Functional Characterization of Replication and Stability Factors of an Incompatibility Group P-1 Plasmid from *Xylella fastidiosa*[∇]

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Xylella fastidiosa **strain riv11 harbors a 25-kbp plasmid (pXF-RIV11) belonging to the IncP-1 incompatibility group. Replication and stability factors of pXF-RIV11 were identified and used to construct plasmids able to replicate in** *X. fastidiosa* **and** *Escherichia coli***. Replication in** *X. fastidiosa* **required a 1.4-kbp region from pXF-RIV11 containing a replication initiation gene (***trfA***) and the adjacent origin of DNA replication (***oriV***). Constructs containing** *trfA* **and** *oriV* **from pVEIS01, a related IncP-1 plasmid of the earthworm symbiont** *Verminephrobacter eiseniae***, also were competent for replication in** *X. fastidiosa***. Constructs derived from pXF-RIV11 but not pVEIS01 replicated in** *Agrobacterium tumefaciens***,** *Xanthomonas campestris***, and** *Pseudomonas syringae***. Although plasmids bearing replication elements from pXF-RIV11 or pVEIS01 could be maintained in** *X. fastidiosa* **under antibiotic selection, removal of selection resulted in plasmid extinction after 3 weekly passages. Addition of a toxin-antitoxin addiction system (***pemI/pemK***) from pXF-RIV11 improved plasmid stability such that >80 to 90% of** *X. fastidiosa* **cells retained plasmid after 5 weekly passages in the absence of antibiotic selection. Expression of PemK in** *E. coli* **was toxic for cell growth, but toxicity was nullified by coexpression of PemI antitoxin. Deletion of N-terminal sequences of PemK containing the conserved motif RGD abolished toxicity.** *In vitro* **assays revealed a direct interaction of PemI with PemK, suggesting that antitoxin activity of PemI is mediated by toxin sequestration. IncP-1 plasmid replication and stability factors were added to an** *E. coli* **cloning vector to constitute a stable 6.0-kbp shuttle vector (pXF20-PEMIK) suitable for use in** *X. fastidiosa***.**

Xylella fastidiosa is a Gram-negative gammaproteobacterium inhabiting the xylem of host plants (42). Infection by *X. fastidiosa* may lead to blockage of xylem transport, resulting in scorching and dieback symptoms of perennial crops, ornamentals, and landscape plants (23). In California, introduction of the polyphagous glassy-winged sharpshooter vector (6, 24) led to epidemics of Pierce's disease in grapes (21, 41, 33) and emergence of leaf scorch/dieback diseases of oleander (25), olive (16), sweetgum (15), and mulberry (17).

X. fastidiosa strains recovered from mulberry trees exhibiting leaf scorch symptoms harbor closely related 25-kbp plasmids sharing extensive nucleotide sequence identity with pVEIS01 (GenBank accession no. CP000543), a 31-kbp plasmid from the earthworm symbiont *Verminephrobacter eiseniae* (35). Annotation of the 25,105-bp sequence of pXF-RIV11 (GenBank accession no. GU938457) revealed that the plasmid contained homologues of incompatibility group P-1 (IncP-1) replication elements (*trfA* and *oriV*). Replication initiation of IncP-1 plasmids requires one plasmid-encoded protein (TrfA) (4). Plasmid replication is initiated by binding of TrfA protein to iterated sequences of *oriV* (19, 34).

Stable inheritance of plasmids may be achieved through toxin-antitoxin (TA) addiction systems (1, 3, 9, 14, 28, 29, 36, 40). The toxin disrupts an essential cellular process; antitoxin neutralizes toxin activity by direct binding (5, 7, 31, 39). Because the toxin is stable and persists longer than labile anti-

toxin, plasmid-free daughter cells are eliminated from the population even in the absence of other means of selection (such as antibiotic resistance or catabolic pathways conferring environmental adaptation). TA modules are grouped into at least eight families based on sequence and structure-function similarity (11, 20). The sequence of pXF-RIV11 includes homologues of the *pemI/pemK* TA system that has been characterized for pR100 (37, 38, 39, 40). PemK toxin encoded by pR100 inhibits protein synthesis by cleaving mRNAs at specific sites; addition of PemI antitoxin negates toxic properties of PemK (43).

Strains of *X. fastidiosa* harbor a variety of other plasmids (8, 13, 22, 26, 32). Derivatives of some these plasmids have been used as shuttle vectors able to replicate in both *Escherichia coli* and *X. fastidiosa* (13, 26). However, constructs bearing replication elements from these endogenous *X. fastidiosa* plasmids were unstable in the absence of antibiotic selection, precluding use in complementation experiments conducted with *X. fastidiosa* in plants. Here, we describe the functional characterization of replication elements from pXF-RIV11 and pVEIS01. We also characterize the *pemI/pemK* TA module from pXF-RIV11 that conferred stable inheritance of shuttle vector plasmids based on minimal IncP-1 replicons in *X. fastidiosa* in the absence of antibiotic selection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strain JM109 (Promega) was grown in TB medium at 37°C. *X. fastidiosa* strain Temecula1 was grown in PD3 medium at 28°C (27). *Agrobacterium tumefaciens* strain GV3101 was grown in LB medium at 28°C. *Pseudomonas syringae* pv. tomato strain DC3000 was grown in King's B medium at 28°C. *Xanthomonas campestris* pv. campestris strain Xcc8004

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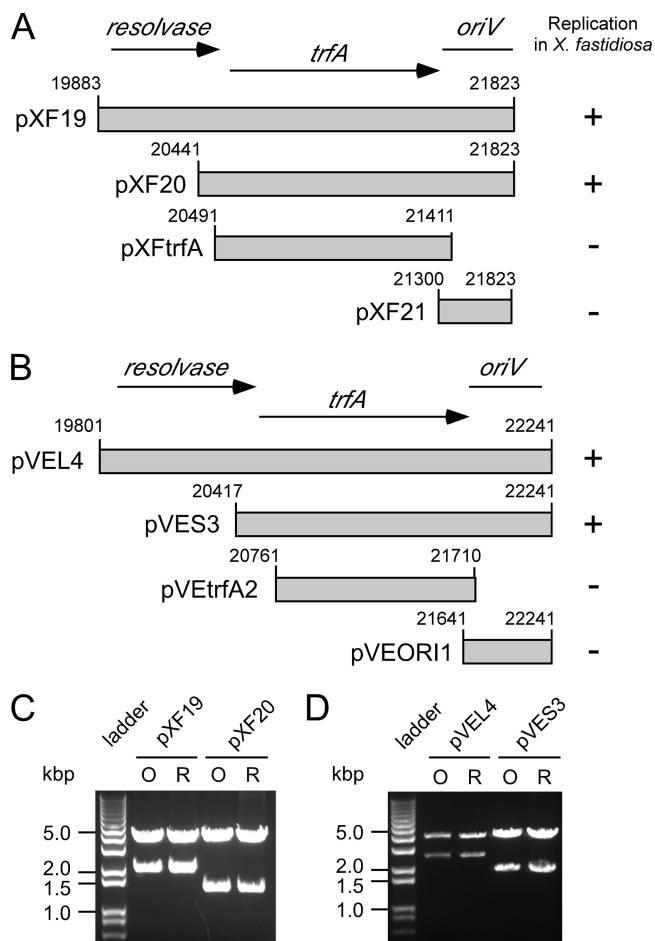
FIG. 1. IncP-1 replicons from *X. fastidiosa* and *V. eiseniae* replicate in *X. fastidiosa*. DNA fragments derived from pXF-RIV11 (A) or pVEIS01 (B) were PCR amplified and inserted into *E. coli* cloning vector pCR2.1 bearing the *nptII* gene for kanamycin resistance (vector sequences not shown). Only constructs bearing both *trfA* and *oriV* were competent for replication in *X. fastidiosa*. Panels C and D show EcoRI digests of plasmids used to transform *X. fastidiosa* (lanes O) or rescued from *X. fastidiosa* by transformation of *E. coli* (lanes R).

was grown in NYG medium (5 g of peptone, 3 g of yeast extract, and 20 g of glycerol per liter) at 28°C. When required, antibiotics were added as follows: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml (for *E. coli*), 30 μg/ml (for *A. tumefaciens*, *P. syringae*, and *X. campestris*), or 5 μg/ml (for *X. fastidiosa*); chloramphenicol, 30 μg/ml; spectinomycin, 50 μg/ml.

Construction of plasmids. For the constructs diagrammed in Fig. 1A and B, the indicated sections of pXF-RIV11 or pVEIS01 were obtained by PCR amplification and cloned into pCR2.1 using a TA cloning kit (Invitrogen). Identities of cloned PCR products were confirmed by sequencing. PCR amplification also was used to obtain and add NotI sites to the fragment containing *pemI/pemK* (pXF-RIV11 nucleotides [nt] 1221 to 1906), with the product cloned into the NotI site in the pCR2.1 portion of plasmids pXF19, pXF20, pVEL4, and pVES3. For *pemK* and *pemI* expression in *E. coli*, various regions of *pemI/pemK* were PCR amplified and cloned into the pET28a vector (Novagen); *pemI* was amplified and cloned into pCDF1b (Novagen). For coexpression experiments, inserts were cloned into pETDuet-1 (Novagen). Proteins expressed from pET28a and pCDF1b included N-terminal His6 tags; proteins coexpressed from pETDuet-1 included N-terminal His6 tags (PemK or green fluorescent protein [GFP]) or a C-terminal S tag (PemI).

Plasmid stability analysis. Transformation of *X. fastidiosa* was performed as described previously (18), using a GenePulser Xcell electroporator (Bio-Rad). Electroporation of *A. tumefaciens*, *P. syringae*, and *X. campestris* was performed according to Bio-Rad protocols for *P. aeruginosa*. *X. fastidiosa* transformants

were picked from PD3-kanamycin plates and grown in PD3 liquid with kanamycin for 7 to 10 days until turbid. Strains were subcultured (1:10 dilution) into PD3 liquid without kanamycin. Every 7 days, culture was dilution plated onto PD3 agar (with and without kanamycin) and subcultured (1:10 dilution) into fresh PD3 liquid. A total of five passages in nonselective PD3 liquid medium were performed. Colonies of *A. tumefaciens*, *P. syringae*, and *X. campestris* transformants were picked directly from kanamycin-supplemented plates into liquid medium without kanamycin and dilution plated and subcultured (1:100 dilution) every other day.

Protein expression, purification, and Western blot analysis. *E. coli* strain BL21(DE3)pLysS (Stratagene) was used for all protein expression experiments. For extraction of recombinant proteins, expression was induced at an optical density at 600 nm (OD600) of 0.6 by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG); cultures were harvested 3 h after induction. For experiments where culture growth was monitored over a 5-h postinduction period, protein expression was induced by adding 1 mM IPTG at an OD600 of 0.3. His6-tagged PemK and His6-tagged GFP were purified from soluble cell extracts using Ni-nitrilotriacetic acid (NTA) affinity chromatography as described by the manufacturer (Qiagen). Protein electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) and Western blot assays were performed using standard methods (30). Primary antibodies, anti-His (Calbiochem) and anti-S (Novagen), were used at a 1:2,000 dilution; horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) was used at a 1:15,000 dilution and detected using a Fast ECL kit (Thermo Scientific Pierce).

RESULTS

Minimal IncP-1 replicons require *trfA* and *oriV* for replication in *X. fastidiosa*. Previously (35), a 5.3-kbp fragment of pXF-RIV11 was shown to be sufficient for plasmid replication in *X. fastidiosa*. As the 5.3-kbp fragment contained homologues (*trfA* and *oriV*) known to be required for replication of other IncP-1 plasmids (4), subclones of pXF-RIV11 (Fig. 1A) or the corresponding region of pVEIS01 (Fig. 1B) were generated and evaluated for replication competence in *X. fastidiosa*. Plasmid replication in *X. fastidiosa* required both *trfA* and *oriV*. Constructs bearing only *trfA* or *oriV* from pXF-RIV11 or pVEIS01 were not competent for replication in *X. fastidiosa*. The open reading frame (ORF) encoding a resolvase homologue and located upstream of *trfA* for both pXF-RIV11 and pVEIS01 was not required for plasmid replication in *X. fastidiosa*. Thus, minimal IncP-1 replicons mapped to pXF-RIV11 nt 20441 to 21823 (1,383 bp) or pVEIS01 nt 20417 to 22241 (1,825 bp). Restriction enzyme digests of plasmids used to transform *X. fastidiosa* and subsequently rescued from *X. fastidiosa* by transformation of *E. coli* yielded identical patterns, indicating that five serial passages (under antibiotic selection) of transformed *X. fastidiosa* bearing IncP-1 replicons derived from pXF-RIV11 (Fig. 1C) or pVEIS01 (Fig. 1D) did not result in gross alteration (large insertions or deletions) of plasmid sequences.

The *pemI/pemK* plasmid addiction system confers stable inheritance of IncP-1 replicons in *X. fastidiosa*. The putative *pemI/pemK* plasmid addiction system from pXF-RIV11 was added to IncP-1 replicons derived from pXF-RIV11 (Fig. 2A) or pVEIS01 (Fig. 2B). Restriction enzyme digests of plasmid samples before transformation of, and after rescue from, *X. fastidiosa* yielded identical patterns. Thus, addition of *pemI/pemK* did not cause gross alteration of IncP-1 replicons derived from pXF-RIV11 (Fig. 2C) or pVEIS01 (Fig. 2D) during passage in *X. fastidiosa*.

To evaluate plasmid inheritance over an extended period in the absence of antibiotic selection, *X. fastidiosa* transformed
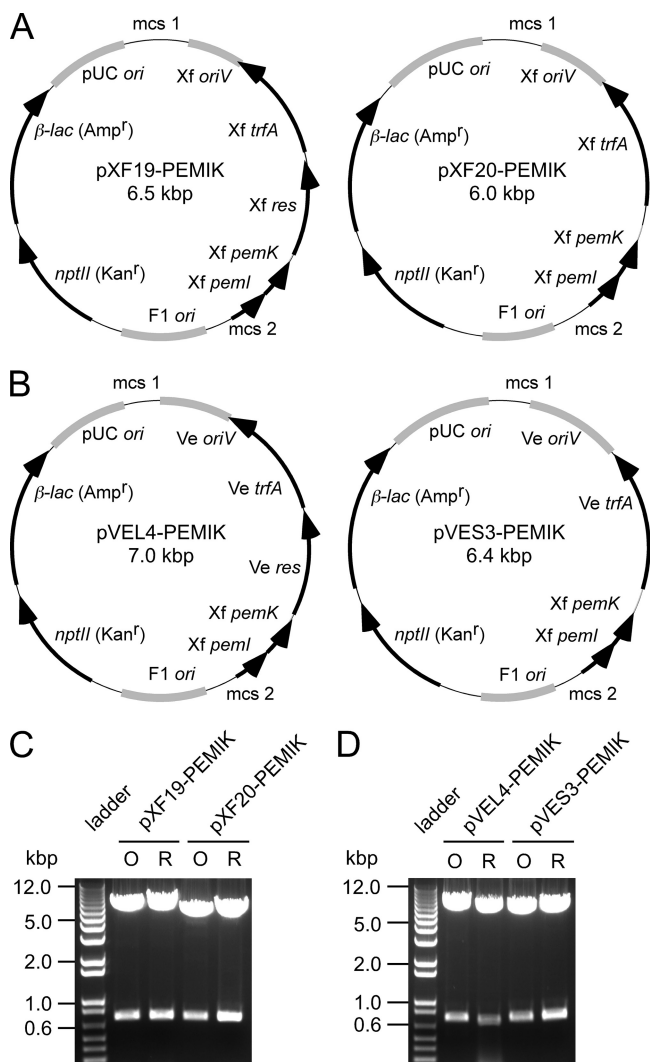
FIG. 2. Inc-P1 replicon constructs bearing the *pemI/pemK* plasmid addiction system. Maps of plasmids derived from pXF-RIV11 (A) or pVEIS01 (B) are depicted. ORFs are denoted by black arrows, and various origins of replication are indicated as gray arcs. The larger constructs (pXF19-PEMIK and pVEL4-PEMIK) differ from the corresponding smaller constructs (pXF20-PEMIK and pVES3-PEMIK) by containing the complete resolvase ORF (*res*) of the respective IncP-1 plasmid donor located upstream of the cognate *trfA* ORF. Locations of multiple cloning sites (mcs1 and mcs2) are indicated. Panels C and D show NotI digests (excising *pemI/pemK*) of plasmids used to transform *X. fastidiosa* (lanes O) or rescued from *X. fastidiosa* by transformation of *E. coli* (lanes R).

FIG. 3. The *pemI/pemK* addiction system (*pemI/K*) confers stable inheritance in the absence of antibiotic selection. *X. fastidiosa* transformed with minimal replicons derived from pXFRIV11 (A) or pVEIS01 (B) with or without *pemI/K* was grown in the absence of antibiotic selection (five passages over a 5-week period). Periodically, samples were plated onto medium with or without kanamycin. Stability of plasmid constructs was estimated by the ratio of the number of CFU on kanamycin-containing medium to the number of CFU on medium lacking kanamycin (% resistant CFU). Error bars denote standard deviations.

with IncP-1 replicons (containing or lacking *pemI/pemK*) were grown in liquid medium without kanamycin for five serial passages (Fig. 3). Stability of plasmid inheritance by daughter cells was assessed by plotting ratios of numbers of CFU recovered by plating aliquots of liquid cultures (lacking kanamycin) onto solid medium containing or lacking kanamycin. After three serial passages in liquid culture without antibiotic selection, the number of kanamycin-resistant *X. fastidiosa* (previously transformed with IncP-1 replicons lacking *pemI/pemK*) CFU recovered declined to zero. In contrast, the ratio of the number of CFU recovered from *X. fastidiosa* (transformed with IncP-1
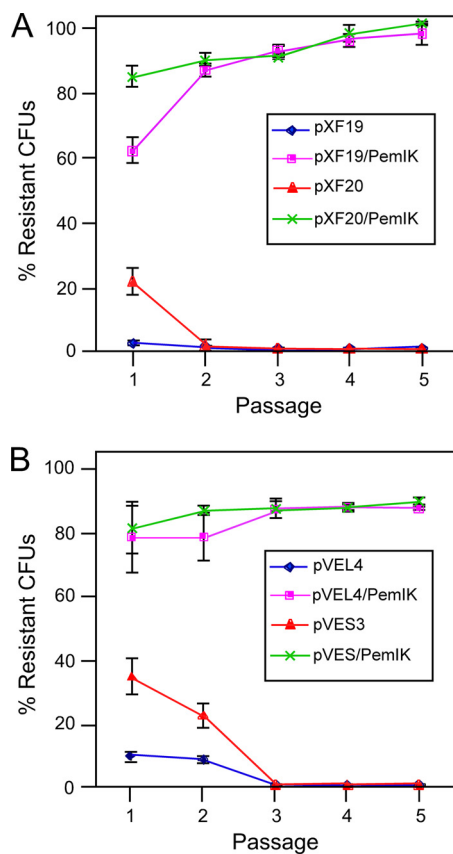
replicons containing *pemI/pemK*) on plates with or without kanamycin remained stable during passages 3 to 5 at >80% to >90%. These results were considered phenotypic evidence that *pemI/pemK* constitutes a plasmid addiction system that is functional in *X. fastidiosa*.

IncP-1 replicons derived from pXF-RIV11 have a broader experimental host range than those derived from pVEIS01. Plant pathogenic bacteria unrelated to *X. fastidiosa* were evaluated as hosts of IncP-1 replicons derived from pXF-RIV11 or pVEIS01 (Table 1). Electroporation of *P. syringae* pv. tomato or *A. tumefaciens* with the minimal IncP-1 replicon (pXF20) derived from pXF-RIV11 resulted in kanamycin-resistant colonies from which the plasmid could be rescued by subsequent transformation of *E. coli*. In contrast, transformation of (and plasmid rescue from) *X. campestris* pv. campestris required a larger construct (pXF19) derived from pXF-RIV11 that included the upstream resolvase ORF in addition to *trfA* and *oriV*. As expected, the plasmid bearing only *oriV* from pXF-RIV11 (pXF21) was unable to replicate in all three species. Transformation of *X. campestris* pv. campestris, *P. syringae* pv.

TABLE 1. Experimental host ranges of IncP-1 plasmids derived from pXF-RIV11 or pVEIS01

Plasmid	*Xanthomonas campestris* pv. campestris Xcc8004		*Pseudomonas syringae* pv. tomato PstDC3000		*Agrobacterium tumefaciens* GV3101	
	Replicates?[a]	Stable?[b]	Replicates?	Stable?	Replicates?	Stable?
pXF19	Yes	No	Yes	No	Yes	No
pXF20	No		Yes	No	Yes	No
pXF21	No		No		No	
pXF19/PemIK	Yes	<5%	Yes	<5%	Yes	<5%
pXF20/PemIK	No		Yes	<5%	Yes	<5%
pVEL4	No		No		No	
pVES3	No		No		No	

[a] Yes, numerous colonies after transformation and successful rescue of plasmid by transformation of *E. coli*. No, few to no colonies after transformation and no rescue of plasmid by transformation of *E. coli*.

[b] No, no kanamycin-resistant colonies recovered after one passage on solid medium lacking kanamycin; <5%, kanamycin-resistant colonies recovered after one passage on solid medium lacking kanamycin, but liquid culture stability tests indicated that ≤5% of the colonies retained resistance to kanamycin after two passages in liquid medium lacking kanamycin.

tomato, and *A. tumefaciens* with IncP-1 replicons derived from pVEIS01 did not yield kanamycin-resistant colonies from which plasmids could be rescued by subsequent transformation of *E. coli*. IncP-1 replicons derived from pXF-RIV11 were unstable in *X. campestris* pv. campestris, *P. syringae* pv. tomato, and *A. tumefaciens* in the absence of antibiotic selection (Table 1). Despite addition of the *pemI/pemK* plasmid addiction system, only a minority of the cells (<5%) retained kanamycin resistance after two passages of growth in liquid medium lacking kanamycin.

Expression of PemI counteracts toxicity of PemK. Expression of His$_6$-tagged PemK in *E. coli* transformed with pET28a: PemK(a) resulted in growth inhibition following IPTG induction (Fig. 4A), a phenotype consistent with that of a toxin expressed in the absence of a cognate antitoxin. In contrast, growth of *E. coli* cotransformed with pET28:PemK(a) and pCDF1b:PemI expressing His$_6$-tagged PemI was not inhibited following IPTG induction. Restoration of growth following IPTG induction was not observed in *E. coli* cotransformed with pET28a:PemK(a) and the expression vector pCDF1b lacking *pemI*. These results indicate that the *pemI/pemK* plasmid addiction system derived from pXF-RIV11 functions as a TA module, with PemI serving as the cognate antitoxin of PemK toxin. To verify the expression of each protein upon IPTG induction, His$_6$-tagged proteins from *E. coli* (transformed individually with each expression vector) were purified from total soluble proteins by Ni-NTA affinity chromatography and detected in Western blot assays using anti-His antibodies (Fig. 4B).

Twelve amino-terminal amino acid residues of PemK are dispensable for toxicity. The *pemK* ORF partially overlaps the upstream *pemI* ORF. There are three in-frame methionine codons that potentially may serve as start codons. Therefore, 5′-terminal nested deletions were constructed in pET28a (Fig. 5A) such that translation of PemK would include methionine at amino acid position 1 (pET28a:PemK$^{1-120}$), 13 (pET28a: PemK$^{13-120}$), or 20 (pET28a:PemK$^{20-120}$) downstream of the His$_6$ tag. Following IPTG induction of *E. coli* transformed with the PemK constructs, all three His$_6$-tagged proteins were purified by Ni-NTA affinity chromatography and detected by Western blotting with anti-His antibodies (Fig. 5B). Cultures transformed with each PemK construct also were cotransformed with pCDF1b:PemI or pCDF1b empty vector (Fig.

5C). Growth of cotransformed cultures following IPTG induction indicated that deletion of PemK amino acids 1 to 12 did not alter the growth inhibition phenotype, whereas deletion of PemK amino acids 1 to 19 resulted in no inhibition of growth.
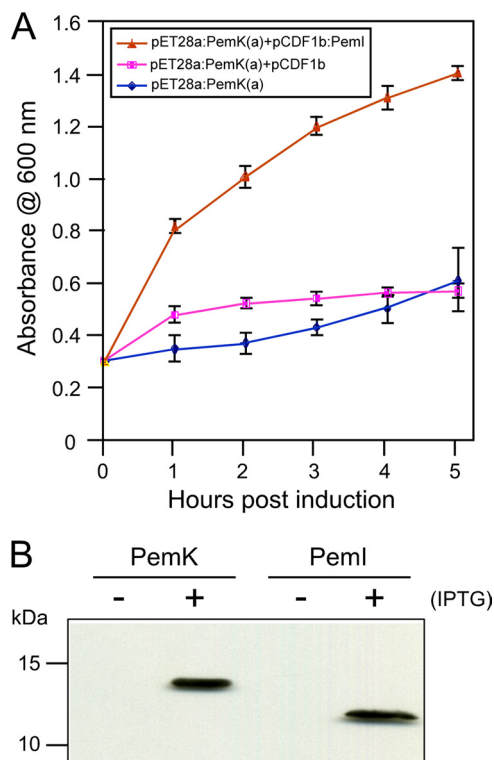

FIG. 4. Effect of PemK and PemI expression on growth of *E. coli*. Growth of *E. coli* transformed with expression plasmids was measured by mean culture absorbance at 600 nm (error bars denote standard deviations) following IPTG induction at 0 h (A). Growth inhibition resulting from expression of His$_6$-tagged PemK toxin alone [pET28a: PemK(a)] was reversed by coexpression with His$_6$-tagged PemI antitoxin (pCDF1b:PemI) but not by coexpression with the empty vector pCDF1b. Western blot assay detection (with anti-His antibodies) of His$_6$-tagged PemK toxin and His$_6$-tagged PemI antitoxin (expressed individually in separate cultures) extracted from *E. coli* with (+) or without (−) IPTG induction and purified by Ni-NTA affinity chromatography (B). Sizes of protein standards are indicated on the left.
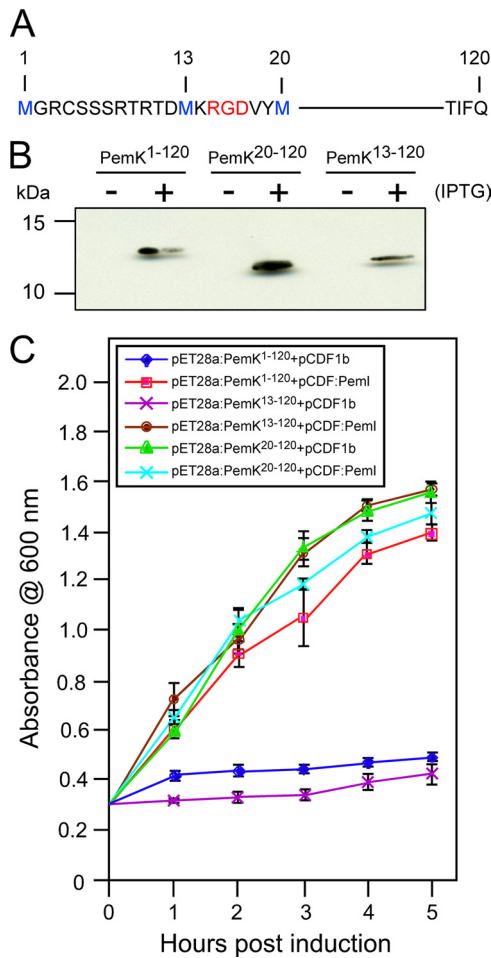
FIG. 5. Expression and growth inhibition properties of amino-terminal deletion mutant forms of PemK toxin. The sequence of the PemK amino-terminal region is presented as one-letter code (A); methionine residues in blue indicate the beginning of PemK sequences expressed as fusions with amino-terminal His_6 tags (not shown); numbers denote amino acid coordinates with the first upstream and in-frame methionine designated 1; red indicates a motif (RGD/E) conserved among PemK homologues. Western blot assay detection (B) with anti-His antibodies of His_6-tagged full-length PemK ($PemK^{1-120}$) or His_6-tagged, truncated PemK ($PemK^{13-120}$, $PemK^{20-120}$) extracted from *E. coli* with (+) or without (−) IPTG induction and purified by Ni-NTA affinity chromatography. Sizes of protein standards are indicated on the left. Growth of *E. coli* transformed with expression plasmids (C) was measured by mean culture absorbance at 600 nm (error bars denote standard deviations) following IPTG induction at 0 h. Growth inhibition resulting from expression of His_6-tagged PemK constructs retaining toxin activity ($PemK^{1-120}$, $PemK^{13-120}$) was reversed by coexpression with His_6-tagged PemI antitoxin (pCDF1b:PemI) but not by coexpression with the empty vector pCDF1b.

FIG. 6. Copurification of $PemK^{13-120}$ and PemI *in vitro*. The dual-expression vector pETDuet-1 was used to coexpress His_6-tagged $PemK^{13-120}$ toxin or His_6-tagged GFP with S-tagged PemI antitoxin (A). Coomassie-stained SDS-PAGE of cell extracts (B) from uninduced cells (−), IPTG-induced cells (+), and Ni-NTA column eluate (E) indicated that S-tagged PemI is retained on the Ni-NTA column only in the presence of His_6-tagged $PemK^{13-120}$. S-tagged PemI is not retained on the column when coexpressed with His_6-tagged GFP. Sizes of protein standards are indicated on the left. Western blot assay detection (with both anti-His and anti-S antibodies) confirms that S-tagged PemI is present in both induced cell extracts (+) but is detected in the Ni-NTA column eluate (E) only when coexpressed with His_6-tagged PemK (C). Western blotting (D) with anti-His and anti-S antibodies singly and then both together confirms the specificity of the antibodies and the identities of His_6-tagged $PemK^{13-120}$ and S-tagged PemI.

As expected, coexpression of PemI abolished growth inhibition. The largest deletion lacked RGD at amino acid positions 15 to 17 (Fig. 5A) that constitute a conserved motif (RGD/E) present in a similar position among numerous PemK homologues identified in GenBank through protein BLAST searches (data not shown).

Copurification of PemK and PemI indicates that the toxin and antitoxin bind to one another. Toxin and antitoxin proteins from other TA systems have been shown to directly in-
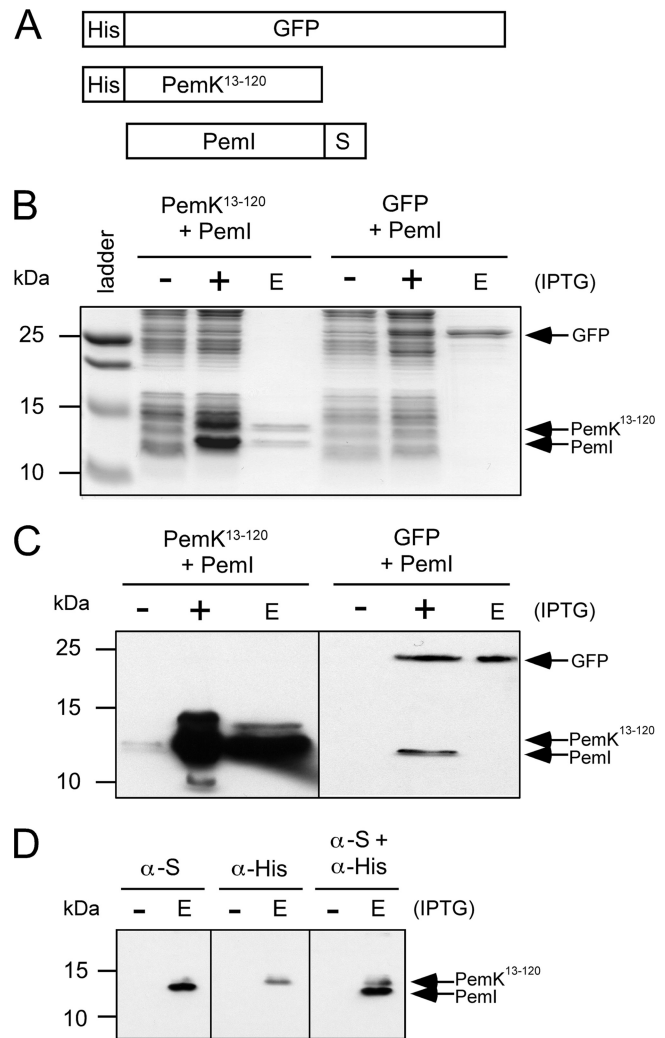
teract. Therefore, interaction of pXF-RIV11 PemK and PemI was tested. The genes were cloned into a pETDuet-1 vector, resulting in N-terminally His_6-tagged $PemK^{13-120}$ and C-terminally S-tagged PemI (Fig. 6A). As a negative control, His_6-tagged GFP was coexpressed with S-tagged PemI, also in pETDuet-1. *E. coli* cell extracts were passed over a Ni-NTA affinity column to bind the His_6-tagged proteins. As demonstrated by SDS-PAGE (Fig. 6B) and confirmed by Western blot assays (Fig. 6C and D), S-tagged PemI was retained on the

Ni-NTA column only in the presence of His$_6$-tagged PemK[13-120]; coexpression with His$_6$-tagged GFP did not result in retention of S-tagged PemI on the Ni-NTA affinity column. Therefore, antitoxin PemI specifically binds to toxin PemK, a result consistent with toxin sequestration as demonstrated in other TA systems (7, 11, 20).

DISCUSSION

Functional analysis of sequences derived from pXF-RIV11 and pVEIS01 indicated that *trfA* and *oriV* are the only plasmid-borne elements necessary for replication in *X. fastidiosa*, as expected for IncP-1 plasmids. Whereas the natural host (*V. eiseniae*) of pVEIS01 is a betaproteobacterium, *X. fastidiosa* is a gammaproteobacterium. As the TrfA proteins encoded by the two plasmids share 87% amino acid sequence identity and since the iterated elements of *oriV* are similar (35), it is perhaps not unexpected that replicons derived from pVEIS01 were able to replicate in *X. fastidiosa*. Whether minimal replicons derived from pXF-RIV11 are competent for replication in *V. eiseniae* was not determined, as a transformation protocol for *V. eiseniae* has not been developed.

Additional host range experiments indicated that the minimal replicon (pXF20) derived from pXF-RIV11, but not the minimal replicon (pVES3) derived from pVEIS01, was able to replicate in two gammaproteobacteria, *P. syringae* pv. tomato and *A. tumefaciens*. However, a larger construct (pXF19) was needed for replication in *X. campestris* pv. campestris. These two constructs differ in that only pXF19 contained the complete resolvase ORF located upstream of *trfA*. Stable maintenance of certain low-copy-number plasmids such as pRP4 (12) requires a partitioning (*par*) system that includes a functional resolvase. Therefore, the simplest interpretation of differential host range data for pXF19 and pXF20 is that *X. fastidiosa*, *P. syringae* pv. tomato, and *A. tumefaciens* (but not *X. campestris* pv. campestris) encode resolvases able to resolve plasmid dimers such that monomeric progeny molecules may be distributed to daughter cells. As inclusion of the resolvase ORF derived from pVEIS01 did not result in replication of a pVEIS01 derivative (pVEL4) in *P. syringae* pv. tomato, *A. tumefaciens*, or *X. campestris* pv. campestris, host range differences of derivatives from pXF-RIV11 versus pVEIS01 were not simply due to the presence or absence of the cognate resolvase gene.

Phenotypic properties of *pemI*/*pemK* homologues of pXF-RIV11 were consistent with that of a TA module: sole expression of PemK toxin inhibited cell growth, addition of PemI antitoxin restored growth, and PemK and PemI directly interacted with one another. Furthermore, addition of the pXF-RIV11 *pemI*/*pemK* module conferred stability of plasmid inheritance in *X. fastidiosa* grown for prolonged periods in the absence of antibiotic selection, precisely as expected for a TA module addiction system. That addition of *pemI*/*pemK* did not substantially increase the stability of plasmids in *A. tumefaciens*, *P. syringae*, and *X. campestris* indicates host background affects the efficiency of stability conferred by the pXF-RIV11 plasmid addiction system. Although the specific mode of action of PemK toxin encoded by pXF-RIV11 remains to be determined, it is likely that pXF-RIV11 PemK toxin acts in a fashion

similar to that of other PemK toxins: an RNase targeting cellular RNAs (2, 43).

The pXF-RIV11 *pemK* ORF (nt 1513 to 1872) partially overlaps that of the 5′-proximal *pemI* ORF (nt 1289 to 1549). Within the 5′-proximal region of the *pemK* ORF, the first in-frame methionine codon (pXF-RIV11 coordinates 1513 to 1515) completely overlaps the *pemI* ORF, the second in-frame *pemK* methionine codon (1549 to 1551) partially overlaps the *pemI* stop codon (1550 to 1552), and the third in-frame *pemK* methionine codon (nt 1570 to 1572) is positioned downstream of the overlapping region. As amino-terminal truncation of PemK (deletion of amino acids 1 to 19) resulted in loss of toxin activity, translation of PemK likely initiates at the first or second in-frame methionine codons. That deletion of the first 12 codons of the *pemK* ORF had no effect on toxin activity suggests that either PemK translation initiates at the second in-frame methionine codon or the first 12 in-frame amino acids are dispensable for PemK toxic properties and binding of PemI antitoxin. The conserved motif RGD (pXF-RIV11 PemK amino acids 15 to 17) was located downstream of sequences dispensable for toxin activity. Interestingly, conserved RGD/E motifs have been found in a variety of cell adhesion proteins (10) but a specific function for this motif in PemK homologues has yet to be identified.

The research reported here was initiated, in part, with a practical objective: development of a plasmid shuttle vector for *X. fastidiosa* that is stably inherited over extended time periods in the absence of antibiotic selection. Such stability is required to conduct mutant complementation assays in plants, as expression of disease symptoms often requires many months after inoculation (23). Although an exogenous plasmid (pBBRIMCS5 bearing a replication origin from *Bordetella*) has been used as a shuttle vector to complement a nonpathogenic *tolC* mutant of *X. fastidiosa*, only 50% of cells retained the plasmid after 60 days *in planta* (27). Furthermore, it is unclear whether pBBRIMCS5 would be stable in plants in cases where the mutant retained pathogenicity.

We selected an endogenous plasmid of *X. fastidiosa* as starting material since pXF-RIV11 could (i) replicate in *X. fastidiosa* and (ii) was stable despite lacking a selectable marker. The *V. eiseniae* plasmid (pVEIS01) was selected as alternative starting material given the extensive sequence identity it shares with pXF-RIV11. We anticipated that stable inheritance would require more than the minimal gene complement needed for replication. As bioinformatic analysis of pXF-RIV11 identified the *pemI*/*pemK* TA homologue (35), we used this endogenous plasmid addiction system to confer stability of shuttle vectors. Because transformation of *X. fastidiosa* with derivatives of pXF-RIV11 was consistently more efficient than that with derivatives of pVEIS01 (data not shown), pXF20-PEMIK (Fig. 2) represents the most suitable shuttle vector construct described herein. Ten unique endonuclease restriction sites are clustered in two multiple cloning regions such that foreign DNA may be readily inserted into pXF20-PEMIK.

Mention of proprietary or brand names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval to the exclusion of others that also may be suitable.

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