A Conjugative 38 kB Plasmid Is Present in Multiple Subspecies of *Xylella fastidiosa*

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Abstract

A ~38kB plasmid (pXF-RIV5) was present in the Riv5 strain of *Xylella fastidiosa* subsp. *multiplex* isolated from ornamental plum in southern California. The complete nucleotide sequence of pXF-RIV5 is almost identical to that of pXFA01 from *X. fastidiosa* subsp. *fastidiosa* strain M23; the two plasmids vary at only 6 nucleotide positions. BLAST searches and phylogenetic analyses indicate pXF-RIV5 and pXFA01 share some similarity to chromosomal and plasmid (pXF51) sequences of *X. fastidiosa* subsp. *pauca* strain 9a5c and more distant similarity to plasmids from a wide variety of bacteria. Both pXF-RIV5 and pXFA01 encode homologues of a complete Type IV secretion system involved in conjugation and DNA transfer among bacteria. Mating pair formation proteins (Trb) from *Yersinia pseudotuberculosis* IP31758 are the mostly closely related non-*X. fastidiosa* proteins to most of the Trb proteins encoded by pXF-RIV5 and pXFA01. Unlike many bacterial conjugative plasmids, pXF-RIV5 and pXFA01 do not carry homologues of known accessory modules that confer selective advantage on host bacteria. However, both plasmids encode seven hypothetical proteins of unknown function and possess a small transposon-associated region encoding a putative transposase and associated factor. Vegetative replication of pXF-RIV5 and pXFA01 appears to be under control of RepA protein and both plasmids have an origin of DNA replication (*oriV*) similar to that of pRP4 and pR751 from *Escherichia coli*. In contrast, conjugative plasmids commonly encode TrfA and have an *oriV* similar to those found in IncP-1 incompatibility group plasmids. The presence of nearly identical plasmids in single strains from two distinct subspecies of *X. fastidiosa* is indicative of recent horizontal transfer, probably subsequent to the introduction of subspecies *fastidiosa* to the United States in the late 19th century.

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Introduction

Horizontal gene transfer plays a critical role in bacterial adaptation and evolution. On average, 81% of the genes in a typical bacterial genome have been involved in a horizontal transfer event at some point in the past [1]. One of the most common mechanisms for DNA exchange is via conjugative plasmids that encode type IV secretion systems (T4SS), a broad class of macromolecular translocation machinery. There are three main types of T4SS: i) conjugation systems that transfer DNA and, in some instances, DNA-binding proteins; ii) effector translocator systems that deliver proteins and other effectors to eukaryotic cells during bacterial infection of eukaryotic hosts; and iii) DNA uptake or release systems that move DNA between the interior of the cell and the extracellular environment [2]. Conjugation systems are found both on self-transmissible or conjugative plasmids, circular DNA molecules that replicate independently of the bacterial chromosome, and on integrative and conjugative elements that integrate into the chromosome, excising and forming a circular intermediate prior to translocation [2]. One well characterized conjugation system is VirB/D4 from *Agrobacterium tumefaciens*, which is composed of a cell-envelope spanning secretion channel and an extracellular pilus that contacts the recipient cell [3]. For VirB/D4, the translocation system consists of VirB2-11 proteins forming the secretion channel, the VirD4 substrate receptor or type IV coupling protein (T4CP), and proteins for pilus formation and DNA substrate processing [2]. Many conjugative plasmids also contain accessory modules encoding cargo proteins which act as virulence factors, confer resistance to antibiotics/heavy metals, or catabolize toxic organic substances [4].

*Xylella fastidiosa* is a fastidious, xylem-limited Gram-negative bacterial phytopathogen causing numerous vascular occlusion and water stress diseases including Pierce’s disease of grape, almond leaf scorch, oleander leaf scorch, and other diseases of perennial crops and landscape plants [5]. Four subspecies of *X. fastidiosa* have been identified based on a multi-locus sequence typing (MLST) phylogeny [6,7]. Subspecies *fastidiosa* contains strains of low genetic diversity that cause Pierce’s disease and sometimes almond leaf scorch in the U. S. and diverse strains from Central America; subspp. *pauca* is thought to have been introduced to the U. S. in the late 19th century [8]. Subspecies *multiplex* is an endemic North American clade capable of infecting numerous hosts (but generally not grapevine) [9–11]. Subspecies *pauca* contains South American strains causing citrus variegated chlorosis and coffee leaf scorch [12]. Subspecies *sandyi* consists of closely related strains isolated from oleander in California and Texas and is thought to have been introduced to the United States approximately 30 years ago [6]. Currently, 5 fully sequenced and annotated *X. fastidiosa* genomes are available (pauca strain 9a5c [13], fastidiosa strain Temecula [14], multiplex strain M12 [15], fastidiosa strain M25 [15], and fastidiosa...
strain GB314 [16]). Two additional sequences (multiplex strain Dixon, GenBank accession number NZ_AAAL00000002; and sandy strain Ann-1, GenBank accession number NZ_AAA M00000000.3) are incomplete, unassembled shotgun sequences; the Ann-1 sequence may have been derived from a mixed culture and appears to be contaminated with sequences from a multiplex strain [17].

Here, we characterize two closely related 38kB conjugative plasmids of _X. fastidiosa_. Plasmid pXF-RIV5 was isolated from the Riv5 strain of _X. fastidiosa_ subspecies _multiplex_ [18]; complete sequence of pXF-RIV5 was determined in this current work. Plasmid pXFAS01 is known only as a circular contig discovered during the complete genome sequencing of _X. fastidiosa_ subspecies _fastidiosa_ strain M23 [15]. While minimal annotation of pXFAS01 accompanies the sequence in GenBank, no analysis of the type IV secretion system, origin of transfer or origin of replication from pXFAS01 has been presented previously. This work presents analyses of gene complement and phylogeny of these two closely related plasmids to reveal i) an evolutionary history of recombination among divergent sources to generate the mosaic backbone shared by pXF-RIV5 and pXFAS01, and ii) evidence of recent translocation of plasmid DNA via conjugation among distinct subspecies of _X. fastidiosa_.

**Materials and Methods**

**Culture and MLST of _X. fastidiosa_ subspecies _multiplex_ strain Riv5**

Isolation of _X. fastidiosa_ strain Riv5 from ornamental plum (_Prunus cerasifera_) was described previously [18]. Strain Riv5 cultures were grown in liquid periwinkle wilt (PW) medium for 7–10 days at 28°C and used to inoculate plates containing solid PW medium. After 7–10 days of growth at 28°C, bacterial colonies were washed from 10 PW plates and extracted for total genomic DNA [11]. Genomic DNA was used as template for PCR amplification of seven housekeeping genes (cysG, gltT, holC, malF, leuA, nuoL, petG) and petU [7]; consensus sequences were determined for each amplified region based on sequences of three independent clones per PCR product. Consensus sequences for each amplified region were concatenated into a single sequence. MLST was performed as described [7] with concatenated Riv5 sequences aligned with the corresponding concatenated sequences from multiple strains representative of each _X. fastidiosa_ subspecies available in GenBank using CLUSTALX. Phylogenetic placement of strain Riv5 was determined based on a neighbor-joining tree (1000 bootstrap replicates) for each protein was constructed based on a multiple alignment of amino acid sequences generated using CLUSTALX. Nodes bearing <70% bootstrap support were considered unreliable and collapsed to polytomies.

**Results**

Riv5 is a strain of subspecies _multiplex_.

Genomic DNA sequences used for MLST of strain Riv5 were deposited as GenBank Accessions JX679700-JX67997. Phylogeny of concatenated sequences of the eight genes examined by MLST indicated that strain Riv5 clustered with _X. fastidiosa_ strains of subspecies _multiplex_, including the fully-sequenced _multiplex_ strain M12 (data not shown). Phylogeny of each individual gene used for MLST also clustered strain Riv5 with strains of subspecies _multiplex_ (data not shown). These results are consistent with phylogenetic placement of strain Riv5 based on 16S-23S rRNA spacer sequences [18].

pXF-RIV5 contains a type IV secretion system

The complete nucleotide sequence of pXF-RIV5 is 38,297 bp in length with a G+C content of 49.2%, which is similar to the 51–52% G+C content of sequenced _X. fastidiosa_ genomes [13,15]. As detailed in Table 1 and Figure 1, pXF-RIV5 has 35 ORFs encoding proteins similar to characterized proteins of known function from other organisms while seven ORFs encode hypothetical proteins for which functions of homologues identified in GenBank are unknown. The two largest groups of genes are the conjugative transfer (tra) and mating pair formation (trb) modules. Together, these two genetic modules encode homologues of all proteins necessary for a functional T4SS [2]. Genes for plasmid replication (repA, repE, and _orfB_ and partition (parC and _orfB_ also are present. Two genes, _orfA_ and _orfB_, encode proteins similar to transposon-associated recombinase and transposase, respectively; no other transposon-like elements on pXF-RIV5 were identified. Unlike many conjugative plasmids from animal pathogens or
Table 1. Annotation of open reading frames from pXF-RIV5.

| Name       | start | stop  | # aa | strand | product                                                                 | most closely related gene product                                                                 |
|------------|-------|-------|------|--------|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| TrbD       | 3     | 323   | 107  | +      | conjugal transfer protein, ATPase, VirB3 family                         | conjugation protein TrbD [Azorarcus sp. 71 EbN1] YP_195564.1                                      |
| TrbE       | 311   | 2875  | 855  | +      | conjugal transfer protein, ABC transporter-like, VirB4 family          | conjugation protein TrbE [Yersinia 69 pseudotuberculosis IP 31758] YP_001393245.1                   |
| TrbF       | 2872  | 3588  | 239  | +      | conjugal transfer protein, inner membrane protein, VirB8 family       | conjugation transfer protein TrbF [Yersinia 61 pseudotuberculosis IP 31758] YP_001393246.1         |
| TrbG       | 3605  | 4495  | 297  | +      | conjugal transfer protein, periplasmic or outer membrane protein, VirB9 family | conjugation transfer protein TrbG [Yersinia 68 pseudotuberculosis IP 31758] YP_001393247.1         |
| TrbH       | 4498  | 4974  | 159  | +      | conjugal transfer protein, putative lipoprotein, VirB7 family          | conjugation transfer protein TrbH [Yersinia 41 pseudotuberculosis IP 31758] YP_001393248.1         |
| Trbl       | 4980  | 6380  | 467  | +      | conjugal transfer protein, inner membrane protein, VirB10 family      | conjugation transfer protein TrbI [Yersinia pseudotuberculosis IP 31758] YP_001393249.1            |
| TrbJ       | 6399  | 7172  | 258  | +      | conjugal transfer protein, periplasmic or outer membrane protein, VirB5 family | conjugative transfer protein TrbJ [Burkholderia pseudomallei Pakistan 9] ZP_03794994.1             |
| pRiv5_008  | 7189  | 7425  | 79   | +      | hypothetical protein, putative lipoprotein attachment site            | lipoprotein [Aggregatibacter actinomycetemcomitans D115-1] YP_03966129.1                           |
| TrbL       | 7447  | 8817  | 457  | +      | conjugal transfer protein, inner membrane protein, VirB6 family       | conjugation transfer protein TrbL/VirB6 [Yersinia pseudotuberculosis IP 31758] YP_001393251.1       |
| TrbN       | 8823  | 9422  | 200  | +      | conjugal transfer protein, lytic transglycosylase domain, possible VirB1 | conjugation transfer protein TrbN [Yersinia 63 pseudotuberculosis IP 31758] YP_001393252.1          |
| pRiv5_011  | 10035 | 10262 | 76   | -      | conserved hypothetical protein, Pfam01565/DUF972 family                | hypothetical protein XM1N_4535 [Xanthomonas citri pv. mangiferaeindicae LMG 941] ZP_09830305.1    |
| pRiv5_012  | 10259 | 10627 | 123  | -      | hypothetical protein                                                   | hypothetical protein EGYY_28500 [Erigerthella sp. YY7918] YP_004712226.1                          |
| resolvase   | 11170 | 11733 | 188  | +      | putative resolvase [Methylomicrobium alcaliphilum]                     | putative resolvase (Methylomicrobium alcaliphilum) YP_004901765.1                              |
| pRiv5_014  | 12064 | 12657 | 198  | +      | hypothetical protein                                                   | hypothetical protein MYA_6037 [Burkholderia sp. KJ006] YP_006337102.1                             |
| TrfB        | 12725 | 13039 | 105  | +      | probable TrfB transcriptional repressor protein                         | hypothetical protein BBR47_02790 [Brevibacillus brevis NBRC 100599] YP_002769760.1              |
| pRiv5_016  | 13216 | 13809 | 198  | +      | hypothetical protein                                                   | hypothetical protein MYA_6037 [Burkholderia sp. KJ006] YP_006337102.1                             |
| pRiv5_017  | 13850 | 14437 | 196  | -      | hypothetical protein                                                   | hypothetical protein Acife_3030 [Acidithiobacillus ferrovorans S53] YP_004785429.1               |
| TraC        | 14505 | 18992 | 1496 | -      | conjugal transfer protein, topoisomerase/primase-like                 | TraC DNA primase [Plasmid QKH54] YP_619864.1                                                  |
| TraD        | 18998 | 19360 | 121  | -      | conjugal transfer protein, inner membrane protein                      | TraD protein [IncP-1 plasmid pKJ5] YP_709180.1                                               |
| TraE        | 19363 | 21420 | 686  | -      | conjugal transfer protein, topoisomerase-primase domain               | TraE [Pseudomonas putida] YP_031626268.1                                                      |
| TraF        | 21449 | 21985 | 179  | -      | conjugal transfer protein, peptidase/pilin processing protease         | TraF protein of DNA transfer system [Methylophaga sp. JAM7] YP_006297569.1                      |
| TraG        | 21982 | 23916 | 645  | -      | conjugal transfer protein, coupling protein, VirD4 family             | conjugation transfer protein TraG [Yersinia 79 pseudotuberculosis IP 31758] YP_001393286.1       |
Relationship of pXF-RIV5 to other X. fastidiosa plasmids

The sequence of pXF-RIV5 is almost identical to pXFAS01 from X. fastidiosa subsp. fastidiosa strain M23 [15]. For consistency, nucleotide coordinates of pXF-RIV5 were assigned to correspond to nucleotide coordinates designated for pXFAS01. Alignment of pXF-RIV5 and pXFAS01 revealed polymorphism, all of which are transitions, at only six nucleotide positions over the entire ~38 kb length. Two transitions are located in intergenic regions at nt 9,556 (between bbv and ORF11) and at nt 36,281 (between ssBP and orfA). The other four transitions are located in the traI gene encoding a conjugative relaxase homologue. Two transitions (nts 24,789 and 24,921) in the traI gene were synonymous substitutions that did not alter predicted protein sequence. The remaining two transitions in the traI gene were nonsynonymous substitutions that altered the codon for amino acid 462 (nt 25,019) from proline (pXFAS01) to serine (pXF-RIV5) or altered the codon for amino acid 386 (nt 25,250) from serine (pXFAS01) to proline (pXF-RIV5), where both were introduced by a single recombination event between pXF-RIV5 or pXFAS01 and a closely related plasmid. Although both nonsynonymous substitutions are not in highly conserved portions of TraI, potential alteration of function cannot be excluded. Clustering of four out of six polymorphic sites in less than 500 bp of a 38 kb plasmid raises the possibility that all substitutions in the traI gene were introduced by a single recombination event between pXF-RIV5 or pXFAS01 and a closely related plasmid.

Table 1. Cont.

| Name    | start | stop  | # aa | strand | product                                                  | most closely related gene product % aa identity |
|---------|-------|-------|------|--------|----------------------------------------------------------|---------------------------------------------|
| TraJ    | 26440 | 26793 | 118  | -      | conjugal transfer protein relaxosome component            | conjugal transfer relaxosome component Trait [Yersinia pseudotuberculosis IP 31758] YP_001393287.1 |
| TraK    | 27018 | 27413 | 132  | +      | conjugal transfer protein, putative oriT binding protein  | TraK protein [IncP-1 plasmid pKJK5] YP_709187.1 |
| TraL    | 27413 | 28138 | 242  | +      | conjugal transfer protein, contains P-loop nucleotide binding domain | TraL protein [Pseudomonas sp. ADP] NP_862455.1 |
| TraM    | 28138 | 28590 | 151  | +      | conjugal transfer protein, transcriptional activator        | traM gene product [Methylmicobium Aureum] YP_004901800.1 |
| TraN    | 28652 | 29248 | 199  | -      | conjugal transfer protein, mating pair stabilization protein | hypothetical protein pKJK5.51 | YP_709190.1 |
| TraO    | 29275 | 29628 | 118  | -      | conjugal transfer protein, putative membrane protein       | putative conjugation protein TraO [Azotobacter sp. EbN1] YP_195564.1 |
| parB-like| 29726 | 30757 | 344  | -      | contains parB-like nuclease domain, putative partition site DNA binding protein | ParB equivalent nuclease [uncultured bacterium] YP_112421.1 |
| parA-like| 30754 | 31833 | 360  | -      | putative ATPase involved in plasmid replication and partition | IncC1 protein [uncultured bacterium] NP_598102.1 |
| orfA    | 32062 | 32286 | 75   | +      | orfA family, site-specific serine recombinase, transposon-associ. | transposon IS605 OrfA [Methylacidiphilum infernorum V4] YP_001941027.1 |
| orfB    | 32829 | 33476 | 399  | +      | orfB family, helix-turn-helix domain, probable transposase   | transposon IS605 OrfB [Methylacidiphilum infernorum V4] YP_001941028.1 |
| KleE    | 33517 | 33837 | 107  | -      | probable KleE stable plasmid inheritance protein            | KleE protein [Plasmid pB3] YP_133959.146 |
| RepA    | 34560 | 35471 | 304  | -      | protein involved in plasmid replication, exact role unknown | RepA [Acidithiobacillus caldus SM-1] YP_004750509.1 |
| pRiv5-036| 35493 | 35849 | 119  | -      | hypothetical protein                                      | hypothetical protein pSB102_p07 [Plasmid pSB102] NP_361021.2 |
| ssBP    | 35893 | 36252 | 120  | -      | single-strand DNA binding protein                          | single-strand DNA-binding protein [uncultured bacterium] YP_112367.1 |
| TrbA    | 36370 | 36726 | 119  | +      | conjugal transfer protein, helix-turn-helix containing oriT regulator | conjugal transfer protein TrbA [Yersinia 73 pseudotuberculosis IP 31758] YP_001393241.1 |
| TrbB    | 36932 | 37892 | 321  | +      | conjugal transfer protein, ATPase, VirB 11 family          | conjugal transfer protein TrbB [Yersinia 70 pseudotuberculosis IP 31758] YP_001393242.1 |
| TrbC    | 37905 | 38297 | 131  | +      | conjugal transfer protein, subunit of bacterial pilus, VirB2 family | conjugal transfer protein TrbC [Yersinia 71 pseudotuberculosis IP 31758] YP_001393243.1 |

The most closely related gene product was identified using BLASTP and excludes other proteins from X. fastidiosa.

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Environmental samples, no accessory modules containing homologues of known virulence factors, antibiotic/heavy metal resistance, or catabolism of toxic organic compounds were encoded by pXF-RIV5.

A Conjugative Plasmid from X. fastidiosa
The plasmid (pXF51) of X. fastidiosa subsp. pauca strain 9a5c encodes a partial trb module [22] which is 96% identical at the nucleotide level over almost 9 kB of pXF-RIV5 and pXFAS01, spanning trbE through trbN (nts 481 – nts 9401). Strain 9a5c also has an extensive cluster of trb genes on the chromosome [22] sharing 97% nucleotide sequence identity with 10 kB of pXF-RIV5 and pXFAS01 (nts 37612 – nts 9417). A small region of pXF-RIV5 and pXFAS01 (140 bp; nts 9276 – nts 9414) shares 89% nucleotide sequence identity to 25 kB IncP-1 plasmids (pXF-RIV11, pXF-RIV16, pXF-RIV19, and pXF-RIV25) from mulberry-infecting strains of X. fastidiosa [18]. In pXF-RIV5 and pXFAS01, the homologous region is within the trbN gene; however, in the IncP-1 plasmids, the complete trbN gene is not present.

DNA replication elements and T4SS components of pXF-RIV5 have distinct evolutionary histories

Most proteins encoded by pXF-RIV5 and pXFAS01 are homologues of proteins encoded by numerous bacterial taxa. As shown in Figure 2, neighbor-joining phylogenetic trees were constructed for representative proteins (TraI and TrbG) from the two T4SS modules and for the replication protein RepA. The trees for TraI and TrbG have similar but not identical topology. The most closely related homologues (excluding those from X. fastidiosa) for both TraI and TrbG are from Yersinia pseudotuberculosis IP31758 and E. coli PA14. Because Tra and Trb proteins must work together to form a functional T4SS, it is not surprising that both T4SS gene clusters have similar phylogeny.

The phylogenetic history inferred for RepA is quite different from that of the T4SS (Figure 2). Most RepA homologues from X. fastidiosa constituted a clade distinct from RepA homologues encoded by all other taxa identified in BLAST P searches. RepA from X. fastidiosa strains Dixon, EB92.1 and Ann-1* were either identical, or nearly identical to that encoded by pXF-RIV5 and pXFAS01. Dixon and Riv5 are subspecies multiplex strains whereas M23 (host of pXFAS01) and EB92.1 are strains of subspecies fastidiosa. It is noted that two distinct RepA sequences are associated with the Ann-1 genome. One RepA sequence from Ann-1 (designated Ann-1*) is identical to that encoded by pXF-RIV5, pXFAS01, and strain Dixon. The second RepA sequence from Ann-1 (designated Ann-1) shared only 53.2% amino acid sequence identity with RepA of pXF-RIV5 and pXFAS01 and clustered in a different clade with RepA from pRSC35 of Ralstonia solanacearum CMR15 as the most closely related homologue identified. As the Ann-1 strain genome sequence is known to be contaminated with a subspecies multiplex genome sequence [17], the simplest interpretation of these results is that RepA Ann-1* represents the multiplex contaminant and that RepA Ann-1 is the divergent homologue resident in the “true” Ann-1 genome of subspecies sandyi. Confirmation of this interpretation will require sequencing of genomes of additional strains of subspecies sandyi.

The presumptive origin of transfer (oriT) of pXF-RIV5 and pXFAS01 (nts 26869–26921) are similar to the experimentally
verified oriT [21] from two E. coli plasmids (pRP4 and pR751). The stem-loop inverted repeat structure that forms the TraK binding site of pRP4 oriT shares 89% nucleotide sequence identity (56 of 63 nts identical) with the oriT homologue of pXF-RIV5 and pXFAS01 (Figure 3A). Two nucleotide substitutions in the oriT sequences between the X. fastidiosa plasmids and pRP4 are compensatory changes in the stem, preserving secondary structure (Figure 3A); the other five substitutions are in loop regions. The oriT region from pR751 shares less nucleotide sequence identity (79.3%; 50 of 63 bp) with pXF-RIV5 and pXF-AS01 and has one additional stem base pair relative to pXF-RIV5, pXFAS01 and pRP4.

The presumptive vegetative origin of replication (oriV) of pXF-RIV5 and pXFAS01 (nts 34039–34504) consists of an approximately 300 bp A+T rich region followed by four tandem repeats (Figure 3B). While the combination of an A+T rich region followed by tandem repeats is present in oriV of many plasmids, more common arrangements have a larger number of repeats (16 to 20) with the 19 nt core of the repeat forming a perfect 10 base pair palindromes [23]. The 19 nt core repeat sequence in pXF-RIV5 is a partial palindromes with only 6 base pairs possible.

Discussion

As with many bacteria, X. fastidiosa harbors a variety of plasmids. Several have been characterized, including an IncP-1 plasmid [18], a small rolling-circle replicon [24], and pXF51 [22].
Here, we have described distinct attributes of pXF-RIV5 and pXFAS01, resident in strains representing multiple subspecies of *X. fastidiosa*. Given the probable conjugative abilities of these plasmids, it is possible that similar plasmids may be found in other as yet uncharacterized strains of *X. fastidiosa* or even in other bacterial species that share ecological niches with *X. fastidiosa*.

While many broad host range conjugative plasmids belong to the IncP-I incompatibility group and contain *tra* homologues for vegetative replication [2,25], replication of pXF-RIV5 and pXFAS01 uses a RepA-dependent process. Therefore, pXF-RIV5 and pXFAS01 are not readily assigned to a classic incompatibility group. Nonetheless, *tra* and *tb* genetic modules of pXF-RIV5 and pXFAS01 do cluster (Figure 2) with a subgroup typified by *E. coli* pRP4 and other IncP*α* group plasmids [2,4]. It is likely that an ancestral recombination event occurred between an IncP*α* group plasmid similar to pRP4 and a plasmid with RepA-dependent vegetative replication to create the plasmid backbone (e.g., modules controlling replication and conjugative transfer) found in pXF-RIV5 and pXFAS01.

In addition to backbone genetic modules, many conjugative plasmids contain accessory modules encoding host-beneficial functions [26]. These accessory modules are often located at the ends of the *tra* and *tb* modules and/or near transposon or resolvase genes. No accessory modules with identifiable function were encoded by pXF-RIV5 and pXFAS01, nor by any other characterized plasmid of *X. fastidiosa*. Interestingly, ORFs for five of seven hypothetical proteins encoded by pXF-RIV5 and pXFAS01 are located downstream of both *tra* and *tb* modules, and in close proximity to a resolvase homologue (Figure 1). Whether these ORFs of unknown function constitute an accessory module conferring selective advantage to *X. fastidiosa* remains to be determined.

pXF-RIV5 and pXFAS01 are the only characterized plasmids of *X. fastidiosa* encoding all known factors (e.g., a complete T4SS) required for transfer of DNA from recipient to donor cells via conjugation. DNA transfer among strains/subspecies of *X. fastidiosa* has occurred, as evidenced by massive introgression events leading to the origin of mulberry-infecting [27] and citrus/ coffee-infecting [28] strains of *X. fastidiosa*. In these cases, the mechanism of DNA transfer leading to homologous recombination appears to be transformation, as lengths of recombinant regions were generally small, albeit numerous. Indeed, recent evidence suggests that *X. fastidiosa* is naturally competent for acquisition of foreign DNA with intrinsic transformation efficiency higher than that of many other bacterial species [29]. Thus, the identification of pXF-RIV5 and pXFAS01, bearing all known hallmarks of a conjugative plasmid, suggests that *X. fastidiosa*, a plant pathogen of significant economic concern, also may transfer large segments of DNA via conjugation. Indeed, the presence of almost identical plasmids in two separate subspecies of *X. fastidiosa* (pXF-RIV5 in *multiplex* and pXFAS01 in *fastidiosa*) implies a recent inter-subspecies translocation event. Subspecies *multiplex* is relatively diverse and, therefore, likely has been present in the U. S. for a considerable time; subspecies *fastidiosa* in the U. S. exhibits limited genetic diversity. It has been hypothesized that all strains of subspecies *fastidiosa* in the U. S. are derived from a single introduction from Central America that occurred circa 1880 [8]. If so, the inter-subspecies plasmid translocation event responsible for host associations of pXF-RIV5 and pXFAS01 occurred more recently than 1880. Collectively, these observations suggest that the introduction of exotic subspecies of *X. fastidiosa* further complicates disease management, as newly introduced *X. fastidiosa* subspecies not only may cause disease(s) previously not known to occur in a region, they also provide a wealth of genetic diversity to be shared with endemic subspecies.

### Supporting Information

Table S1 GenBank protein ID numbers for all proteins appearing in phylogenetic trees (Figure 2).

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### Author Contributions

Conceived and designed the experiments: DCS. Performed the experiments: DCS. Analyzed the data: EER DCS. Wrote the paper: EER DCS.

### References

1. Dagan T, Arzy-Randrup Y, Martin W (2008) Modular networks and cumulative impact of lateral transfer in prokaryote genome evolution. Proceedings of the National Academy of Sciences of the United States of America 105: 10039–10044.

2. Alcántara-Martínez CE, Christie PJ (2009) Biological diversity of prokaryotic type IV secretion systems. Microbiol Mol Biol Rev 73: 775–808.

3. Christie PJ (2004) Type IV secretion: the Agrobacterium VirB/D4 and related conjugation systems. Biochim Biophys Acta 1694: 219–234.

4. Garcia-Bascuñán MP, Francia MV, de la Cruz F (2009) The diversity of conjugation systems. Biochim Biophys Acta 1694: 219–234.

5. Hopkins DL, Purcell AH (2003) Xylella fastidiosa: cause of Pierce’s disease of grapevine and other emergent diseases. Plant Disease 86: 1056–1066.

6. Yuan X, Morano L, Bromley R, Spring-pearson S, Stouthamer R, et al. (2010) Multiplex inter-subspecies translocation event. Subspecies *multiplex* is rela-

7. Alcántara-Martínez CE, Christie PJ (2009) Biological diversity of prokaryotic type IV secretion systems. Microbiol Mol Biol Rev 73: 775–808.

8. Alcántara-Martínez CE, Christie PJ (2009) Biological diversity of prokaryotic type IV secretion systems. Microbiol Mol Biol Rev 73: 775–808.

9. Melanson RA, Sanderlin RS, McTaggart AR, Ham JH (2012) A systematic study reveals that *Xylella fastidiosa* strains from pecan are part of *X. fastidiosa* subsp. *multiplex*. Plant Disease 96: 1123–1134.

10. Davis MJ, French WJ, Schaad NW (1981) Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Current Microbiology 6: 309–314.

11. Chen J, Groves R, Civerolo EL, Viveros M, Freeman M, et al. (2005) Two *Xylella fastidiosa* genotypes associated with almond leaf scorch disease on the same location in California. Phytopathology 95: 708–714.

12. Almeida RPP, Nascimento FE, Chau J, Prado SS, Tsai CW, et al. (2008) Genetic structure and biology of *Xylella fastidiosa* strains causing disease in citrus and coffee in Brazil. Applied and Environmental Microbiology 74: 5700–5701.

13. Simpson AG, Relnach F, Arruda P, Abreu FA, Accinio M, et al. (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*: The *Xylella fastidiosa* consortium of the organization for nucleotide sequencing and analysis, Sao Paulo, Brazil. Nature 406: 151–157.

14. Yu, Shao MA, De Oliveira MG, Monteiro-Vitorello GB, Miyaki CY, Furlan LR, et al. (2003) Comparative analyses of the complete genome sequences of Pierce’s disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. Journal of Bacteriology 185: 1018–1026.

15. Chen J, Xie G, Han S, Chertkov O, Sims D, et al. (2010) Whole genome sequences of two *Xylella fastidiosa* strains (M12 and M23) causing almond leaf scorch disease in California. Journal of Bacteriology 192: 4534.
16. Zhang S, Flores-Cruz Z, Kumar D, Chakrabarty P, Hopkins DL, et al. (2011) The Xylella fastidiosa biocontrol strain EB92-1 is very similar and syntenic to Pierce’s disease strains. Journal of Bacteriology 193: 5576–5577.

17. Nunney L, Elékikh S, Stouthamer R (2012) The importance of multilocus sequence typing: cautionary tales from the bacterium Xylella fastidiosa. Phytopathology 102: 456–460.

18. Stenger DC, Lee MW, Rogers EE, Chen J (2010) Plasmids of Xylella fastidiosa mulberry-infecting strains share extensive sequence identity and gene complement with pVEIS01 from the earthworm symbiont Verminephrobacter eiseniae. Physiological and Molecular Plant Pathology 74: 238–245.

19. Alscher SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389–3402.

20. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, et al. (2011) CDD: a conserved domain database for the functional annotation of proteins. Nucleic Acids Research 39: D225–229.

21. Pansgrau W, Zieglin G, Laitka E (1988) The origin of conjugative IncP plasmid transfer: interaction with plasmid-encoded products and the nucleotide sequence at the relaxation site. Biochimica et Biophysica Acta 951: 365–374.

22. Marques MV, da Silva AM, Gomes SL (2001) Genetic organization of plasmid pXF51 from the plant pathogen Xylella fastidiosa. Plasmid 45: 194–199.

23. Klockgether J, Reva O, Larbig K, Tsammler B (2003) Sequence Analysis of the Mobile Genome Island pKLCl02 of Pseudomonas aeruginosa C. Journal of Bacteriology 186: 518–534.

24. Guilhabert MR, Stewart VJ, Kirkpatrick BC (2006) Characterization of putative rolling-circle plasmids from the Gram-negative bacterium Xylella fastidiosa and their use as shuttle vectors. Plasmid 55: 70–80.

25. Stenger DC, Lee MW (2011) Phylogeny of replication initiator protein TrfA reveals a highly divergent clade of incompatibility group P1 plasmids. Appl Environ Microbiol 77: 2522–2528.

26. Van der Auwera GA, Krol JE, Suzuki H, Foster B, Van Houdt R, et al. (2009) Plasmids captured in C. metallidurans CH34: defining the PromA family of broad-host-range plasmids. Antonie Van Leeuwenhoek 96: 193–204.

27. Nunney L (2011) Homologous recomination and the invasion of a new plant host by the pathogenic bacterium, Xylella fastidiosa. Phytopathology 101: S130.

28. Nunney L, Yuan X, Bromley RE, Stouthamer R (2012) Detecting genetic introgression: high levels of intersubspecific recombination found in Xylella fastidiosa in Brazil. Applied and Environmental Microbiology 78: 4702–4714.

29. Kung SH, Almeida RP (2011) Natural competence and recombination in the plant pathogen Xylella fastidiosa. Appl Environ Microbiol 77: 5276–5284.