon. Moreover, roughly 70% of each element is composed of sequences with > 95% nucleotide similarity and which encode many of the structural genes predicted to be involved in ICE proliferation and integration. These conserved genes include predicted integrases/recombinases, pilus proteins and ATPases, regulator proteins, a topoisomerase (topB) and helicase, chromosome partitioning proteins (parB), DNA coupling proteins (traD), an NTPase (traG), and numerous loci annotated as “integrative conjugative element proteins.” We have included annotations for all genes within these two ICEs in Table 3.

![Image](image_url)

Despite relatively high sequence similarity across these two closely related ICEs positioned successively in the genome of PmaES4326, gene comparisons suggest the presence of three distinct cargo regions that clearly differentiate these elements (Figure 1). The first ICE contains type III effector proteins hopAO and hopAB3-1 (aka hopPmal) in two different predicted cargo regions, and so we name this region ICE-ABAO. The second ICE contains loci for type III effectors hopD and hopQ as well as the lytic transglycosylase hopAI1, and so we name this ICE ICE-DQ. Cargo region one contains four predicted ORFs in PmaECE-ABAO and fourteen predicted ORFs in ICE-DQ. Cargo region two contains two predicted ORFs in ICE-ABAO and seven predicted ORFs in ICE-DQ, including the effectors hopAO, hopD, and hopQ. Cargo region three contains six predicted ORFs in ICE-ABAO and two in ICE-DQ. Aside from the type III effectors, many of loci potentially encoded by these cargo regions are annotated as hypothetical proteins or as parts of IS elements and transposons.

Differential Contexts for the Genomic Island Containing hopD and hopQ Across PmaES4326 and PtoDC3000

The type III effectors HopD and HopQ are nearly sequence identical in two phytopathogens commonly used as laboratory
models for studying *P. syringae* virulence, *PtoDC3000* and *PmaES4326* (Baltrus et al., 2011). Furthermore, these effectors are found in roughly the same genomic locations in the two strains, bordered on one side by *clpB* and on the other by the type III effector *hopR* (Figures 1, 2). Broad comparisons between *PtoDC3000* and *PmaES4326* further suggest that these effectors form a genomic island along with a third effector (*hopR*) across these two strains (Figure 2), an island which has been referred to as effector cluster IV in the *PtoDC3000* genome (Kvitko et al., 2009). Moreover, the size of the region between *clpB* and *hopR* in *PtoDC3000* is roughly 70 kb, which closely approximates the size of each ICE in *PmaES4326*. While this region within *PtoDC3000* does contain hints that it previously housed a functional ICE, there are other sections that are quite divergent from that in *PmaES4326* (Figure 2). Specifically, the predicted recombinase in *PtoDC3000* is truncated due to a frameshift mutation and multiple other loci that encode functions important for ICE conjugation and transfer are disrupted by IS elements (Figure 2). Lastly, we note that conservation of nucleotide and protein sequences between PmaICEs coupled with nucleotide diversity in *PtoDC3000* at this genomic location renders accurate evaluation and comparison of evolutionary histories between *PtoDC3000* and the ICEs of *PmaES4326* challenging. However, the most parsimonious explanation does appear that an ICE element housed both *hopQ* and *hopD* within an ancestor of strain *PtoDC3000* and that this ICE was locked in place through both IS element insertions and loss of recombinase activity.

### An Evictable Integrative Conjugative Element Containing HopQ and HopD in *PmaES4326*

ICEs are categorized by their ability to cleanly excise from the genome, and we confirmed the prediction that the type three effectors *hopQ* and *hopD* are contained in an excisable region in *PmaES4326* in two distinct ways. As a first step, we characterized the size of the region excised during intentional creation of a *hopQ*- mutant. To do this, we generated a merodiploid strain in which plasmid pCPP5729 was recombined into a region adjacent to *hopQ*, and then selected for resolution of the merodiploid through plating on sucrose. Presence of sacB in plasmid pCPP5729 renders the merodiploid sensitive to killing by sucrose. Notably, this merodiploid also contains regions sequence identical to those surrounding *hopQ* in *PmaES4326* and so we originally expected that the *hopQ* gene could be locally deleted through RecA-dependent homologous recombination. We isolated sucrose resistant isolates after plating this merodiploid, and identified strains that were neither WT revertants nor clean *hopQ* deletions by PCR genotyping using previously validated genotyping primers. Interestingly, while no clean *hopQ* deletion mutants were identified after sucrose counter-selection of merodiploid strains, WT revertants were. We then performed whole genome sequencing to confirm whether the ICE-DQ genomic region was lost under these selective conditions. We refer to this strain recovered by *in vitro* selection hereafter as *Pma* DQ3. As one can see in Figure 3, *hopQ* and the surrounding regions that are predicted to be part of ICE-DQ have been lost in the targeted deletion strain *Pma* DQ3, confirming that this region can be cleanly and completely excised from the chromosome in a manner consistent with the action of site-specific recombinases contained by ICEs. Searches of both the raw Illumina and Nanopore reads for remnants of *hopQ* or *hopD* yielded no hits despite extensive depth (~51 × for Illumina reads and ~27 × for Nanopore reads), which suggests that ICE-DQ was fully lost and not retained as an episome in *Pma* DQ3 (data not shown).

### Loss of PmaICE-DQ Enables Virulence of *PmaES43436* on *Nicotiana benthamiana*

The presence of *hopQ* in *Pma* would be expected to elicit the ETI response in *Nicotiana benthamiana* accessions containing the R-gene *Roq1*, and a lack of compatible infection and disease. Given this information, we tested whether *Pma* DQ3 would...
| Number | Name                                             | Type     | Minimum | Maximum | Length | Direction |
|--------|--------------------------------------------------|----------|---------|---------|--------|-----------|
| 1      | Tyrosine-type recombinase/integrase               | CDS      | 3,625   | 5,064   | 1,440  | Reverse   |
| 2      | DNA-binding domain-containing protein             | CDS      | 5,061   | 6,992   | 1,932  | Reverse   |
| 3      | DUF3742 family protein                           | CDS      | 7,804   | 8,005   | 402    | Forward   |
| 4      | traG conjugal transfer protein                   | CDS      | 8,047   | 9,585   | 1,539  | Reverse   |
| 5      | Hypothetical protein                             | CDS      | 9,582   | 9,950   | 369    | Reverse   |
| 6      | Integrating conjugative element protein           | CDS      | 9,953   | 11,323  | 1,371  | Reverse   |
| 7      | TIGR03756 family integrating conjugative elementprotein | CDS  | 11,344  | 12,282  | 939    | Reverse   |
| 8      | TIGR03757 family integrating conjugative elementprotein | CDS      | 12,279  | 12,755  | 477    | Reverse   |
| 9      | Hypothetical protein                             | CDS      | 13,053  | 13,241  | 189    | Reverse   |
| 10     | Hypothetical protein                             | CDS      | 13,259  | 13,798  | 540    | Reverse   |
| 11     | Hypothetical protein                             | CDS      | 13,839  | 14,393  | 555    | Reverse   |
| 12     | Hypothetical protein                             | CDS      | 14,752  | 15,297  | 546    | Reverse   |
| 13     | Thiorodoxin domain-containing protein            | CDS      | 15,809  | 16,307  | 699    | Reverse   |
| 14     | Hypothetical protein                             | CDS      | 16,304  | 16,603  | 300    | Reverse   |
| 15     | Conjugative transfer ATPase                      | CDS      | 16,600  | 19,347  | 2,748  | Reverse   |
| 16     | TIGR03751 family conjugal transfer lipoprotein   | CDS      | 19,557  | 24,010  | 456    | Reverse   |
| 17     | TIGR03752 family integrating conjugative elementprotein | CDS      | 19,990  | 21,486  | 1,497  | Reverse   |
| 18     | TIGR03749 family integrating conjugative elementprotein | CDS      | 21,476  | 22,399  | 924    | Reverse   |
| 19     | TIGR03746 family integrating conjugative elementprotein | CDS      | 22,396  | 23,064  | 669    | Reverse   |
| 20     | TIGR03750 family conjugal transfer protein       | CDS      | 23,061  | 23,453  | 393    | Reverse   |
| 21     | TIGR03745 family integrating conjugative elementmembrane protein | CDS   | 23,469  | 23,837  | 369    | Reverse   |
| 22     | TIGR03758 family integrating conjugative elementprotein | CDS      | 23,857  | 24,096  | 240    | Reverse   |
| 23     | Conjugal transfer protein                        | CDS      | 24,053  | 24,458  | 366    | Reverse   |
| 24     | DUF4177 domain-containing protein                 | CDS      | 24,538  | 24,870  | 333    | Reverse   |
| 25     | Hypothetical protein                             | CDS      | 25,041  | 25,472  | 432    | Forward   |
| 26     | hopA4 type III effector                          | CDS      | 25,861  | 26,877  | 1,017  | Reverse   |
| 27     | UvD-helicase domain-containing protein           | CDS      | 27,062  | 28,519  | 1,458  | Reverse   |
| 28     | TIGR03747 family integrating conjugative elementmembrane protein | CDS      | 28,529  | 29,278  | 750    | Reverse   |
| 29     | traD                                             | CDS      | 29,318  | 31,477  | 2,160  | Reverse   |
| 30     | Integrating conjugative element protein          | CDS      | 31,489  | 31,992  | 504    | Reverse   |
| 31     | Transglycosylase SLT domain-containing protein   | CDS      | 31,989  | 32,546  | 558    | Reverse   |
| 32     | TIGR03759 family integrating conjugative elementprotein | CDS  | 32,531  | 33,277  | 747    | Reverse   |
| 33     | Hypothetical protein                             | CDS      | 33,286  | 33,990  | 705    | Reverse   |
| 34     | dcm                                             | CDS      | 34,335  | 35,390  | 1,056  | Forward   |
| 35     | vxn                                             | CDS      | 35,329  | 35,786  | 458    | Reverse   |
| 36     | DNA mismatch repair protein                      | CDS      | 35,850  | 37,871  | 2,022  | Reverse   |
| 37     | DCCD/DEAH-box helicase family protein            | CDS      | 37,973  | 40,231  | 2,258  | Reverse   |
| 38     | Class I SAM-dependent methyltransferase         | CDS      | 40,335  | 41,786  | 1,452  | Reverse   |
| 39     | Hypothetical protein                             | CDS      | 41,816  | 42,415  | 600    | Reverse   |
| 40     | DUF3275 family protein                           | CDS      | 42,536  | 43,147  | 612    | Reverse   |
| 41     | Hypothetical protein                             | CDS      | 43,248  | 43,610  | 363    | Reverse   |
| 42     | Hypothetical protein                             | CDS      | 43,672  | 43,986  | 315    | Reverse   |
| 43     | DUF3577 domain-containing protein                | CDS      | 44,290  | 44,859  | 570    | Reverse   |
| 44     | Hypothetical protein                             | CDS      | 44,879  | 45,403  | 525    | Forward   |
| 45     | Hypothetical protein                             | CDS      | 45,480  | 45,848  | 369    | Reverse   |
| 46     | Regulator                                       | CDS      | 46,036  | 46,589  | 554    | Reverse   |
| 47     | hopB                                            | CDS      | 47,650  | 49,671  | 2,022  | Reverse   |
| 48     | Hypothetical protein                             | CDS      | 49,794  | 50,363  | 570    | Reverse   |
| 49     | Hypothetical protein                             | CDS      | 50,657  | 51,094  | 438    | Reverse   |
| 50     | ATP-dependent helicase                           | CDS      | 51,329  | 53,281  | 1,953  | Reverse   |
| 51     | Hypothetical protein                             | CDS      | 53,284  | 54,849  | 1,566  | Reverse   |
| 52     | CspP family protein                              | CDS      | 55,287  | 55,466  | 180    | Reverse   |
| 53     | Hypothetical protein                             | CDS      | 55,471  | 55,644  | 174    | Reverse   |
| 54     | AAA family ATPase                                | CDS      | 55,991  | 56,941  | 951    | Reverse   |
| 55     | hopAB type III effector                          | CDS      | 56,958  | 58,115  | 1,158  | Reverse   |
| 56     | IS911 family transposase                        | CDS      | 58,481  | 59,101  | 621    | Reverse   |
| 57     | RuB protein                                     | CDS      | 59,132  | 59,200  | 69     | Reverse   |
| 58     | ISA481 family transposase                       | CDS      | 59,217  | 60,878  | 1,662  | Reverse   |
| 59     | Recombinase family protein                      | CDS      | 60,859  | 61,434  | 576    | Reverse   |
| 60     | Hypothetical protein                             | CDS      | 61,751  | 62,224  | 474    | Reverse   |

(Continued)
| Number | Name                                      | Type          | Minimum | Maximum | Length | Direction |
|--------|-------------------------------------------|---------------|---------|---------|--------|-----------|
| 61     | Single-stranded DNA-binding protein CDS   |               | 62,425  | 62,886  | 462    | Reverse   |
| 62     | Phage regulatory protein CDS              |               | 62,898  | 63,730  | 833    | Reverse   |
| 63     | DUF3158 family protein CDS                |               | 63,736  | 64,257  | 522    | Reverse   |
| 64     | TIGR03761 family integrating conjugative element protein CDS | | 64,294 | 65,073 | 780 | Reverse |
| 65     | Hypothetical protein CDS                  |               | 65,066  | 65,662  | 597    | Reverse   |
| 66     | Hypothetical protein CDS                  |               | 65,992  | 67,353  | 1,362  | Reverse   |
| 67     | DUF2857 family protein CDS                |               | 67,350  | 68,087  | 738    | Reverse   |
| 68     | parB chromosome partitioning protein CDS  |               | 68,124  | 69,884  | 1,761  | Reverse   |
| 69     | Anc family DNA-binding protein CDS        |               | 69,887  | 70,204  | 318    | Reverse   |
| 70     | Hypothetical protein CDS                  |               | 70,382  | 70,786  | 405    | Reverse   |
| 71     | Hypothetical protein CDS                  |               | 70,788  | 71,117  | 330    | Reverse   |
| 72     | Hypothetical protein CDS                  |               | 71,437  | 72,123  | 687    | Reverse   |
| 73     | Hypothetical protein CDS                  |               | 72,563  | 73,153  | 591    | Reverse   |
| 74     | dnaB chromosome partitioning protein CDS  |               | 73,150  | 74,496  | 1,347  | Reverse   |
| 75     | AAA family ATPase CDS                    |               | 74,554  | 75,414  | 861    | Reverse   |
| 76     | tRNA-Pro                                  |               | 75,651  | 75,727  | 77     | Reverse   |
| 77     | Tyrosine-type repressor/integrase CDS     |               | 76,041  | 77,495  | 1,455  | Reverse   |
| 78     | DNA-binding domain-containing protein CDS |               | 77,492  | 79,423  | 1,932  | Reverse   |
| 79     | tsig conjugal transfer protein CDS        |               | 80,037  | 80,438  | 402    | Reverse   |
| 80     | Hypothetical protein CDS                  |               | 80,499  | 82,028  | 1,533  | Reverse   |
| 81     | Integrating conjugative element protein CDS |               | 82,087  | 83,772  | 1,686  | Reverse   |
| 82     | TIGR03756 family integrating conjugative element protein CDS | | 83,793 | 84,731 | 939 | Reverse |
| 83     | TIGR03757 family integrating conjugative element protein CDS | | 84,728 | 85,159 | 432 | Reverse |
| 84     | DUF1-140 domain-containing protein CDS    |               | 85,713  | 86,201  | 489    | Reverse   |
| 85     | Lytic murein transglycosylase CDS         |               | 86,453  | 87,694  | 1,242  | Reverse   |
| 86     | Hypothetical protein CDS                  |               | 88,186  | 89,818  | 978    | Reverse   |
| 87     | Carbon storage regulator CDS              |               | 90,586  | 90,810  | 225    | Forward   |
| 88     | TrafD/DksA family transcriptional regulator CDS | | 90,846 | 91,262 | 417 | Forward |
| 89     | Hypothetical protein CDS                  |               | 91,297  | 91,518  | 222    | Forward   |
| 90     | GNAT family N-acetyltransferase CDS       |               | 92,339  | 92,830  | 492    | Reverse   |
| 91     | AAA family ATPase CDS                     |               | 93,208  | 93,489  | 282    | Forward   |
| 92     | tnpB CDS                                  |               | 93,794  | 94,150  | 357    | Forward   |
| 93     | IS66 family transposase CDS               |               | 94,167  | 95,699  | 1,533  | Forward   |
| 94     | IS5 family transposase CDS                |               | 95,741  | 96,382  | 642    | Forward   |
| 95     | AAA family ATPase CDS                     |               | 96,453  | 97,271  | 819    | Forward   |
| 96     | Hypothetical protein CDS                  |               | 98,266  | 99,648  | 699    | Forward   |
| 97     | i(p)ppGpp synthetase CDS                  |               | 98,961  | 99,260  | 300    | Forward   |
| 98     | Thioester domain-containing protein CDS    |               | 99,924  | 100,024 | 2,977  | Reverse   |
| 99     | Hypothetical protein CDS                  |               | 100,214 | 100,669 | 456 | Reverse |
| 100    | Conjugal transfer ATPase CDS              |               | 100,647 | 101,433 | 1,497 | Reverse |
| 101    | TIGR03751 family conjugal transfer lipoprotein CDS | | 104,133 | 105,056 | 924 | Reverse |
| 102    | TIGR03752 family integrating conjugative element protein CDS | | 105,053 | 105,721 | 669 | Reverse |
| 103    | TIGR03749 family integrating conjugative element protein CDS | | 105,718 | 106,110 | 393 | Reverse |
| 104    | TIGR03750 family conjugal transfer protein CDS | | 106,126 | 106,494 | 369 | Reverse |
| 105    | TIGR03745 family integrating conjugative element membrane protein CDS | | 106,514 | 106,753 | 240 | Reverse |
| 106    | TIGR03758 family integrating conjugative element protein CDS | | 106,750 | 107,115 | 366 | Reverse |
| 107    | TIGR03759 family integrating conjugative element protein CDS | | 107,195 | 107,525 | 331 | Reverse |
| 108    | TIGR03760 family integrating conjugative element protein CDS | | 108,102 | 109,286 | 1,185 | Reverse |
| 109    | TIGR03761 family integrating conjugative element protein CDS | | 109,422 | 109,907 | 516 | Forward |
| 110    | TIGR03762 family integrating conjugative element protein CDS | | 110,235 | 111,335 | 1,101 | Forward |
| 111    | Serine kinase - nikkomycin CDS            |               | 111,422 | 112,675 | 1,254 | Forward |
| 112    | Conjugative protein CDS                   |               | 112,728 | 113,972 | 1,245 | Forward |
| 113    | hopD type III effector protein CDS        |               | 114,438 | 116,555 | 2,118 | Forward |
| 114    | hopO type III effector protein CDS        |               | 116,671 | 118,014 | 1,344 | Forward |
| 115    | Phosphonoilokinase CDS                    |               | 118,048 | 118,365 | 318 | Forward |
| 116    | UvD helicase domain-containing protein CDS |               | 118,603 | 120,060 | 1,458 | Reverse |
| 117    | TIGR03747 family integrating conjugative element membrane protein CDS | | 120,070 | 120,819 | 750 | Reverse |
| 118    | TraD CDS                                  |               | 120,859 | 123,018 | 2,160 | Reverse |
| 119    | Integrating conjugative element protein CDS |               | 123,030 | 123,536 | 507 | Reverse |
| 120    | TIGR03759 family integrating conjugative element protein CDS | | 123,533 | 124,060 | 558 | Reverse |
| 121    | TIGR03759 family integrating conjugative element protein CDS | | 124,075 | 124,821 | 747 | Reverse |

(Continued)
### TABLE 3 | (Continued)

| Number | Name                                | Type  | Minimum | Maximum | Length | Direction |
|--------|-------------------------------------|-------|---------|---------|--------|-----------|
| 124    | Hypothetical protein                | CDS   | 124,830 | 125,534 | 705    | Reverse   |
| 125    | IS5 family transposase              | CDS   | 125,711 | 126,561 | 851    | Reverse   |
| 126    | Hypothetical protein                | CDS   | 126,700 | 127,581 | 882    | Reverse   |
| 127    | Hypothetical protein                | CDS   | 127,745 | 128,107 | 363    | Reverse   |
| 128    | DEAD/DEAH box helicase family protein | CDS  | 128,202 | 130,460 | 2,259  | Reverse   |
| 129    | Class I SAM-dependent methyltransferase | CDS | 130,564 | 132,015 | 1,452  | Reverse   |
| 130    | Hypothetical protein                | CDS   | 132,045 | 132,644 | 600    | Reverse   |
| 131    | DUF3275 family protein              | CDS   | 132,770 | 133,281 | 612    | Reverse   |
| 132    | Hypothetical protein                | CDS   | 133,482 | 133,844 | 363    | Reverse   |
| 133    | DUF3577 domain-containing protein   | CDS   | 134,172 | 134,726 | 555    | Reverse   |
| 134    | Hypothetical protein                | CDS   | 134,746 | 135,270 | 525    | Forward   |
| 135    | Hypothetical protein                | CDS   | 135,347 | 135,715 | 369    | Reverse   |
| 136    | Regulator                           | CDS   | 135,923 | 136,456 | 534    | Reverse   |
| 137    | topB                                | CDS   | 135,171 | 139,538 | 2,022  | Reverse   |
| 138    | Hypothetical protein                | CDS   | 136,661 | 140,230 | 570    | Reverse   |
| 139    | Hypothetical protein                | CDS   | 140,774 | 141,457 | 684    | Reverse   |
| 140    | ATP-dependent helicase              | CDS   | 141,779 | 143,731 | 1,953  | Reverse   |
| 141    | Hypothetical protein                | CDS   | 143,734 | 145,259 | 1,526  | Reverse   |
| 142    | CrpF family protein                 | CDS   | 145,697 | 145,897 | 201    | Reverse   |
| 143    | IS6 family transposase              | CDS   | 146,020 | 147,260 | 1,241  | Forward   |
| 144    | Hypothetical protein                | CDS   | 147,274 | 147,519 | 246    | Reverse   |
| 145    | Single-stranded DNA-binding protein | CDS   | 147,725 | 148,186 | 462    | Reverse   |
| 146    | Phage regulatory protein            | CDS   | 148,198 | 149,031 | 834    | Reverse   |
| 147    | DUF3158 family protein              | CDS   | 149,037 | 149,600 | 564    | Reverse   |
| 148    | TIGR03781 family integrating conjugative element protein | CDS | 149,597 | 150,376 | 780    | Reverse   |
| 149    | Hypothetical protein                | CDS   | 150,369 | 150,918 | 550    | Reverse   |
| 150    | Hypothetical protein                | CDS   | 151,362 | 152,723 | 1,362  | Reverse   |
| 151    | DUF2857 family protein              | CDS   | 152,720 | 153,457 | 738    | Reverse   |
| 152    | parB chromosome partitioning protein| CDS   | 153,494 | 155,254 | 1,761  | Reverse   |
| 153    | Arc family DNA-binding protein      | CDS   | 155,257 | 155,574 | 318    | Reverse   |
| 154    | Hypothetical protein                | CDS   | 155,510 | 155,755 | 246    | Reverse   |
| 155    | Hypothetical protein                | CDS   | 155,752 | 156,156 | 405    | Reverse   |
| 156    | Hypothetical protein                | CDS   | 156,158 | 156,487 | 330    | Reverse   |
| 157    | Hypothetical protein                | CDS   | 156,797 | 157,387 | 591    | Reverse   |
| 158    | dnaB                                | CDS   | 157,384 | 158,730 | 1,347  | Reverse   |
| 159    | AAA family ATPase                   | CDS   | 158,788 | 159,648 | 861    | Reverse   |
|        | tRNA-Pro                            | tRNA  | 159,887 | 159,963 | 77     | Forward   |

### FIGURE 2

The **clpB-hopR** Region in PtoDC3000. Here we visualize the region between **clpB** and **hopR** from the PtoDC3000 genome, and compare this region to both predicted ICE elements from PmaES4326. Each predicted locus is colored as purple (present in one of the PmaES4326 ICE elements), blue (present in both PmaES4326 ICE elements as per nucleotide identity), and green (only present in PtoDC3000). Predicted tRNA loci are shown in magenta. We also show two nucleotide positions within this region: top number is from the start of **clpB** and the bottom number is from the start of the genome (from **dnaA**). We label all identified type III effector loci and **clpB**, as well as all annotated IS elements (with an "IS") and genes that have predicted frameshifts (with an "*"). All genes potentially implicated in ICE function have been labeled with numbers corresponding to potential orthologs from strain PmaES4326 ICEs and listed in Table 3.
gain compatibility with *N. benthamiana*. The *Pma* DQ3 strain did in fact gain disease compatibility with *N. benthamiana* in a manner similar to the gain-of-compatibility phenotypes previously observed for *PtoDC3000 ΔhopQ1-1* mutants and *Xanthomonas euvesicatoria* 85–10 ΔxopQ mutants (Wei et al., 2007; Adlung et al., 2016). Furthermore, *N. benthamiana* incompatibility could be restored in *PmaICEADQ3* by hopQ complementation in trans and *PmaES4326* incompatibility was not observed in *roq1-1* CRISPR-edited *N. benthamiana* as shown in Figure 4 (Qi et al., 2018).

**Loss of PmaICE-DQ Under Selection in planta**

Observing that we could readily recover strains containing spontaneous evictions of the ICE-DQ containing a single crossover pK18 merodiploid under the selective pressure of sucrose counter-selection, and that the targeted *Pma* DQ3 strain gained disease compatibility with *N. benthamiana*, we were curious whether the selective pressure of ETI would also result in recovery of strains with ICE-DQ evicted. We inoculated *N. benthamiana* with *PmaES4326-C* and *PtoDC3000* at approximately 5 × 10^5 CFU/mL establishing three lineages for each strain (Lineage A, LA; Lineage B, LB; Lineage C, LC). Six to seven days post inoculation bacteria from each lineage were recovered and used to create new inoculum to establish each strain outlined in white boxes underneath the alignment. Each contiguous segment is oriented so that clpB is found on the left side of the segment and so that hopR is found on the right side. Sections within the alignment colored red are those that are aligned into contiguous blocks by Mauve and are shared by all three strains. A white section within the wild type *PmaES4326* alignment represents a region (*PmaICE-DQ*) that is not present in the other two strains. Mauve alignments of these regions demonstrate that identical evictions of ICE-DQ occurred in each strain.

![Figure 3](image_url) | Identical deletions of PmaICE-DQ following laboratory selection and passage under selective conditions in planta. We compared genomic regions bordering ICE-DQ in a strain where the region containing hopQ was intentionally deleted (*Pma DQ3*) as well as a strain that arose after five passages in planta in *N. benthamiana* (*Pma LAP5-20*). Genomic segments from each of the three strains are arranged vertically, with each line labeled by strain name and genes within each strain outlined in white boxes underneath the alignment. Each contiguous segment is oriented so that clpB is found on the left side of the segment and so that hopR is found on the right side. Sections within the alignment colored red are those that are aligned into contiguous blocks by Mauve and are shared by all three strains. A white section within the wild type *PmaES4326* alignment represents a region (*PmaICE-DQ*) that is not present in the other two strains. Mauve alignments of these regions demonstrate that identical evictions of ICE-DQ occurred in each strain.

**Transfer of PmaICE-DQ Between Strains**

Aside from being able to excise from the genome, ICE elements are also categorized by their ability to transfer across bacterial strains, and we therefore tested whether ICE-DQ could undergo conjugation to a naïve strain. To do this, ICE-DQ was marked with chloramphenicol resistance by generating a single-crossover merodiploid with a pK18 derivative plasmid (*Pma ICE-DQ:pK18*). Three recipient strains, *PtoDC3000* and two independent *PmaΔICE-DQ* strains (the targeted *Pma DQ3* strain and the passage derived *Pma LAP5-20*) were labeled with a Tn7 3xmCherry resistant transposon to confer spectinomycin resistance as well as visually differentiate them from the donor strain. The *Pma* ICE-DQ:pK18, Km^R^ marked donor strain was co-cultured with each of the three Tn7
FIGURE 4 | PmaES4326 gains disease compatibility with N. benthamiana in the absence of hopQ/Roq1-mediated ETI. The PmaES4326 WT strain, Pma DQ3 (ΔICE-DQ), DQ3 complemented with pCPP3372hopQ (DQ3pQ), and the non-pathogenic T3SS- strain hrcN:Tn5 were infiltrated into leaves of the N. benthamiana Lab accession (A) or the roq1-1 CRISPR mutant derivative line (B) at $3 \times 10^4$ CFU/mL. Eight days post inoculation leaves were photographed to document symptoms and bacterial load from four 4 mm diameter leaf discs from each infiltrated area by dilution plating was determined as CFUs/cm² leaf tissue. Values are the means and standard deviations of LOG transformed CFUs/cm² from three inoculated plants. *p < 0.05 compared to PmaES4326 WT strain as determined by unpaired one-tailed t-test.

FIGURE 5 | A subset of PmaES4326 isolates passaged through N. benthamiana gain disease compatibility. (A) Two isolates out of 22 PmaES4326 lineage A (LA) passage 5 (P5) isolates produce disease symptoms in the N. benthamiana LAB accession comparable to the targeted Pma DQ3 (ΔICE-DQ) strain. Leaves were photographed eight days post inoculation with $3 \times 10^4$ CFU/mL bacterial suspensions. (B) Tenfold dilution series 10 µL spot plate for assessing bacterial load of select strains highlighted in white in panel A.

3xmCherry SpR recipients. Conjugations between these strains were then plated on kanamycin and spectinomycin selective media (Figure 6A). No kanamycin and spectinomycin resistant mutants or exconjugants were recovered from the PtoDC3000 conjugation or from the single parent control experiments. However, we were able to recover kanamycin and spectinomycin
FIGURE 6 | ICE-DQ can be readily transferred into PmaES4326 strains that lack the ICE element. (A) Schematic representation of the conjugation strategy for monitoring ICE-DQ transfer between strains. The ICE-DQ of the donor strain was marked with kanamycin resistance by single crossover integration the pK18ms plasmid (Pma ICE-DQ:pK18). The recipient strains Pma DQ3 (∆ICE-DQ targeted), Pma LAP5-20 (∆ICE-DQ passage-derived) and PtoDC3000 were marked by introduction of a spectinomycin resistance Tn73xmCherry transposon. Donor and recipients were co-cultured on LM media and ICE-DQ:pK18 mCherry expressing ICE-DQ exconjugants were recovered on kanamycin and spectinomycin. (B) ICE-DQ:pK18 exconjugant recovery frequencies/10⁹ recipients were determined based on kanamycin and spectinomycin resistant recipients as determined by dilution plating. Values are means and standard deviations of three biological replicates. *p < 0.05 compared to Pto WT strain as determined by unpaired one-tailed t-test.

DISCUSSION

Integrative Conjugative Elements (ICEs) are important drivers of evolutionary dynamics within and across bacterial populations and communities because of their potential to disseminate genes and pathways through horizontal transfer (Johnson and Grossman, 2015). Genes encoded by ICEs often impart phenotypes critical for survival under specific environmental conditions, including antibiotic resistance loci as well as phage defense systems (Botelho and Schulenburg, 2021; LeGault et al., 2021). In the phytopathogen Pseudomonas syringae, plasticity of ICE elements encoding genes involved in heavy metal resistance, type III effectors, and carbon metabolism has been identified as the main difference between epidemic strains causing disease across kiwi orchards in New Zealand and with previous outbreaks (Colombi et al., 2017; McCann et al., 2017; Poulter et al., 2018). Despite the accumulation of numerous complete genomes sequences for P. syringae and related species and the likelihood of ICEs to impart traits that affect growth in agricultural settings, to date there have been relatively few ICEs identified and vetted for this pathogen.

Placement of genes and pathways in ICEs may be especially important under conditions of fluctuating selection pressures where selection pressures on cargo genes in an ICE switch from positive to negative depending on the environment. One such scenario involves the presence of type three effector genes, which are bacterial proteins delivered from symbionts into eukaryotic host cells and which are critical for pathogenesis of P. syringae strains in planta (Grant et al., 2006). Type three effectors are particularly well studied in phytopathogens such as P. syringae, where they can be of exceptional benefit on some host plants by enabling strains to overcome host immune responses but may also directly trigger immune responses based on the presence of cognate immune receptors on other host genotypes (Dillon et al., 2019a; Laflamme et al., 2020). The presence of type three effectors on ICEs would enable the rapid transmission of these critical virulence genes across strains, while also enabling genes to be rapidly lost from lineages if host genotypes shift such that the effectors are recognized by R gene pathways. Extending this thought, effectors in ICEs that are recognized by hosts are potentially lost more readily through ICE element excision than through mutation (given dedicated excision machinery of the ICEs), which may facilitate adaptation if a strain encounters a resistant host background. While not an extensive experiment, our passage experiment where a subset of PmaES4326 but not PtoDC3000 clones becomes compatible with N. benthamiana does demonstrate that parameter space exists for such a scenario. Moreover, effectors that are inactivated through mutation can only be reactivated by reversion mutations resistant mCherry + exconjugants in PmaES4326ΔICE-DQ strains at rates of 2.50 × 10⁻⁷ stdev ± 1.23⁻⁷ and 3.05 × 10⁻⁷ stdev ± 1.51⁻⁷ exconjugants per recipient respectively in Pma DQ3 and Pma LAP5-20 recipient strains (Figure 6B) which strongly suggests that the ICE-DQ element is readily transmissible into PmaES4326 strains lacking ICE-DQ.
or through horizontal transfer and acquisition from different strains. In contrast, if effectors are found on ICEs and lost through excision, it’s possible that a small resident pool of strains containing these ICEs will remain on other host plants or throughout the environment, facilitating rapid reacquisition of effectors that could be beneficial under different contexts. Lastly, there are a plethora of type III effectors in *P. syringae* that carry out a variety of functions across different host plants and resistance backgrounds (Dillon et al., 2019a). Some of these effectors can be considered “core” and found in syntenic locations across strains while others are more variably present. It’s possible that presence of effectors in ICEs could itself reflect something about the characteristics of these effectors, in that the proteins found as cargo on ICEs may be subject to higher levels of fluctuating selection across hosts than those found in the core set.

HopQ and HopD are often found together on genomic islands in *P. syringae* genomes, and have been associated with adaptation to agriculturally important crop plants (Wei et al., 2007; Baltrus et al., 2011; Monteil et al., 2016). Presence of *hopQ* in an active ICE in strain *PmaES4326* sets up an interesting scenario because a nearly identical version of this effector is present in a somewhat syntenic position in a relatively closely related strain *PtoDC3000* except that this effector is not part of a functional ICE. Therefore, this genomic context suggests that *hopQ* would experience more evolutionary flexibility in *PmaES4326* than in *PtoDC3000* because it can be more easily lost and/or transferred from this strain due to its presence in a region of the chromosome that can excised at a relatively high rate. To test for differences in evolutionary flexibility, we passaged replicate populations of both *PmaES4326* and *PtoDC3000* in an accession of *N. benthamiana* which can recognize and mount an immune response to HopQ. As such, wild type versions of *PmaES4326* and *PtoDC3000* are recognized by this accession and trigger an immune response which prevents disease. However, we found that passage of *PmaES4326* from plant to plant resulted in recovery of strains of *PmaES4326* that didn’t trigger the immune response. Therefore, presence of this type III effector inside of an ICE enables rapid loss of the recognized effector under conditions of negative selection and there is relatively less flexibility for this effector to be lost in a strain where it is not present in an active ICE.

One other curious feature of the genome of *PmaES4326* is that it encodes two distinct but highly similar ICE elements (Figure 1). The configuration is particularly interesting as two distinct ICE elements have integrated successively into the *PmaES4326* genome and many of the genes involved in ICE functions are quite conserved between the two. It also appears as though integration of the first element recreated a tRNA with a proline anticodon and that the second ICE element can use this as a further integration point. Indeed, excision of ICE-DQ in both the targeted *Pma* DQ3 and passage-derived *Pma* LAP5-20 strains reported here also deletes one of the predicted proline tRNAs. It may therefore be no coincidence that two ICE elements that are highly similar (but which contain different cargo regions) can integrate successively into the genome as it’s straightforward to imagine that highly specific recombinases would have similar target sequences.

Although not the main focus of this manuscript, we also report on plasticity of the *PmaES4326* genome writ large across lab derived strains (Table 2). As the number of complete *P. syringae* genome sequences accumulates, it is becoming more apparent that strain *PmaES4326* is notable compared to the rest of the species complex because it contains so many secondary replicons as well as additional elements that contribute to genomic plasticity (Stavrinides and Guttman, 2004; Stavrinides et al., 2012). Many of these plasmids appear to contain genes involved in virulence and so acquisition of these replicons through horizontal gene transfer has likely contributed to the pathogenic ability of this strain across multiple hosts (Stavrinides and Guttman, 2004). However, there is a clear downside to the large plasmid repertoire of *PmaES4326* as demonstrated by genome sequences reported here. Although we can’t definitively catalog passage histories, genome sequences from isolates from the Dangl and Collmer labs are likely not diverged by > 10 passages total. In this time, the Dangl isolate (*PmaES4326*-D) has lost two different plasmids that were originally reported by Stavrinides et al. and which are contained in the Collmer lab isolate (*PmaES4326*-C). Indeed, genome assemblies for the targeted ICE-DQ excision strain *Pma* DQ3 suggest that this strain has also lost at least one plasmid in addition to the ICE-DQ after only three additional passages in culture. Lastly, even though this strain was extensively surveyed for plasmids through gel electrophoresis and Sanger sequencing, we report the existence of an additional large plasmid present within all sequenced isolates of this strain.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

**AUTHOR CONTRIBUTIONS**

DB and BK conceived, conceptualized, analyzed the experiments, and co-wrote the manuscript. QF conducted a subset of experiments described in the manuscript. All authors contributed to the article and approved the submitted version.

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