Plasmid Vectors for *Xylella fastidiosa* Utilizing a Toxin-Antitoxin System for Stability in the Absence of Antibiotic Selection

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ABSTRACT

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The phytopathogen *Xylella fastidiosa* causes disease in a variety of important crop and landscape plants. Functional genetic studies have led to a broader understanding of virulence mechanisms used by this pathogen in the grapevine host. Plasmid shuttle vectors are important tools in studies of bacterial genetics but there are only a limited number of plasmid vectors available that replicate in *X. fastidiosa*, and even fewer that are retained without antibiotic selection. Two plasmids are described here that show stable replication in *X. fastidiosa* and are effective for gene complementation

both in vitro and in planta. Plasmid maintenance is facilitated by incorporation of the PemI/PemK plasmid addiction system, consisting of PemK, an endoribonuclease toxin, and its cognate antitoxin, PemI. Vector pXf20pemIK utilizes a native *X. fastidiosa* replication origin as well as a high-copy-number pUC origin for propagation in *Escherichia coli* cloning strains. Broad-host-range vector pBBR5pemIK is a medium- to low-copy-number plasmid based on the pBBR1 backbone. Both plasmids are maintained for extended periods of time in the absence of antibiotic selection, as well as up to 14 weeks in grapevine, without affecting bacterial fitness. These plasmids present an alternative to traditional complementation and expression vectors which rely on antibiotic selection for plasmid retention.

Xylella fastidiosa is the causal agent of Pierce's disease of grapevine, citrus variegated chlorosis, and leaf scorch diseases in almond, oleander, and a number of other cultivated plant species (Hopkins 1989; Hopkins and Purcell 2002; Purcell et al. 1999). *X. fastidiosa* is a fastidious organism limited to growth in specific niches, mainly xylem vessels of the host plant and foregut of the insect vector. A slow rate of replication under laboratory conditions and an extended timeframe for in planta virulence trials make genetic studies of pathogenesis and transmission challenging.

Genetic determinants of virulence in *X. fastidiosa* have been identified through random or site-directed mutagenesis and confirmed by complementation analysis (Clifford et al. 2013; Guilhabert and Kirkpatrick 2005; Guilhabert et al. 2001; Reddy et al. 2007). An effective complementation strategy using insertion of a gene of interest into a neutral region of the chromosome has been widely used in grape-pathogenic *X. fastidiosa* strains (Matsumoto et al. 2009). This method relies on homologous recombination between a suicide vector and the bacterial chromosome, limiting use to strains with high sequence similarity. An alternative is to express the gene of interest on a plasmid. However, most commonly used cloning and expression vectors are unable to replicate in *X. fastidiosa*. Plasmid-based complementation methods also rely on stability of the plasmid vector, which varies depending on experimental conditions, selective pressure, and genes expressed (Guilhabert and Kirkpatrick 2003; Matsumoto et al. 2012; Reddy et al. 2007).

Use of antibiotic selection for plasmid maintenance is impractical for certain experimental culture conditions or for in planta studies.

Toxin-antitoxin (TA) systems are an alternative mechanism for plasmid stability in a wide range of bacterial species in the natural environment. Also known as plasmid addiction systems, TA systems consist of a stable toxin molecule and a cognate antitoxin (either protein or RNA) which prevents toxin-mediated growth inhibition or cell death (Unterholzner et al. 2013). For plasmid-encoded TA systems, maintenance of the plasmid is necessary to ensure continuous production of an unstable antitoxin. TA systems are categorized based on antitoxin mode of action. Type II TA systems are defined by a protein antitoxin that interacts with the toxin posttranslation, inhibiting toxin activity (Blower et al. 2011; Syed and Lévesque 2012). Type II TA systems can be found on plasmids or on bacterial chromosomes and, in some cases, regulate bacterial growth rate and cell dormancy (Syed and Lévesque 2012).

PemI/PemK is a type II TA system originally described for its role in stable inheritance of *Escherichia coli* plasmid R100 (Tsuchimoto et al. 1988, 1992). Toxin PemK inhibits cell growth by acting as an endoribonuclease, and antitoxin PemI prevents PemK activity by direct binding (Lee et al. 2012; Zhang et al. 2004). PemI is a target for cellular proteases, and requires continuous production to be maintained at a functional level (Zhang et al. 2004). A PemI/PemK homolog found on a large plasmid in *X. fastidiosa* strain Riv11 functions in a similar manner, contributing to plasmid stability in *X. fastidiosa* (Lee et al. 2010, 2012). The previously described shuttle vector pXf20pemIK (Lee et al. 2010) contains an oriV origin of replication and replication initiator protein TrfA for propagation in *X. fastidiosa* (Stenger and Lee 2011). Addition of the PemI/PemK TA system caused pXf20pemIK to be maintained in *X. fastidiosa* subsp. *fastidiosa* Temecula-1 for multiple generations in the absence of antibiotic selection in vitro (Lee et al. 2010). Suitability of pXf20pemIK for complementation and in planta experiments has not been determined previously.

In this study, we expanded characterization of the PemI/PemK plasmid addiction system as a stability factor for plasmid complementation vectors to be used in *X. fastidiosa* virulence studies in grapevine. Two vectors described here, pXf20pemIK and broad-host-range

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vector pBBR5pemIK, are effective for mutant complementation in *X. fastidiosa* and also are able to replicate in multiple bacterial species, providing a versatile tool for genetic studies in vivo.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and transformation.

All bacterial strains and plasmid constructs are listed in Table 1. Competent cells, cloning vectors, and reagents were purchased from Thermo Fisher Scientific unless otherwise specified. Restriction endonucleases were purchased from New England Biolabs. *E. coli* Top10 was used as a cloning host and for plasmid stability analysis. *E. coli* ccdB Survival 2 T1^R was used for propagation of Gateway vectors. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C with addition of antibiotics when necessary. For *E. coli*, antibiotics were used at the following concentrations: ampicillin at 100 µg/ml, kanamycin at 30 µg/ml, chloramphenicol at 35 µg/ml, and gentamycin at 10 µg/ml. *X. fastidiosa* strains Stag's Leap (subsp. *fastidiosa*) and Dixon (subsp. *multiplex*), and mutant derivatives were grown in PD3 medium at 28°C (Davis et al. 1981). For *X. fastidiosa* strains, PD3 was supplemented when necessary with kanamycin, gentamycin, or chloramphenicol, at a concentration of 5 µg/ml. *Xanthomonas campestris* pv. *campestris* strain 8004 and *Pseudomonas syringae* pv. *tomato* DC3000 were grown at 28°C in LB medium supplemented with kanamycin (30 µg/ml) or gentamycin (10 µg/ml) when necessary.

Xylella fastidiosa was transformed by electroporation (Guilhabert et al. 2001). Plasmid DNA was treated with M.SssI CpG methyltransferase (New England Biolabs) prior to transformation to improve efficiency (Matsumoto and Igo 2010). *Xanthomonas campestris* pv. *campestris*,

P. syringae pv. *tomato*, and *E. coli* were transformed by electroporation using cells prepared as follows. Cells were grown in LB medium to a cell density of optical density at 600 nm (OD₆₀₀) = 0.3 and harvested by centrifugation. Cells were washed twice in sterile distilled H₂O and twice in 10% glycerol and resuspended in 10% glycerol to 1/10 of the original volume.

Plasmid vector construction. Previously described plasmids pBBR1-MCS5 (Kovach et al. 1995) and pXf20pemIK (Lee et al. 2010) were modified in the following manner. pBBR5pemIK was constructed by insertion of a 700-bp fragment containing the *pemI/pemK* open reading frames and native promoter excised from pXf20pemIK using *EcoRI*. Gateway cloning versions of pXf20pemIK and pBBR5pemIK vectors were created using a Gateway vector conversion kit following the manufacturer's instructions. Briefly, Gateway cassette version A was cloned into the *EcoRV* site of pXf20pemIK or the *SmaI* site of pBBR5pemIK to create pXf20pemIK-GW and pBBR5pemIK-GW, respectively. All plasmids were sequenced in entirety to confirm all modifications and locations of unique restriction endonuclease sites.

Mutant $\Delta csp1$ complemented strains. A cold shock protein homolog (Csp1) was previously characterized (Burbank and Stenger 2016) for its role in cold response and virulence in *Xylella fastidiosa*. A knockout mutant ($\Delta csp1$) was used to evaluate effectiveness of plasmid vectors for complementation analysis in both in vitro and in planta experiments. All polymerase chain reaction (PCR) primer sequences used are listed in Supplementary Table S1 and PCR conditions are listed in Supplementary Appendix S1. The genome sequence of *X. fastidiosa* Temecula-1 (Van Sluys et al. 2003) was used for reference in the absence of the full genome sequence of the closely

TABLE 1. Strains and plasmids used in this study

Strains and plasmids	Characteristics ^a	Source or reference
Bacterial strains		
<i>Xylella fastidiosa</i> subsp. <i>fastidiosa</i>		
Stag's Leap	Pathogen causing Pierce's disease of grapevine	Krivanek et al. 2005
<i>X. fastidiosa</i> subsp. <i>multiplex</i> Dixon	Pathogen causing almond leaf scorch	Chen et al. 2005
$\Delta csp1$	Stag's Leap knockout mutant in cold shock protein <i>csp1</i> , Cm ^R	(Burbank and Stenger 2016)
$\Delta csp1/csp1^+$	$\Delta csp1$ complemented by chromosomal insertion, Cm ^R , Gm ^R	(Burbank and Stenger 2016)
$\Delta dinJrelE$	Stag's Leap knockout mutant in toxin-antitoxin system <i>dinJ/relE</i> , Cm ^R	This study
$\Delta dinJrelE/dinJrelE^+$	$\Delta dinJrelE$ complemented by chromosomal insertion, Cm ^R , Gm ^R	This study
<i>Escherichia coli</i> Top10	Cloning host, F ⁻ <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$, $\Phi80lacZ\Delta M15$, $\Delta lacX74$, <i>recA1</i> , <i>araD139</i> , $\Delta(araIeu)7697$, <i>galU</i> , <i>galK</i> , <i>rpsL</i> , (Str ^R), <i>endA1</i> , <i>nupG</i>	Thermo Fisher Scientific
<i>E. coli</i> ccdB Survival 2 T1 ^R	Cloning host for propagation of Gateway vectors, F- <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\Phi80lacZ\Delta M15$ $\Delta lacX74$ <i>recA1</i> <i>araD139</i> $\Delta(ara-leu)7697$ <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i> <i>fhuA::IS2</i>	Thermo Fisher Scientific
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	NCPPB 1108	Cuppels 1986
<i>Xanthomonas campestris</i> pv. <i>campestris</i> 8004	Spontaneous rifampicin-resistant strain derived from <i>X. campestris</i> pv. <i>campestris</i> NCPPB number 1145	Daniels et al. 1984
Plasmids		
pCR8/GW/TOPO	Gateway entry vector for cloning PCR products, Sp ^R	Thermo Fisher Scientific
pCR8-csp1	Entry vector carrying <i>csp1</i> open reading frame and native promoter, Sp ^R	This study
pCR8-dinJrelE::Chl	Entry vector carrying chloramphenicol resistance marker flanked by 1kb upstream and downstream regions of <i>dinJ/relE</i> , Sp ^R	This study
pCR8-dinJrelE	Entry vector carrying <i>dinJ/relE</i> open reading frames and native promoter, Sp ^R	This study
pAX1-Gm/GW	Gateway compatible chromosomal insertion vector, Gm ^R	(Burbank and Stenger 2016)
pAX1-dinJrelE	Chromosomal insertion vector carrying <i>dinJ/relE</i> open reading frames and native promoter, Gm ^R	This study
pXf20pemIK	Complementation vector, pUC ori, oriV, <i>pemI/pemK</i> stability factor, Kan ^R , Amp ^R	(Lee et al. 2010)
pXf20pemIK-GW	Gateway cloning compatible, requires ccdB strain for propagation. GenBank accession KX036765	This study
pXf20pemIK-csp1	Carries <i>Xylella fastidiosa</i> <i>csp1</i> with native promoter, Kan ^R , Amp ^R	This study
pXf20pemIK-dinJrelE	Carries <i>X. fastidiosa</i> <i>dinJ/relE</i> with native promoter, Kan ^R , Amp ^R	This study
pBBR1-MCS5	Broad-host-range cloning vector, pBBR1 replication, Gm ^R	(Kovach et al. 1995)
pBBR5pemIK	Complementation vector, pBBR1 replication, <i>pemI/pemK</i> stability factor, Gm ^R	This study
pBBR5pemIK-GW	Gateway cloning compatible, requires ccdB strain for propagation, GenBank accession KX036764	This study
pBBR5pemIK-csp1	Carries <i>X. fastidiosa</i> <i>csp1</i> with native promoter, Gm ^R	This study
pBBR5pemIK-dinJrelE	Carries <i>X. fastidiosa</i> <i>dinJ/relE</i> with native promoter, Gm ^R	This study

^a Cm^R, Sp^R, Amp^R, Kan^R, and Gm^R indicate resistance to chloramphenicol, spectinomycin, ampicillin, kanamycin, and gentamycin, respectively.

related Stag's Leap strain. Construction of knockout mutant $\Delta csp1$ and complemented strain $\Delta csp1/csp1^+$ has been previously described (Burbank and Stenger 2016). For plasmid complemented strains, the open reading frame of *csp1* (Temecula-1 locus tag PD1380) was PCR amplified and cloned into pCR8/GW/TOPO to create plasmid pCR8-csp1. This construct was recombined with pXf20pemIK-GW and pBBR5pemIK-GW to create plasmids pXf20pemIK-csp1 and pBBR5pemIK-csp1, respectively. Complementation plasmids were transformed into $\Delta csp1$ by electroporation (Guilhabert et al. 2001).

$\Delta dinJrelE$ mutant and complemented strains. To further assess plasmid effects on gene expression level, an unrelated mutant ($\Delta dinJrelE$) and plasmid complemented strains were evaluated for gene expression level. This double knockout mutant was created in two adjacent genes, *dinJ* and *relE* (locus tag PD1099-PD1100) encoding a TA system previously characterized in *X. fastidiosa* Temecula-1 (Lee et al. 2014). DNA fragments (1 kb) upstream and downstream of *dinJrelE* were PCR amplified using Stag's Leap

genomic DNA as template and primer pairs dinJupfwd/dinJupRevChl and reLEDnFwdChl/reLEDnRev. A chloramphenicol resistance cassette from the Tn5 HyperMu transposon (Epicentre) was PCR amplified separately using primers Chlfwd and Chlrev. Primers dinJupRevChl and reLEDnFwdChl contain 15 bp of sequence homologous to the chloramphenicol resistance marker. All three DNA fragments (upstream and downstream flanking regions and chloramphenicol resistance marker) were annealed and assembled using overlap extension PCR (Heckman and Pease 2007). PCR conditions are listed in Supplementary Appendix S1. The combined PCR product of approximately 3 kb was gel purified and cloned into vector pCR8/GW/TOPO, following the manufacturer's instructions, to create pCR8-dinJrelE::chl. This plasmid was transformed into *X. fastidiosa* by electroporation. Homologous recombination and gene replacement were selected for by plating transformants on PD3 with chloramphenicol. Chloramphenicol-resistant colonies were screened by colony PCR using primer sets RST31/RST33 (Minsavage et al. 1994) (*X. fastidiosa*-specific primers)

Fig. 1. Map and characteristics of pXf20pemIK. **A.** Plasmid pXf20pemIK containing the minimum replicon from native plasmid isolated from strain Riv11, and *Escherichia coli* cloning vector replication elements and antibiotic markers from pCR2.1 (Thermo Fisher Scientific); and **B.** Gateway cloning-compatible derivative of pXf20pemIK. All listed restriction sites are unique. Plasmid maps were created using Vector NTI Advance software (Thermo Fisher Scientific).

and *dinJrelE*checkfwd/*dinJrelE*checkrev (gene-specific primers). Correct mutants (Δ *dinJrelE*) were further confirmed by cloning and sequencing the site of recombination. For complementation of mutant Δ *dinJrelE*, a DNA fragment containing the *dinJrelE* open reading frames and 250 bp upstream and downstream (to include all native promoter and terminator sequences) was PCR amplified using primers *dinJrelE*fwd/*dinJrelE*rev and cloned into vector pCR8/GW/TOPO following the manufacturer's instructions, creating pCR8-*dinJrelE*. This plasmid was recombined with Gateway-compatible insertion vector pAX1-Gm-GW (Burbank and Stenger 2016; Matsumoto et al. 2009) according to the manufacturer's protocol for Gateway cloning to create construct pAX1-*dinJrelE*. This suicide plasmid was transformed into mutant Δ *dinJrelE* by electroporation; recombination into a chromosome neutral site was confirmed by cloning and sequencing the site of recombination. The resulting complemented strain was called Δ *dinJrelE*/*dinJrelE*⁺. Plasmid complemented strains bearing *dinJrelE* were created in the same manner as described for *cspI* complemented strains. pCR8-*dinJrelE* was recombined with pXf20p*emIK* and pBBR5p*emIK* to create pXf20p*emIK*-*dinJrelE* and pBBR5p*emIK*-*dinJrelE*, respectively.

Plasmid stability in vitro. To determine plasmid stability in *X. fastidiosa*, wild-type strains (Stag's Leap and Dixon) carrying empty-vector plasmids pBBR5p*emIK* and pXF20p*emIK* were grown in PD3 medium with or without antibiotics and subcultured every 7 days for a total of five consecutive passages. Aliquots were

diluted and plated on PD3 with and without antibiotic selection. Colonies were quantified after 10 days of incubation at 28°C. Percent plasmid retention was determined based on number of antibiotic-resistant CFU divided by total CFU. To determine plasmid stability in *E. coli*, *P. syringae* pv. *tomato*, and *Xanthomonas campestris* pv. *campestris*, cells were grown in LB medium with or without antibiotics and subcultured every 24 h for *E. coli* or 48 h for *P. syringae* pv. *tomato* and *X. campestris* pv. *campestris*. After each subculture, percentage of cells retaining the plasmid were quantified in the same manner as for *Xylella fastidiosa*.

Copy number. *X. fastidiosa* strains carrying empty vectors pXf20p*emIK* or pBBR5p*emIK* were grown in PD3 for 1 week. *E. coli*, *P. syringae* pv. *tomato*, and *Xanthomonas campestris* pv. *campestris* were grown in LB medium overnight (for *E. coli*) or for 48 h (*P. syringae* pv. *tomato* and *X. campestris* pv. *campestris*). Total DNA was extracted from all strains using a Dneasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. DNA samples were diluted 1:10 and 2- μ l aliquots were used as template for quantitative PCR (qPCR) with AB SYBR Green Master Mix. Plasmid quantity was determined based on a standard curve of known concentrations of the purified plasmids using primers *pemIK*fwd/*pemIK*rev, targeting the *pemIK* open reading frame for amplification. For strains carrying both pXf20p*emIK* and pBBR5p*emIK*, alternate sets of primers were used to target the unique antibiotic markers on the different plasmids (*kan*fwd/*kan*rev and *gm*fwd/*gm*rev). Primers used for genome quantification targeted a gene or region known to be

Fig. 2. Map and characteristics of pBBR5p*emIK*. **A**, pBBR5p*emIK*, created by addition of the *pemI/pemK* toxin-antitoxin system sequence to broad-host-range vector pBBR1-MCS5 and **B**, Gateway compatible derivative. All listed restriction sites are unique. Plasmid maps were created using Vector NTI Advance software (Thermo Fisher Scientific).

present in a single copy on the bacterial chromosome: *gyrB* for *Xylella fastidiosa* and *P. syringae*, *rpoB* for *Xanthomonas campestris*, and *dxs* for *E. coli*. Primers to determine genome quantity were as follows: XfgyrBfwd/XfgyrBrev (*Xylella fastidiosa*), dxsfwd/dxsrev (*E. coli*), rpoBfwd/rpoBrev (*Xanthomonas campestris* pv. *campestris*), and PsgyrBfwd/PsgyrBrev (*P. syringae* pv. *tomato*). Copy number per genome was calculated based on size and quantity of both the bacterial chromosome and plasmid, as described (Lee et al. 2006).

Gene-expression analysis. Total RNA was extracted from cells after 7 days of growth on PD3 plates with or without the appropriate antibiotics using the hot phenol extraction method described by Jahn et al. (2008). RNA samples were treated with Turbo Dnase and RNA integrity was evaluated by agarose gel electrophoresis. Total RNA (1 µg) was used to make cDNA using an iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using AB SYBR Green Master Mix and primer pairs *csp1*fwd/*csp1*rev and *dinJ*fwd/*dinJ*rev for quantification of *csp1* and *dinJ*, respectively. Relative gene expression was normalized to reference gene *dnaQ* (primers *dnaQ*fwd/*dnaQ*rev). *DnaQ* expression was observed to be stable in different mutants tested and also has been described previously as a reference gene in *Xylella fastidiosa* gene-expression studies (Zaini et al. 2008). Gene-expression analysis was performed on at least three independent biological replicates for each strain, with three PCR replicates for each.

Cold tolerance. *X. fastidiosa* strains were grown on PD3 plates for 7 days at 28°C. Cells were harvested by scraping off plates and resuspended in sterile 1× phosphate-buffered saline (PBS). Starting cell density was normalized by measuring OD₆₀₀ and adjusting the concentration to OD₆₀₀ = 0.1 with 1× PBS. A 1-ml aliquot of cells from each strain was removed and serial dilutions were plated on PD3 to serve as an untreated control. Cold-treated samples were kept at 4°C for 24 h and then serial dilutions were plated on PD3. Colonies were counted for quantification after 10 days of incubation at 28°C. Percent survival indicated number of CFU in treated samples divided by number of CFU in the untreated control, presented as the mean of at least three independent experiments, each consisting of three biological replicates.

Virulence assay. Grapevines (*Vitis vinifera* ‘Chardonnay’) were inoculated with wild-type Stag’s Leap, Δ *csp1*, Δ *csp1/csp1*⁺, Δ *csp1* pBBR5pemIK-*csp1*, Δ *csp1* pXf20pemIK-*csp1*, and empty-vector controls using the needle inoculation method previously described (Hill and Purcell 1995; Roper et al. 2007). Plants were observed for symptom development weekly and assigned a disease rating on a scale of 0 to 5 (Roper et al. 2007). Bacterial quantity was determined by qPCR. Total DNA was extracted from grapevine petioles 15 cm above the point of inoculation 12 weeks postinoculation. qPCR was performed using AB SYBR Green Master Mix following the manufacturer’s instructions, with 5 µl of total DNA as template and primer set XF145-60fwd/XF145-60rev for *X. fastidiosa* detection (Ledbetter and Rogers 2009). PCR conditions are listed in Supplementary Appendix S1. Quantity of *X. fastidiosa* was determined based on a standard curve of wild-type Stag’s Leap genomic DNA, and normalized to total DNA concentration.

Plasmid stability in planta. Grapevines were inoculated with wild-type *X. fastidiosa* Stag’s Leap as well as strains carrying empty-vector plasmids pBBR1-MCS5, pBBR5pemIK, and pXf20pemIK using the same method as described for virulence assays. Petioles (3 petioles/plant) were removed from symptomatic leaves after 14 weeks postinoculation and tissue weight was recorded. Petioles were surface sterilized by successive washing in 70% ethanol for 30 s and 20% bleach for 30 s, and rinsed twice in sterile water for 30 s. Tissue was ground in 2 ml of sterile 1× PBS and serial dilutions were plated on PD3 plates with and without antibiotics. Colonies were quantified after 1 week of growth at 28°C and normalized to original tissue weight. Percentage of cells retaining the plasmid was determined by the number of antibiotic-resistant CFU divided by total CFU. Ten plants were sampled for each strain and dilution plating was performed on triplicate samples from each plant.

Tests for statistical significance. Significance of quantitative differences among treatments was determined using one-way analysis of variance followed by a Tukey-Kramer multiple comparison test ($\alpha = 0.05$). All statistical analyses were performed using Origin 2015 graphing and analysis software (OriginLab Inc.).

RESULTS

Expression of PemI/PemK on plasmid vectors increases stability. A modified version of pXf20pemIK (Lee et al. 2010) (pXf20pemIK-GW, GenBank accession KX036765) was created by adding a Gateway cloning cassette (Walhout et al. 2000) to increase versatility of the vector by eliminating the need for restriction endonuclease cloning (Fig. 1). In addition, a broad-host-range vector was constructed to further explore utility of the PemI/PemK addition system for plasmid stability in a wider range of bacterial species, using pBBR1-MCS5 (Kovach et al. 1995) as a vector backbone. pBBR1-MCS5 alone is able to replicate in *X. fastidiosa*, although it is lost after extended incubation or growth in planta (Matsumoto et al. 2009, 2012; Reddy et al. 2007). Addition of the *pemI/pemK* sequence downstream of the multiple cloning site created pBBR5pemIK (Fig. 2). A Gateway cloning-compatible version (pBBR5pemIK-GW, GenBank accession KX036764) also was created by insertion of a Gateway cassette into the multiple cloning site (Fig. 2). These two plasmids, pXf20pemIK and pBBR5pemIK, as well as Gateway-compatible derivatives were used for characterization as complementation vectors in *X. fastidiosa*.

Vectors pXf20pemIK and pBBR5pemIK are both stable in *X. fastidiosa* but vary in host range and copy number. Vectors were transformed into several gram-negative bacterial species to assess host range and plasmid stability without antibiotic selection. Plasmid pXf20pemIK was stable for replication in *E. coli* and *X. fastidiosa* Stag’s Leap but otherwise had a limited host range, especially in the absence of antibiotic selection (Table 2), which is consistent with previous findings (Lee et al. 2010). pXf20pemIK also did not replicate in *X. fastidiosa* Dixon, a strain belonging to the *multiplex* subspecies which causes almond leaf scorch (Table 2). pBBR5pemIK was able to replicate in all species and strains tested. Copy numbers of *pemI/pemK* carrying vectors were evaluated in different host species using a qPCR method (Lee et al. 2006). pXf20pemIK replicates at a high copy number in *E. coli* due to the pUC origin (Table 3). In *X. fastidiosa*, pXf20pemIK replicates at a lower copy number based on indigenous *X. fastidiosa* oriV. pBBR5pemIK, in addition to having a broad host range, consistently replicates at a medium to low copy number in all host species studied, both with and without antibiotic selection (Table 3).

Vectors pBBR5pemIK and pXf20pemIK are compatible in *E. coli* and *X. fastidiosa*. Compatibility of pBBR5pemIK and pXf20pemIK for cotransformation was assessed in both *E. coli* and *X. fastidiosa*. *E. coli* already carrying pBBR5pemIK was transformed with pXf20pemIK by electroporation. *E. coli* transformants resistant to both kanamycin and gentamycin (from resistance encoded by pBBR5pemIK and pXf20pemIK, respectively) were selected and colonies were found to contain both plasmids based on restriction endonuclease digestion (Supplementary Figure S1). *E. coli* strains containing both plasmids were subcultured every 24 h for a total of five times without antibiotic selection and evaluated for retention of the plasmids by plating on selective media containing either kanamycin or gentamycin. Retention of pBBR5pemIK was close to 100% whereas pXf20pemIK was only present in a small percentage of cells (13%) (Fig. 3). In the presence of antibiotic selection, the copy number of pXf20pemIK was 113 ± 38 in *E. coli* carrying both plasmids, based on qPCR. Under the same conditions, the copy number of pBBR5pemIK was 22 ± 4.3 .

To determine compatibility of pXf20pemIK and pBBR5pemIK in *X. fastidiosa*, wild-type Stag’s Leap carrying pXf20pemIK was transformed with pBBR5pemIK by electroporation. Transformants were recovered from selective plates containing both kanamycin

and gentamycin and the presence of both plasmids was confirmed by restriction endonuclease digestion. Similar to what was observed in *E. coli*, pBBR5pemIK was still present in 100% of *X. fastidiosa* colonies. pXf20pemIK was retained by 50% of *X. fastidiosa* colonies after five subcultures (Fig. 3). The copy number of pXf20pemIK was reduced to 1.5 ± 0.74 in *X. fastidiosa* carrying both plasmids grown with antibiotic selection. pBBR5pemIK copy number was not affected by the presence of pXf20pemIK. In both *E. coli* and *X. fastidiosa*, the presence of the two plasmids simultaneously was confirmed by plasmid extraction and restriction endonuclease digestion (Supplementary Fig. S1).

pBBR5pemIK and pXf20pemIK are effective complementation vectors in *X. fastidiosa*. Suitability of pXf20pemIK and pBBR5pemIK for complementation was evaluated using a previously characterized *X. fastidiosa* knockout mutant, $\Delta csp1$ (Burbank and Stenger 2016). Mutant $\Delta csp1$ contains a deletion in a cold shock protein homolog and is less tolerant of cold temperatures, showing reduced survival rates after incubation at 4°C. To compare the merit of plasmid complementation with previously described methods, complemented strain $\Delta csp1/csp1^+$ was included, which contains a chromosomal insertion of *csp1* at a neutral location (Matsumoto et al. 2009). Plasmids pXf20pemIK and pBBR5pemIK, containing a wild-type copy of *csp1* (including its native promoter), were evaluated for phenotypic complementation of $\Delta csp1$. Survival rates of wild-type Stag's Leap, $\Delta csp1$, and plasmid-complemented strains were quantified following cold treatment at 4°C for 24 h (Fig. 4). Both plasmid-complemented strains did not differ significantly from the wild-type survival rate (Fig. 4), equivalent to the complemented strain $\Delta csp1/csp1^+$ (Burbank and Stenger 2016). Strain $\Delta csp1$ carrying empty-vector pBBR5pemIK and pXf20pemIK did not show complementation. All strains were grown without antibiotic selection for the duration of the experiment. These results demonstrate the utility of PemI/PemK-stabilized vectors for complementation experiments in vitro.

Plasmid impact on gene expression. Gene expression at the transcriptional level was compared for *csp1* in wild-type Stag's Leap, $\Delta csp1$ pBBR5pemIK-*csp1*, $\Delta csp1$ pXf20pemIK-*csp1*, and $\Delta csp1/csp1^+$ to determine whether there were effects due to expression on the

plasmids (Table 4). Although *csp1* expression in $\Delta csp1/csp1^+$ was equivalent to wild-type levels, expression in $\Delta csp1$ pBBR5pemIK-*csp1* and $\Delta csp1$ pXf20pemIK-*csp1* was 50-fold higher (Table 4). However, this increase is most likely due to the regulation of *csp1* specifically, because this gene shows constitutively high expression at the translational level as well (Burbank and Stenger 2016). Expression analysis of a different gene (*dinJ*) showed a much smaller increase (fivefold) in plasmid-complemented strains compared with the wild type or a chromosomal insertion (Table 4). DinJ is an antitoxin that functions as part of the DinJ/RelE TA system (Lee et al. 2014). DinJ homologs have demonstrated autoregulation by direct binding to the *dinJ* promoter region. This regulatory feedback would reduce the increase in expression caused by a multicopy plasmid.

PemI/PemK conveys long-term stability to plasmids in planta. Stability of pBBR5pemIK and pXf20pemIK in wild-type *X. fastidiosa* Stag's Leap were evaluated after 14 weeks postinoculation in grapevines inoculated by a pin prick on the stem. The original pBBR1-MCS5 vector without addition of the *pemI/pemK* sequence also was included for comparison. pXf20 (prior to addition of *pemI/pemK*) was not stable after several generations in vitro (Lee et al. 2010) and, therefore, was not tested in planta. Bacterial populations isolated from plants inoculated with wild-type Stag's Leap, pBBR1-MCS5, pBBR5pemIK, and pXf20pemIK were compared to determine whether carrying the plasmids had any fitness cost during growth in the plant (Fig. 5A). There was no significant difference in bacterial population between the different strains, suggesting that the plasmid empty vectors do not affect growth and replication of *X. fastidiosa* in the plant (Fig. 5A). Percentage of bacterial population retaining the plasmid was quantified by growing *X. fastidiosa* populations isolated from infected plant tissue in the presence and absence of antibiotics. Restriction enzyme digestion confirmed the presence of the plasmid in antibiotic-resistant colonies (data not shown). pXf20pemIK and pBBR5pemIK were retained at 98 and 95%, respectively (Fig. 5B), while pBBR1-MCS5 lacking *pemI/pemK* was not present in any isolated colonies. These results indicate that PemI/PemK is an effective addition system for plasmid maintenance during bacterial growth in the plant host.

pXf20pemIK and pBBR5pemIK are effective complementation vectors in planta. pXf20pemIK and pBBR5pemIK were evaluated for suitability as complementation vectors for virulence studies in *X. fastidiosa* using the $\Delta csp1$ mutant, which is still pathogenic in grapevine but shows reduced symptom development. After 12 weeks postinoculation, symptom development was quantified using a 0-to-5 rating scale (Roper et al. 2007). Plants

TABLE 2. Plasmid host range and stability in vitro^a

Bacteria	pXf20pemIK		pBBR5pemIK	
	+Kan	-Kan	+Gm	-Gm
<i>Xylella fastidiosa</i> Stags Leap	+	+	+	+
<i>X. fastidiosa</i> Dixon	-	-	+	+
<i>Escherichia coli</i>	+	+	+	+
<i>Pseudomonas syringae</i>				
pv. <i>tomato</i>	+	-	+	+
<i>Xanthomonas campestris</i>				
pv. <i>campestris</i>	-	-	+	+

^a Symbols: + indicates $\geq 80\%$ of colonies still contain plasmid after five consecutive subcultures and - indicates $\leq 40\%$ contain plasmid after three subcultures.

TABLE 3. Plasmid copy number^a

Bacteria	pXf20pemIK		pBBR5pemIK	
	+Kan	-Kan ^a	+Gm	-Gm ^a
<i>Xylella fastidiosa</i>				
Stags Leap	8 ± 1.0	9 ± 1.8	10 ± 1.7	10 ± 2.6
<i>X. fastidiosa</i> Dixon	...	...	11 ± 3.8	16 ± 3.8
<i>Escherichia coli</i>	220 ± 50	85 ± 14^b	21 ± 1.6	19 ± 1.6
<i>Pseudomonas syringae</i>				
pv. <i>tomato</i>	17 ± 2.6	18 ± 1.1	16 ± 0.15	13 ± 5.0
<i>Xanthomonas campestris</i>				
pv. <i>campestris</i>	...	...	3.9 ± 1.3	3.3 ± 0.18

^a Copy number is reported for growth after one generation.
^b Significantly different from copy number in the presence of antibiotic selection based on Student's *t* test ($P \leq 0.05$).

Fig. 3. Plasmid stability following cotransformation of pXf20pemIK and pBBR5pemIK. Percentage of antibiotic-resistant colonies after five consecutive subcultures without antibiotic selection of *Xylella fastidiosa* and *Escherichia coli* carrying both plasmids pXf20pemIK (KanR) and pBBR5pemIK (GmR). Percentage of cells still retaining the plasmids were determined by antibiotic-resistant CFU divided by total CFU. Bars represent the mean of at least three independent biological replicates plated in duplicate. Error bars indicate standard deviation.

inoculated with $\Delta csp1$ carrying complementation constructs pXf20pemIK-*csp1* or pBBR5pemIK-*csp1* had average disease scores, equivalent to wild-type inoculated plants (Fig. 6A). Plants inoculated with $\Delta csp1$ transformed with empty vectors pXf20pemIK or pBBR5pemIK did not differ significantly from those inoculated with $\Delta csp1$, confirming that empty vectors alone do not have a phenotypic effect on *X. fastidiosa* in the plant (Fig. 6A). Mutant $\Delta csp1$ also has been shown to have a lower bacterial quantity in inoculated plants as compared with wild-type *X. fastidiosa* Stag's Leap (Burbank and Stenger 2016). Bacterial populations were quantified by qPCR of total DNA extracted from petioles of inoculated plants (Fig. 6B). Plants inoculated with $\Delta csp1$ pXf20pemIK-*csp1* and $\Delta csp1$ pBBR5pemIK-*csp1* showed an increase in bacterial quantity as compared with the mutant $\Delta csp1$. Plants inoculated with $\Delta csp1$ carrying empty vectors pXf20pemIK or pBBR5pemIK had bacterial quantities equivalent to $\Delta csp1$ alone (Fig. 6B). These results demonstrate the practical use of plasmids pXf20pemIK and pBBR5pemIK for complementation of *X. fastidiosa* mutants in planta.

DISCUSSION

Plasmid vectors that can be maintained by the cell in a stable manner under a variety of experimental conditions are essential to studies of bacterial genetics. Disease development in susceptible grapevines following inoculation with *X. fastidiosa* generally takes several months. For complementation of mutants altered in virulence or characterization of *X. fastidiosa* strains expressing exogenous proteins, use of plasmid vectors is limited to those that are stable without antibiotic selection for long periods of time in the plant environment. Very few plasmids have been characterized that are supported for replication in *X. fastidiosa*, and even fewer are retained in the absence of selective pressure. Native plasmids are present in several environmental isolates of *X. fastidiosa* and are generally stable but, unmodified, are not suitable for use as cloning vectors because of

size and lack of selectable markers (Lee et al. 2010; Rogers and Stenger 2012; Stenger et al. 2010). Combination of *X. fastidiosa* replication elements with an *E. coli*-compatible cloning vector presents one solution. Minimum replicon and PemI/PemK stability elements derived from a native *X. fastidiosa* plasmid combined with commercially available cloning vector pCR2.1 (Thermo Fisher Scientific) was characterized previously as a potential vector for use in *X. fastidiosa* (pXf20pemIK) in vitro (Lee et al. 2010). We demonstrate here that pXf20pemIK is stable for up to 14 weeks during infection in grapevine, and can be used for phenotypic complementation both in vitro and in planta. This vector is somewhat limited in host range, possibly due to the presence of multiple replication origins. As a broad-host-range alternative, pBBR5pemIK, which utilizes the same TA system for plasmid retention, was developed. This vector is based on the pBBR1-MCS5 backbone, derived from a small native plasmid found in *Bordetella bronchiseptica* (Antoine and Locht 1992). Plasmids based on pBBR1 have been demonstrated to replicate in *E. coli*, *B. pertussis*, *Vibrio cholerae*, *Rhizobium meliloti*, *P. putida*, *X. fastidiosa*, *P. stewartii*, and multiple *Brucella* spp. (Antoine and Locht 1992; Elzer et al. 1995; Kovach et al. 1995; Mohammadi et al. 2012; Reddy et al. 2007). Although relatively stable in the absence of antibiotic selection in some bacterial species (Elzer et al. 1995), during long-term growth of *X. fastidiosa* in planta, pBBR1-MCS5 is lost without selective pressure (Matsumoto et al. 2012). Therefore, greater stability of pBBR5pemIK due to the PemI/PemK TA system enhances its practical use for in planta experiments. Additionally, pBBR1-derived plasmids do not belong to any well-established incompatibility groups and, thus, may coexist with a number of other plasmids, including commonly used cloning and expression vectors (Antoine and Locht 1992). Ability of pBBR5pemIK and pXf20pemIK to be cotransformed in *X. fastidiosa* allows for the possibility of exogenous expression of more than one gene simultaneously. Different strains of *X. fastidiosa* vary in plant host range and presence of different mobile genetic elements (de Mello Varani et al. 2008; Van Sluys et al. 2003). *X. fastidiosa* Stag's Leap belongs to subspecies *fastidiosa*, which includes strain Temecula-1 and other strains causing Pierce's disease of grapevine. Molecular virulence mechanisms of other subspecies such as *multiplex* (causing almond leaf scorch) and *sandyi* (causing oleander leaf scorch) are less well studied. pXf20pemIK was not stable after transformation into Dixon, suggesting a difference in native plasmids present in different *X. fastidiosa* subspecies that could interfere with replication of pXf20pemIK. In comparison, pBBR5pemIK has a broad host range, allowing for use in other *X. fastidiosa* strains beyond those of subspecies *fastidiosa*. Gene expression level is an important consideration for complementation vectors because phenotypic anomalies can be created by over- or underexpression of the gene of interest. Significantly higher gene expression (50-fold) was observed for *csp1* carried on both

Fig. 4. Complementation of $\Delta csp1$ knockout mutant using plasmids vectors. Percent cell survival of *Xylella fastidiosa* was quantified based on counts of CFU before and after 24 h at 4°C. Mean percent cell survival was compared for wild-type Stag's Leap (WT), a deletion mutant of *csp1* ($\Delta csp1$), $\Delta csp1$ complemented by chromosomal insertion ($\Delta csp1/csp1^+$), and plasmid complemented strains $\Delta csp1$ pXf20pemIK-*csp1* and $\Delta csp1$ pBBR5pemIK-*csp1*. $\Delta csp1$ pXf20pemIK and $\Delta csp1$ pBBR5pemIK are empty-vector controls. Bars represent means ($\pm$ standard error) combined from three independent experiments, each consisting of three replicates plated in duplicate. Means with different letters are significantly different based on analysis of variance followed by a Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

TABLE 4. Relative gene expression

Strain	Relative quantity ^a	
	<i>csp1</i>	<i>dinJ</i>
Wild-type Stag's Leap	1 $\pm$ 0.00	ND
$\Delta csp1$	0.02 $\pm$ 0.02 ^b	ND
$\Delta csp1/csp1^+$	1.13 $\pm$ 0.78	ND
$\Delta csp1$ pBBR5pemIK- <i>csp1</i>	54.26 $\pm$ 4.70 ^b	ND
$\Delta csp1$ pXf20pemIK- <i>csp1</i>	50.24 $\pm$ 4.85 ^b	ND
Wild-type Stag's Leap	ND	1 $\pm$ 0.00
$\Delta dinJrelE$	ND	0.015 $\pm$ 0.01 ^b
$\Delta dinJrelE/dinJrelE^+$	ND	1.66 $\pm$ 0.70
$\Delta dinJrelE$ pBBR5pemIK- <i>dinJrelE</i>	ND	5.54 $\pm$ 1.82 ¹
$\Delta dinJrelE$ pXf20pemIK- <i>dinJrelE</i>	ND	5.32 $\pm$ 2.02 ¹

^a ND = not determined.
^b Significantly different relative to the wild-type expression level based on a Student's *t* test (*P* $\leq$ 0.05).

plasmids pBBR5pemIK and pXf20pemIK compared with the wild type. However, this is most likely due to transcriptional regulation of *csp1* specifically because the effect was not seen with a different gene (*dinJ*). It is probable that *csp1* does not have feedback regulation at the transcriptional level such that high expression results from multiple gene copies controlled by a constitutive promoter. Similar expression levels were seen regardless of which plasmid was used, suggesting that this increase was not due to readthrough activity from another upstream promoter. Equally high expression levels also were observed for *csp1* expressed on pBBR1-MCS5 (data not shown), implying that PemI/PemK activity is not affecting transcription of *csp1*. Expression of *dinJ*, which is autoregulated at the transcriptional level, was fivefold higher in plasmid complemented strains compared

with wild-type gene expression. This suggests that repression through native promoter sequences is able to partially compensate for increased gene dosage caused by expression on a multicopy plasmid. In instances where overexpression of exogenous genes is desirable, this could be a benefit; however, in specific cases where it is essential to have very tightly regulated gene expression at the transcriptional level, the vectors described here may need further modification to adjust promoter strength.

Fig. 5. Stability of pXf20pemIK and pBBR5pemIK in planta. Bacterial populations were isolated from petioles of symptomatic leaves after 14 weeks postinoculation. Petiole samples were surface sterilized and ground in 1× phosphate-buffered saline. Serial dilutions were plated on PD3 medium with and without antibiotics. CFU were quantified and normalized to original tissue weight. **A**, Total CFU of strains carrying different plasmids were compared with CFU from wild-type (WT) inoculated plants to determine whether plasmids had any effect on bacterial fitness in the plant. **B**, Percentage of cells still retaining the plasmids were determined by antibiotic-resistant CFU divided by total CFU. Bars represent the mean of 10 samples taken from different plants and plated in triplicate. Error bars indicate standard deviations. Bars with the same letters are not significantly different based on analysis of variance ($\alpha = 0.05$).

Fig. 6. Complementation of $\Delta csp1$ mutant in planta using plasmid vectors. Susceptible grapevines (*Vitis vinifera* Chardonnay) were needle inoculated with wild-type Stag's Leap (WT), a *csp1* deletion mutant ($\Delta csp1$), or complemented strains $\Delta csp1/csp1^+$, $\Delta csp1$ pXf20pemIK-*csp1*, and $\Delta csp1$ pBBR5pemIK-*csp1* in addition to empty-vector control strains $\Delta csp1$ pXf20pemIK and $\Delta csp1$ pBBR5pemIK. Plants inoculated with phosphate-buffered saline served as negative controls. After 12 weeks postinoculation, **A**, symptoms were quantified using a 0-to-5 rating scale (Roper et al. 2007) and **B**, bacterial quantity was determined by quantitative polymerase chain reaction based on a standard curve of WT genomic DNA and normalized to total DNA concentration. Quantity of *Xylella fastidiosa* DNA was determined. Bars represent means ($\pm$ standard error); means with different letters are significantly different based on analysis of variance followed by a Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

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