Detection of a Single-Copy Plasmid, pXFSL21, in *Xylella fastidiosa* Strain Stag's Leap with Two Toxin-Antitoxin Systems Using Next-Generation Sequencing

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ABSTRACT

Plasmids are important genetic elements contributing to bacterial evolution and environmental adaptation. *Xylella fastidiosa* is a nutritionally fastidious Gram-negative bacterium causing economically devastating diseases such as Pierce's disease (PD) of grapevine. In this study, the plasmid status of a highly virulent PD strain, Stag's Leap, originally isolated from Napa Valley, CA, was studied using sequencing and bioinformatics tools. DNA samples extracted from a pure culture in periwinkle wilt medium (in vitro DNA) and a PD-symptomatic grapevine artificially inoculated in the greenhouse (in planta DNA) were subject to next-generation sequencing (NGS) analyses (Illumina MiSeq or HiSeq). Sequence analyses and polymerase chain reaction experiments revealed the presence of a circular plasmid, pXFSL21, of 21,665 bp. This plasmid

existed as a single copy per bacterial genome under both in vitro and in planta conditions. Two toxin-antitoxin (T-A) systems (*ycdD-ycdE* and *higB-higA*) were detected in pXFSL21, a possible mechanism for the long-term survival of this single-copy plasmid in the bacterial population. BLAST searches against the GenBank database (version 222) detected homologs of the two T-A systems in chromosomes or plasmids of some *X. fastidiosa* strains. However, double T-A systems were found only in pXFSL21. pXFSL21 was not found in other known PD strains and, therefore, could serve as a molecular marker for strain Stag's Leap monitoring and tracking. The NGS-based technique outlined in this article provides an effective tool for identifying single- or low-copy-number plasmids in fastidious prokaryotes.

Plasmids are self-replicating, extrachromosomal genetic elements responsible for horizontal gene transfer that contribute to bacterial evolution and environmental adaptation, including virulence (Coplin 1989). High-copy-number plasmids are well known for biotechnological manipulations such as DNA fragment cloning. However, many bacterial plasmids such as those associated with environmental adaptation could be at a low copy number or even single copy. Low-copy plasmids are advantageous for survival by lowering host metabolic burdens (Sternberg and Austin 1981). Virulence plasmids are often large (>20 kbp), because they carry genes for functions of replication, dissemination, and maintenance (RDM) in addition to those conferring advantageous phenotypes (CAPs) (Sengupta and Austin 2011).

Xylella fastidiosa is a Gram-negative, xylem-limited, bacterial pathogen causing diseases in many economically important plants; for example, Pierce's disease (PD) of grapevine in California and the southeastern United States (Hopkins 1989), citrus variegated chlorosis (CVC) in Brazil (Chang et al. 1993), and olive quick decline syndrome (OQDS) in Italy (Giampetruzzi et al. 2015; Saponari et al. 2013). Due to its nutritional fastidiousness, research on *X. fastidiosa* has been highly challenging. A 1.3-kbp plasmid was observed in an *X. fastidiosa* periwinkle wilt (PW) strain (Chen et al. 1992) and sequenced by Sanger's method (Pooler and Hartung 1997). Concurrent with a significant increase in *X. fastidiosa* research since the 2000s due to outbreaks of PD, CVC, and OQDS, in total, 46 plasmids ranging from 1.3 to 64 kbp in size have been

identified, annotated, and deposited in the GenBank database (version 222) (Supplementary Table S1). Most of the plasmid discoveries were derived from whole-genome sequencing efforts, particularly the use of next-generation sequencing (NGS) technology. Thirty-three of the *X. fastidiosa* plasmids are >20 kbp in size. In all cases, copy numbers of the sequenced plasmids were not estimated, with the exception of the PW plasmid, estimated to be at least 60 copies per genome (Chen et al. 1992).

Although in low copy number, large plasmids have intricate mechanisms for their maintenance in bacterial populations. One mechanism is the toxin-antitoxin (T-A) system (Gerdes et al. 1986; Hayes 2003). In the T-A system, a plasmid encodes a stable toxin and a labile antitoxin. Daughter cells without the plasmid will be killed by the toxin because the unstable antitoxin is degraded. Such a phenomenon is also called postsegregational killing (Gerdes et al. 1986). The presence of T-A systems in *X. fastidiosa* were detected through genome sequence analyses (Pandey and Gerdes 2005), and functions of the plasmid-borne T-A system (*pemI/pemK*) have been confirmed in vitro (Lee et al. 2014; Stenger et al. 2010). T-A systems have also been found in bacterial chromosomes and received extensive research in light of mechanisms of virulence and programming cell death (Burbank and Stenger 2016; Pandey and Gerdes 2005).

The Stag's Leap strain of *X. fastidiosa* subsp. *fastidiosa* was originally isolated from Napa Valley in California (Buzkan et al. 2005) and has been commonly used as a model for PD research because of its high virulence (Krivanek and Walker 2005; Krugner et al. 2014; Wallis et al. 2013). We are interested in further studying the biology of this model strain using NGS technology and bioinformatics tools. Toward this end, a draft whole-genome sequence of the Stag's Leap strain was obtained (Chen et al. 2016). Based on available genomic sequence data, this study evaluated the plasmid status of this strain through both in vitro and in planta

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experiments and analyses. A single-copy plasmid, pXFSL21, with two T-A systems was identified. Implications of using an NGS approach to study plasmids in *X. fastidiosa* and other fastidious prokaryotes are also discussed.

MATERIALS AND METHODS

Bacterial strains and DNA samples. *X. fastidiosa* strain Stag's Leap was maintained on PW medium (Davis et al. 1981) at 28°C, with regular transfer every 20 to 30 days since 2010. Bacterial DNA was obtained from both in vitro and in planta sources. For the in vitro sample, cells were collected from culture (14 days) and DNA was extracted according to a standard procedure (Maniatis et al. 1982). For in planta samples, a grapevine (*Vitis vinifera*), cultivar Thompson Seedless, grown in the greenhouse for 4 months, was inoculated twice on the stem using the pinprick method with 10-µl drops of *X. fastidiosa* cell suspension using a 20-gauge syringe needle (Hopkins 2001). Twelve weeks after inoculation, PD symptoms on leaves (marginal necrosis) were observed and the most severe symptoms were near the site of inoculation. Leaf petiole samples were collected and macerated with an automated tissue homogenizer (Geno/Grinder; SPEX SamplePrep, Metuchen, NJ). DNA was extracted using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), quantified and used for polymerase chain reaction (PCR) or sequencing experiments. The presence of *X. fastidiosa* in the sample was confirmed by standard PCR with primer sets Teme150fc-Teme454rg and Dixon454fc-Dixon1261rg (Chen et al. 2005). Additionally, primer set Teme150fc-Teme454rg was used for quantitative real-time PCR (qPCR) with SYBR green.

DNA sequencing and whole-genome assembly. NGS experiments were performed through a commercial service. A brief description on sequencing the Stag's Leap genome from in vitro culture DNA with the Illumina MiSeq 2 × 300 platform has been published (Chen et al. 2016). The in planta sample (mixture of both *X. fastidiosa* and grapevine DNA) was sequenced by Illumina HiSeq 2 × 100 (Illumina, Inc., San Diego, CA). With sequence reads from either MiSeq (in vitro) or HiSeq (in planta), both referenced and de novo assemblies were performed using CLC Genomics Workbench software (v. 10.1.1). For reference assembling of HiSeq reads (in planta), default parameters were used. The published Stag's Leap genome sequence (NZ_LSMJ000000000.1) (Chen et al. 2016) was used as a reference. For de novo assembling, parameters were as follows; word size = 64, bubble size = 200, minimum contig length = 250, mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.6, and similarity fraction = 0.9.

Plasmid detection. Two approaches were used for plasmid screening in the draft Stag's Leap genome sequence (NZ_LSMJ000000000.1). (i) One approach identified contigs with high similarity to the known *X. fastidiosa* plasmids. The 46 *X. fastidiosa* plasmid sequences in GenBank were downloaded. The 15 contigs in the draft Stag's Leap genome were used as queries to BLAST search against the 46 *X. fastidiosa* plasmids (word size = 7, e-value = 1e⁻¹⁰). Contigs with high bit score were selected as candidate plasmids. (ii) Another approach analyzed contigs for typical plasmid structures. Annotation of the 15 contigs in NZ_LSMJ000000000.1 through the RAST server (<http://rast.nmpdr.org/>) (Aziz et al. 2008) were examined for the simultaneous presence of genes associated with both RDM and CAP (Coplin 1989).

Plasmid sequence enclosure, annotation, and protein three-dimensional structure analyses. Contig 15 of NZ_LSMJ000000000.1 was identified as a plasmid candidate. Two approaches were used to prove circularity. (i) One approach involved in silico read mapping, as previously described (Zheng et al. 2018). Briefly, a 5,000-bp section (A) at the 5' end of contig 15 was cut and attached to the 3' end of the remaining region (B) to make sequence B-A. As a control, a 5,000-bp sequence randomly selected from nucleotide position 564,797 to 569,797 bp of contig 1 (designated as C) was cut and attached to region B to replace A and make sequence B-C.

Both sequences B-A and B-C were used as the references for mapping with both MiSeq and HiSeq reads and check for smooth read transition (Supplementary Fig. S1). (ii) Another approach involved PCR and sequencing. Primers were designed from both the 5' and 3' ends of contig 15 with outward directionality (primer sets 21250F-137R and 21124F-617R) (Supplementary Table S3). Primer3Plus (Untergasser et al. 2007) was used to assist primer designs. Amplicons of standard PCR were further sequenced through the Sanger method on an Applied Biosystems 3130xl Genetic Analyzer. To search for chromosomal integration, contig 15 was used as a query to BLAST search against the other 14 contigs in NZ_LSMJ000000000.1 for highly similar regions. CLC Genomics software was used to view gene orientation and annotation and to generate the genetic map.

Protein structure analysis of selected genes was initially predicted in the Phyre server (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>) using a profile-profile alignment algorithm (Kelley et al. 2015). Final three-dimensional (3D) structure was optimized by using PyMOL Molecular Graphics System, version 2.0 (Schrödinger LLC).

Copy number estimation. Two methods were used to estimate plasmid copy number. (i) The first method was comparison of average nucleotide coverages (ANCs) between plasmid candidate and chromosomal contig sequences, as well as selected plasmid and chromosomal genes, through read mapping using MiSeq and HiSeq data. The ANC ratio of plasmid/chromosomal sequence were calculated. (ii) The second method was comparison of cycle threshold (Ct) values between plasmid-specific gene primer sets and chromosome-specific gene primer sets. Primer sets were designed to generate 200-bp amplicons. The single-copy glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as a reference to estimate the copy number of the target plasmid gene by the ratio of ($E_{\text{target}}^{\text{Ct}(\text{target})} / E_{\text{reference}}^{\text{Ct}(\text{reference})}$) (Pfaffl 2001) based on six replications.

PCR procedures and statistical analyses. Standard PCR was performed according to a previous report (Chen et al. 2005). Briefly, PCR (20 µl) was carried out using the Takara Taq (Hot Start Version) kit (Takara Bio Inc., Otsu, Japan). The reaction mixture contained 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; 100 µM each dNTP; 0.5 µM each primer; 1 unit of Taq DNA polymerase; and 1 µl of cell suspension. Amplification was conducted in a thermocycler (Model PTC-100; MJ Research, Waltham, MA) with an initial denature at 98°C for 5 min followed by 30 cycles consisting of denaturing at 96°C for 30 s, annealing at 55°C for 15 s, and extension at 72°C for 60 s. The amplification products were then stored at 4°C. The amplified DNAs were resolved through 1.5% agarose gel electrophoresis and visualized after staining.

For qPCR, the DNA sample was diluted to 2 ng/µl in pure water. Using Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA), qPCR reactions were set up containing 2 µl of DNA template, 2× Fast SYBR Green Master Mix (containing AmpliTaq Fast DNA polymerase, dNTPs, SYBR Green I dye, ROX dye passive reference, MgCl₂, and stabilizers), and 0.25 µM each of forward and reverse primers for a final reaction volume of 20 µl.

Homologs of T-A system genes. Each T-A system gene sequence was used as query to BLAST search against the GenBank nr and whole-genome shotgun sequence database (version 222) that included 46 plasmids and 41 whole-genome sequences of *X. fastidiosa* (Supplementary Table S2) with word size = 11 and expected threshold = 10. Homologs were evaluated by query coverage and sequence identity. Similarly, related non-*X. fastidiosa* bacteria were identified.

RESULTS

DNA sequencing and whole-genome assembly. Selected metrics of Illumina MiSeq and HiSeq sequencing and the derived assemblies are shown in Table 1. MiSeq generated 6 million 301-bp

reads with a total of nearly 2 giga bp. HiSeq generated >300 million 101-bp reads with a total of nearly 32 giga bp. However, the HiSeq data were a mixture of sequences of both grape and *X. fastidiosa*. The *Xylella* DNA was estimated to be approximately 2% [(2,316,903 × 508 ANC)/31,935,159,902] (Tables 1 and 2). Metrics of *X. fastidiosa*-only were shown under reference mapping, with 493 contigs (2,272,127 bp) from MiSeq and 235 contigs (2,316,903 bp) from HiSeq covering the draft Stag's Leap genome sequence (NZ_LSMJ000000000.1). Adding all de novo contigs with ≥1,000 bp, the draft genome size from in vitro DNA (2,170,878 bp in 77 contigs) and the draft genome size from in planta DNA (2,297,788 bp in 104 contigs) were comparably similar but not identical, likely due to variations in assembling processes.

Plasmid detection. Contig 15 of NZ_LSMJ000000000.1 (Table 2) was identified to be a plasmid because (i) according to annotation, the contig with 27 open reading frames (ORFs) contained both RDM genes (14 ORFs: ORF1, 5, 8, 9, and 18 to 27) and CAP genes (2 ORFs: ORF13 and 14) (Table 3); (ii) contig 15

had high BLAST bit scores (>8,000) to four *X. fastidiosa* plasmids (pXF-P4.OLS0478, pXF-P4.OLS0479, pXF-De Donno, and unnamed from CoDiRO); (iii) MiSeq read mapping showed continuity in the connection of 5' and 3' ends; and (iv) PCR primer sets 21250F-137R and 21124F-617R (Fig. 1B) amplified the expected DNA bands (Fig. 1C). The enclosure was further confirmed by Sanger sequencing of the amplicons. Therefore, contig 15 was designated as pXFSL21 (Fig. 1B). pXFSL21 derived from two de novo contigs of MiSeq data (in vitro DNA) or four de novo contigs of HiSeq data (in planta DNA) (Table 1). The sequence of pXFSL21 has been deposited in GenBank under the accession number MG879030.

Among the 27 ORFs, 22 had predicted gene functions, including 3 DNA primase, 2 resolvase/integrase, 8 in the *trb* conjugative transfer family, 4 that formed two pairs of T-A systems (*ycdD-ycdE*, and *higB-higA*), and 4 in a multidrug efflux pump system involving acriflavine resistance (Table 3). The 3D structures of the two T-A systems proteins are shown in Figure 2A to D.

Copy number estimation. ANC of both MiSeq and HiSeq data to the 15 contigs of the draft Stag's Leap genome sequences were listed in Table 2. Note that contig 15 was renamed as pXFSL21 (GC% = 50.1). Contig 12 had a size of 21,851 (GC% = 50.4), similar to that of pXFSL21. ORF analyses of contig 12 did not reveal typical RDM and CAP genes. Instead, four tRNA genes (tRNA-arg, tRNA-pro, tRNA-his, and tRNA-lys), typically chromosome borne, were found. Therefore, contig 12 was used as a chromosome reference to pXFSL21 for ANC ratio calculations. For in vitro experiments, ANC-pXFSL21/ANC-contig 12 was 1.09 (441/403) (Table 2). For in planta experiments, ANC-pXFSL21/ANC-contig was 0.98 (481/492) (Table 2). The near one-to-one ANC ratios suggested that pXFSL21 is single copy per genome of *X. fastidiosa* under both in vitro and in planta conditions.

Results of ANC analyses at a gene level are shown in Table 4. Using the double-copied chromosomal gene of 16S ribosomal RNA (rRNA) (*rrs*) as a reference, all selected pXFSL21 genes assembled from MiSeq (in vitro DNA) data had ANC ratios to *rrs* of approximately 0.5 (0.37 to 0.59), with the exception of *dnaG1*. BLAST search with pXFSL21 as query detected an identical copy of *dnaG1* in contig 2 (Fig. 1A). Therefore, this gene is double copied, one in the chromosome and the other in pXFSL21 (Fig. 1B).

In the HiSeq (in planta DNA) data mapping, ANC of *rrs* was extraordinarily high (55,770×) (Table 4). This was not surprising due to the conserved nature of 16S rRNA sequences between the

TABLE 1. Comparison of selected metrics of MiSeq (in vitro) and HiSeq (in planta) experiments and genome assembly of *Xylella fastidiosa* strain Stag's Leap

Parameter	In vitro – MiSeq	In planta – HiSeq
	<i>Xylella</i>	<i>Xylella</i> + <i>Vitis</i>
Total bases	1,982,951,278	31,935,159,902 ^a
Total reads	6,587,878	316,189,702
Read length	301	101
De novo assembly		
Number of contigs	952	418,979
Minimum contig length	263 bp	192 bp
Maximum contig length	300,673 bp	230,620 bp
Contig N50	93,115 bp	2,512 bp
Number of contigs ≥ 1,000 bp	127	97,751
Referenced mapping		
With NZ_LSMJ000000000.1 (15 contigs)		
Number of de novo contigs mapped	493 (2,272,127 bp)	235 (2,316,903 bp) ^b
Contigs ≥ 1,000 bp	77 (2,170,878 bp)	104 (2,297,788 bp)
With pXFSL21		
Number of de novo contigs mapped	2 (21,868 bp)	4 (21,718 bp)

^a Only the forward reads were used.

^b Average nucleotide coverage = 508×.

TABLE 2. Comparison of average nucleotide coverages (ANCs) on mapping the draft genome sequence of *Xylella fastidiosa* strain Stag's Leap (NZ_LSMJ000000000.1) between MiSeq (in vitro) and HiSeq (in planta) data^a

Contig	GenBank accession	Contig size (bp)	MiSeq (in vitro)		HiSeq (in planta)	
			Reads	ANC (×)	Reads	ANC (×)
1	LSMJ01000001	401,798	682,914	503	2,426,255	592
2	LSMJ01000002	731,756	731,744	445	3,356,023	463
3	LSMJ01000003	26,067	32,288	367	115,953	448
4	LSMJ01000004	12,441	18,689	445	53,789	436
5	LSMJ01000005	117,337	179,211	452	591,431	508
6^b	LSMJ01000006	19,107	31,637	490	178,448	940
7	LSMJ01000007	123,993	200,294	478	675,558	549
8	LSMJ01000008	10,125	15,774	462	51,046	505
9	LSMJ01000009	559,515	857,269	454	2,775,462	500
10	LSMJ01000010	68,784	107,072	461	342,883	503
11	LSMJ01000011	393,232	623,516	470	1,882,431	483
12^c	LSMJ01000012	21,851	29,759	403	10,6921	492
13	LSMJ01000013	1,820	1,804	294	8,746	478
14	LSMJ01000014	1,307	1,680	381	3,309	255
15 = pXFSL21	LSMJ01000015	21,665	32,236	441	10,3330	481
Total	...	2,510,798	3,914,185	...	12,671,585	...
Mean	...	...	...	436	...	508

^a Contigs in bold were the focus of this study.

^b Contig 6 has an almost doubled ANC value (940×) compared with those of other contigs in the in planta data.

^c Contig 12 is a reference to pXFSL21 for ANC comparison because of the similar size.

plant chloroplast and bacteria. The double-copied *dnaG1* gene sequence was then used as reference for the ANC ratio calculation. All selected pXFSL21 genes showed the ratio of approximately 0.5 (0.44 to 0.56) (Table 4). As a control, all selected single-copy

chromosomal genes also showed a ratio of approximately 0.5 (0.36 to 0.56).

PCR experiments comparing plasmid genes *tetR* and *acrB* to the single-copied chromosomal *GAPDH* gene showed copy numbers

TABLE 3. General information of pXFSL21 from *Xylella fastidiosa* strain Stag's Leap

ORF ^a	Gene name	Position	Base pairs	Amino acids	Annotation ^b
1	<i>dnaG1</i>	129–1,202	1,074	357	DNA primase
2	ORF	1,318–1,773	456	151	Hypothetical protein
3	ORF	2,042–2,596	555	184	Hypothetical protein
4	ORF	2,628–3,092	465	154	Hypothetical protein
5	<i>nik</i>	3,571–3,684	114	37	Nickase
6	ORF	3,814–4,281	468	155	Hypothetical protein
7	ORF	4,505–4,657	153	50	Hypothetical protein
8	<i>invA</i>	4,856–5,050	195	64	DNA invertase
9	<i>rsvB</i>	5,054–5,437	384	127	DNA resolvase
10	<i>ycdD</i>	5,593–5,775	183	60	Antitoxin
11	<i>ycdE</i>	5,884–6,198	315	104	Toxin
12	<i>oar1</i>	6,865–7,671	807	268	3-oxoacyl-reductase
13	<i>acrB</i>	7,717–10,779	3,063	1,020	Acriflavine resistance protein
14	<i>acrA</i>	10,776–11,882	1,107	368	Multidrug efflux protein
15	<i>tetR</i>	11,964–12,581	618	205	Transcriptional regulator
16	<i>higA</i>	12,834–13,124	291	96	Antitoxin
17	<i>higB</i>	13,142–13,420	279	92	Toxin
18	<i>trbJ</i>	13,479–13,685	207	68	Conjugative transfer protein
19	<i>trbI</i>	13,698–14,546	849	282	Conjugative transfer protein
20	<i>trbF</i>	14,608–15,324	717	238	Conjugative transfer protein
21	<i>trbE</i>	15,321–17,132	1,812	603	Conjugative transfer protein
22	<i>trbE</i>	17,714–17,884	171	56	Conjugative transfer protein
23	<i>trbD</i>	17,872–18,138	267	88	Conjugative transfer protein
24	<i>trbC</i>	18,195–18,587	393	130	Conjugative transfer protein
25	<i>trbB</i>	18,600–19,481	882	293	Conjugative transfer protein
26	<i>dnaG2</i>	19,512–19,886	375	124	DNA primase
27	<i>dnaG3</i>	19,966–20,769	804	267	DNA primase

^a ORF = open reading frame. Rows of ORFs 10, 11, 16, and 17 are in bold to highlight the toxin-antitoxin system genes.

^b Annotation was performed through the RAST server (<http://rast.nmpdr.org/>).

Fig. 1. Schematic descriptions of pXFSL21. **A**, Chromosomal integration of *dnaG1*. Annotation of selected genes: *gdhA* = NAD-specific glutamate dehydrogenase, *nadK* = NAD kinase, *Nt5* = 5'-nucleotidase, *hyp* = hypothetical protein, *romA* = outer membrane protein, and *exoI* = exodeoxyribonuclease I. **B**, Circular pXFSL21 with annotated genes and open reading frames. The two toxin-antitoxin systems (*ycdD-ycdE* and *higB-higA*) are highlighted. Solid single-line arrows are relative positions of primers used for polymerase chain reaction (PCR) enclosure experiments. **C**, Agarose gel electrophoresis of PCR amplicons from enclosure primer sets (21250F-137R and 21124F-617R) from in vitro and in planta experiments.

of 1.16 and 0.84, respectively, with the in vitro DNA, and 1.13 and 1.16, respectively, with the in planta DNA (Table 5). These results further supported the conclusions from in silico analyses that pXFSL21 was a single-copy plasmid in strain Stag's Leap.

Homologs of pXFSL21 T-A systems. Homologs of *ycdD-ycdE* were detected in 26 *X. fastidiosa* GenBank sequence records (10 plasmids and 16 whole-genome sequences) (Table 6). Based on sequence similarity, a gene in plasmid PP1 of *Pseudomonas syringae* was highly related to *ycdD*, and a gene in plasmid pP2 of *Xanthomonas citri* was highly similar to *ycdE*. The predicted 3D structure of *ycdE* protein, or YdcE, was not similar to the representative structure of the YdcE/MazF/PemK family of endoribonuclease

functions (Pellegrini et al. 2005). Homologs of *higB-higA* were detected in only two *Xylella fastidiosa* strains, Ann-1 and CO33 (Table 7). The closest bacterium with the *higB-higA* homolog was *Sphingobium cloacae* with its plasmid pSCLO_4. The predicted 3D structures of both HigB and HigA were very similar to the those of a *Proteus vulgaris* strain (Schureck et al. 2014).

DISCUSSION

Traditionally, plasmids are identified by their unique physical features (compared with chromosomal DNA) such as supercoiled conformation and small size through appropriate experimental

Fig. 2. Predicted three-dimensional structure, by Phyre2, of proteins encoded by **A**, *YdcD*; **B**, *YdcE*; **C**, *HigB*; and **D**, *HigA*. N = N terminal and C = C terminal. The helix-turn-helix motif in *higA* is the DNA binding site.

TABLE 4. Average nucleotide coverage (ANC) analyses of selected genes from pXFSL21 and *Xylella fastidiosa* strain Stag's Leap chromosome^a

Gene and annotation ^b	Size (bp)	MiSeq (in vitro)			HiSeq (in planta)		
		Reads	ANC (×)	ANC/ <i>rrs</i>	Reads	ANC (×)	ANC/ <i>dnaG</i>
pXFSL21							
<i>dnaG1</i> , DNA primase	1,074	5,479	860	0.88	5,413	579	1.00
<i>ycdE</i> , programmed cell death toxin	315	661	360	0.37	744	252	0.44
<i>acrB</i> , acriflavine resistance protein	3,063	9,004	573	0.59	6,798	290	0.50
<i>tetR</i> , transcriptional regulator	618	1,581	433	0.44	1,669	311	0.54
<i>trbE</i> , conjugal transferase E	1,812	4,892	463	0.47	4,825	327	0.56
<i>trbB</i> , conjugal transferase B	882	2,327	424	0.43	2,393	317	0.55
Chromosome							
<i>rrs</i> , 16S ribosomal RNA (rRNA)	1,545	7,943	975	1.00	391,918	55,770	n/a
<i>rimM</i> , 16S rRNA processing protein	513	1,501	484	0.50	1,046	247	0.43
<i>aroB</i> , 3-dehydroquinate synthase	1,113	2,638	517	0.53	2,480	296	0.51
<i>dnaG1</i> , DNA primase	1,074	5,480	860	0.88	5,420	579	1.00
<i>gcvB</i> , glycine dehydrogenase	2,946	7,810	483	0.50	6,732	285	0.49
<i>yfcG</i> , glutathione S-transferase	618	1,612	480	0.49	1,358	265	0.46
<i>L23p</i> , large subunit ribosomal protein	303	743	494	0.51	566	209	0.36
<i>cysJ</i> , NADPH Sulfite reductase	1,776	4,916	499	0.51	4,190	288	0.50
<i>Ank1</i> , ankyrin-like protein	3,288	9,200	561	0.58	7,426	278	0.48
<i>wbiE</i> , glycosyl transferase	828	2,175	434	0.45	1,830	265	0.46
<i>ppk1</i> , phosphomethyl pyrimidine kinase	810	2,274	454	0.47	2,355	324	0.56

^a Reads = mapped reads and n/a = not applied because of the extraordinarily high ANC from plant chloroplast genome interference.

^b *dnaG1* and *rrs* are two-copy genes as used as copy-number references. Data in bold were from two-copy genes.

procedures such as agarose gel electrophoresis. Copy number could be estimated based on intensity of stained DNA bands (Chen et al. 1992; Projan et al. 1983). However, visualization of low-copy-number plasmids in agarose gels is difficult if the plasmid quantity is not significantly enriched. Adding to the problem in this study is the slow-growth nature of *X. fastidiosa* in vitro. Preparation of large quantities of DNA from slow-growing bacteria is challenging. The Southern blot procedure could increase detection sensitivity

(Chen et al. 1992); however, preknowledge of plasmid sequence or DNA is required, which was not available for the strain Stag's Leap.

In this study, we successfully applied NGS technology to detect a single-copy plasmid in strain Stag's Leap. The key to success was the high-throughput capacity of NGS. From a microliter level of DNA sample, NGS (HiSeq or MiSeq) generated a high volume of short-sequence reads that evenly or almost evenly covered the whole *X. fastidiosa* genome over hundreds of times (Table 2). The

TABLE 5. pXFSL21 copy-number (CN) analysis by quantitative polymerase chain reaction^a

ORF	Gene	Origin	Primer set ^b	Amp (bp)	In vitro		In planta	
					Ct value	CN	Ct value	CN
13	<i>acrB</i>	Plasmid	acrB_F-acrB_R	195	17.05 ± 0.08	0.84	29.95 ± 0.07	1.16
15	<i>tetR</i>	Plasmid	tetR_F-tetR_R	190	15.43 ± 0.08	1.16	26.22 ± 0.06	1.13
	<i>GAPDH</i>	Chromosome	GAPDH_F-GAPDH_R	191	15.55 ± 0.07	1.0	26.61 ± 0.03	1.0

^a ORF = open reading frame, Amp = amplicon size, and Ct = cycle threshold. CN was calculated based on Pfaffl (2001).

^b Primer efficiencies for *tetR*, *acrB*, and *GAPDH* were 97, 83, and 99%, respectively.

TABLE 6. Homologs of *ycdD-ycdE* of pXFSL21 in *Xylella fastidiosa* and representative bacteria in GenBank database (version 222)^a

		ydcD		ydcE		Accession	Group
		Query (%)	Seq (%)	Query (%)	Seq (%)		
nr/nt Database							
1	<i>X. fastidiosa</i> Ann-1	100	99	100	99	CP006696.1	1
2	<i>X. fastidiosa</i> Hib4	100	98	100	98	CP009885.1	1
3	<i>X. fastidiosa</i> Fb7	48	76	99	70	CP010051.1	2
4	<i>X. fastidiosa</i> FB7 plasmid unnamed	48	76	99	70	CP014330.1	2
5	<i>X. fastidiosa</i> J1a12 plasmid pXF51-J1	48	76	99	70	CP009825.1	2
6	<i>X. fastidiosa</i> U24D plasmid pXF51ud	48	76	99	70	CP009791.1	2
7	<i>X. fastidiosa</i> 9a5c plasmid pXF51	48	76	99	70	AE003851.1	2
8	<i>X. fastidiosa</i> Pr8x plasmid pXF39	48	75	99	71	CP009827.1	2
9	<i>X. fastidiosa</i> M12	48	75	100	73	CP000941.1	2
	<i>Pseudomonas syringae</i> pv. <i>cerasicola</i> CFBP6109 plasmid PP1	100	70	n/a	n/a	NZ_LT963392.1	
	<i>Xanthomonas citri</i> subsp. <i>citri</i> 03-1638-1-1 plasmid pP2	n/a	n/a	99	77	CP023287.1	
	<i>X. citri</i> subsp. <i>malvacearum</i> MS14003 plasmid unnamed1	n/a	n/a	99	77	CP023160.1	
Whole-genome shotgun database							
	<i>Xylella fastidiosa</i> subsp. <i>fastidiosa</i> Stag's Leap	100	100	100	100	LSMJ01000015.1	1
1	<i>X. fastidiosa</i> CO33	100	99	100	100	LJZW01000027.1	1
2	<i>X. fastidiosa</i> Ann-1	100	99	100	99	AAAM04000239.1	1
3	<i>X. fastidiosa</i> subsp. <i>multplex</i> CFBP8416	48	76	100	73	LUYC01000012.1	2
4	<i>X. fastidiosa</i> CVC0256 plasmid pXF-BHR.CVC0256	48	76	99	70	LRVF01000125.1	2
5	<i>X. fastidiosa</i> CVC0251 plasmid pXF-BHR.CVC0251	48	76	99	70	LRVE01000127.1	2
6	<i>X. fastidiosa</i> subsp. <i>pauca</i> 11399 plasmid pXF51	48	76	99	70	JNBT01000036.1	2
7	<i>X. fastidiosa</i> strain BB01 BBA	48	75	100	74	MPAZ01000005.1	2
8	<i>X. fastidiosa</i> subsp. <i>multplex</i> CFBP8417	48	75	100	73	LUYB01000013.1	2
9	<i>X. fastidiosa</i> subsp. <i>multplex</i> CFBP8418	48	75	100	73	LUYA01000012.1	2
10	<i>X. fastidiosa</i> COF0324 plasmid pXF-BHR-COF0324	48	75	99	71	LRVG01000140.1	2
11	<i>X. fastidiosa</i> CFBP8073	48	75	100	73	LKES01000047.1	2
12	<i>X. fastidiosa</i> CO33	48	75	100	73	LJZW01000035.1	2
13	<i>X. fastidiosa</i> sycamore Sy-VA	48	75	100	73	JMHP01000002.1	2
14	<i>X. fastidiosa</i> 6c plasmid pXF6c	48	75	99	71	AXBS02000019.1	2
15	<i>X. fastidiosa</i> subsp. <i>multplex</i> Griffin-1	48	75	100	73	AVGA01000012.1	2
16	<i>X. fastidiosa</i> subsp. <i>multplex</i> ATCC 35871	48	75	100	73	AUAJ01000014.1	2
17	<i>X. fastidiosa</i> Dixon ctg82	48	75	100	73	AAAL02000009.1	2

^a Query = query coverage, Seq = sequence identity, and n/a = not applied.

^b Plasmid records are highlighted in bold.

TABLE 7. Homologs of *higA-higB* of pXFSL21 in *Xylella fastidiosa* and representative bacteria in GenBank database (version 222)^a

ORF	Description	<i>higA</i>		<i>higB</i>		Accession number
		Query (%)	Seq (%)	Query (%)	Seq (%)	
	nr/nt Database					
1	<i>Xylella fastidiosa</i> strain Ann-1 whole genome	100	99	100	99	CP006696.1
	<i>Sphingobium cloacae</i> plasmid pSCLO_4	93	71	99	69	CP000689.1
	Whole-genome shotgun database					
	<i>X. fastidiosa</i> strain Stag's Leap	100	100	100	100	LSMJ01000015.1
1	<i>X. fastidiosa</i> strain Ann-1	100	99	100	99	AAAM04000239.1
2	<i>X. fastidiosa</i> strain CO33	100	99	20	98	LJZW01000027.1

^a ORF = open reading frame, Query = query coverage, and Seq = sequence identity.

short sequence reads were assembled to form long sequence contigs. Candidate plasmid contigs were predicted based on bioinformatics information such as genes involving RDM and CAP. Plasmid circularity was evaluated through read mapping, and confirmed by PCR-Sanger sequencing. Plasmid copy number was readily estimated through ANC analyses and supported by qPCR experiments.

Another significant advantage of the NGS approach is that pXFSL21 was detected and characterized not only from pure in vitro *X. fastidiosa* cultures but also from infected in planta grapevine tissue. This approach is particularly useful for fastidious prokaryote research where pure culture is difficult, or even not possible, to obtain. Indeed, the NGS approach is playing a major role in detection and characterization of circular plasmids or prophages in a nonculturable bacterium, '*Candidatus Liberibacter asiaticus*', associated with citrus huanglongbing (Zheng et al. 2018). Interestingly, the culturable property of the *X. fastidiosa* allowed us to validate both in vitro and in planta methods simultaneously (Tables 2 and 4).

Growth of *X. fastidiosa* in grapevine is a natural system in which to study pathogen–host interactions of PD. NGS technology provided an opportunity to study the behavior of the *X. fastidiosa* genome directly in a plant host. pXFSL21 (contig 15) was clearly single copy under both in vitro and in planta conditions (Tables 2 and 4). However, this was not the case for contig 6 (Table 2). The 19,107-bp contig 6 doubled its copy number under in planta condition (ANC = 940) compared with that under in vitro condition (ANC = 490). Annotation showed that contig 6 contained 24 ORFs that encoded exclusively phage or hypothetical proteins, including a phage lysozyme. Detailed analyses on contig 6 are outside the scope of this study and deserve future attention. Yet analyses of pXFSL21 and contig 6 have demonstrated the potential research power of an NGS approach.

T-A systems are mechanisms well known for their role in maintaining low-copy-number plasmids in a bacterial population (Dmowski and Jagura-Burdzy 2013; Unterholzner et al. 2013; Yang and Walsh 2017). A single T-A system in a *X. fastidiosa* DNA was described previously (Lee et al. 2014; Stenger et al. 2010). This study detected two T-A system in pXFSL21. The Stag's Leap culture hosting pXFSL21 in this study has been subcultured in PW medium numerous times since 2010. It is apparent that pXFSL21 was consistently maintained or survived through all the subculturing as well as in planta manipulations. For this reason, we assumed that the double T-A systems could have provided a double dosage effect that strengthened the survivability of pXFSL21. However, further experimental validations are needed to prove this theory.

The T-A systems of *ycdD-ycdE* and *higB-higA* have been described in multiple bacterial systems (Budde et al. 2006; Pellegrini et al. 2005; Schureck et al. 2014). Both *ycdD-ycdE* and *higB-higA* are type II T-A systems (i.e., the toxin is neutralized by binding of the partner antitoxin protein). The proteinaceous nature of the type II system makes it a feasible target for artificial activation of the toxin for pathogen control. Identification of compounds that disrupt T-A complexes would directly activate the toxin, leading to bacterial cell killing (Unterholzner et al. 2013). The two toxin genes are relatively small: *ycdE* is 315 bp coding for a 104-amino-acid (aa) protein (Fig. 2B) and *higB* is 279 bp coding for a 92-aa protein (Fig. 2C). The identification of these T-A system genes on a unique plasmid creates the opportunity to clone and express these toxin genes in planta to potentially control PD. The knowledge obtained through NGS along with bioinformatics analyses provides the necessary sequence and genomic structural information to generate targeted experiments on the biology and gene function of an organism.

Due to application of NGS, the GenBank database has accumulated >40 *X. fastidiosa* plasmid sequences and >40 of the bacterial whole-genome sequences. The large sequence collections provided a convenient and efficient means for *X. fastidiosa* characterization population wide. For example, the *ycdD-ycdE*

system was found in *X. fastidiosa* strains from different geographical origins and formed two groups: one with *ycdD* almost identical (100% length coverage, 98 to 100% identity) to that in pXFSL21, and the other much different *ycdD* (48% length coverage, 70 to 76% identity) (Table 6). In contrast, *higB-higA* was limited only to strain Ann-1 and CO33. The two T-A systems could be either plasmid- or chromosome-borne, as in other bacteria (Pandey and Gerdes 2005). Many non-*Xylella* sequences similar to the four T-A system genes were found. Phylogenetic trees constructed from these sequences were not informative. Instead, the most similar bacteria were listed for phylogenetic reference. Along the line of plasmid characterization, we were not able to identify *ori* of pXFSL21 with high confidence.

Furthermore, among the five GenBank documented PD strains (i.e., Temecula1, M23, GB514, ATCC 35879, and Stag's Leap), pXFSL21 was only found in the Stag's Leap strain. Given the genetic content of this plasmid, particularly the CAP genes *acrA* and *acrB*, predicted to be involved in resistance to toxic compounds, pXFSL21 may be a promising lead for targeted experiments into the function of plasmid located genes and their effect on the biology of *X. fastidiosa*. This plasmid may play a role in the prevalence or virulence specific to the Stag's Leap strain. However, direct experimental evidence is currently lacking. Nevertheless, pXFSL21 could serve as a molecular marker, or an initial point, to study the uniqueness or its role in virulence of the Stag's Leap strain.

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