PLASMID ADDICTION AS A NOVEL APPROACH FOR DEVELOPING
A STABLE PLASMID VECTOR FOR XYLELLA FASTIDIOSA

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ABSTRACT
The lack of genetic and molecular tools that can be used to study the biology of Xylella fastidiosa (Xf) has made it extremely difficult for researchers to use genetic methods to establish the importance of a particular gene in the development of Pierce’s disease (PD). During the period under review, we have focused on developing plasmid vectors that are stably maintained in Xf throughout the infection cycle. To increase the stability of autonomously replicating plasmid vectors, we have introduced two different types of stabilizing elements into plasmid vectors pXF004, pRL1342, and pBRR1MCS-5. These stabilizing elements include the plasmid addiction systems, hok/sok and parDE, and the active partitioning system, parA. We are currently examining how addition of these stability elements affects plasmid maintenance both in vitro and en planta. We have also developed two integration vectors, which will allow researchers to introduce genes into two different nonessential regions of the Xf chromosome. We are currently evaluating the properties of the insertion strains en planta to make sure that these strains still exhibit the normal PD infectious cycle and have begun to examine the usefulness of both of these vectors for complementation analysis in Xf.

INTRODUCTION
Xylella fastidiosa (Xf) is the causative agent of numerous economically important plant diseases, including Pierce’s disease (PD) of grapevine (Hopkins and Purcell 2002). An important feature of the Xf infectious cycle is the ability of this pathogen to colonize and interact with the xylem tissue of plants and the foregut of insect vectors. Successful colonization of these hosts is dependent on the ability of Xf to subvert host defense networks and to acquire essential nutrients. The virulence determinants of Xf include proteins involved in adhesion and biofilm formation, extracellular enzymes, and toxins.

A fundamental strategy for investigating virulence in bacterial pathogens is to generate mutations and examine the impact of the absence of these gene products on pathogenicity. Over the past five years, many research laboratories have been generating insertion mutations in specific Xf genes and examining the impact of these mutations on the development of PD (Guilhabert and Kirkpatrick 2003, Feil et al. 2003, Reddy et al. 2004, Roper et al. 2004, Meng et al. 2005, Hernandez-Martinez et al. 2006). These studies have led to the identification of a number of mutant strains that do not show the normal PD infection cycle. Although the simplest explanation for these phenotypes is that the gene containing the insertion mutation is required for the normal development of PD, it is also possible that a secondary mutation was acquired during the construction of the original mutation and that the secondary mutation is responsible for the phenotype.

The classic approach to overcoming this type of objection is to perform complementation analysis. If the reintroduction of a wild-type copy of the gene into the mutant strain restores the normal PD infection cycle en planta, the researcher can conclude that the specific gene is important for the development of PD. One common strategy used to reintroduce the wild-type copy of a gene in Gram-negative bacteria involves the use of autonomously replicating plasmid vectors that carry antibiotic resistance genes and multiple cloning sites. Plasmid vectors with these features have been developed that are capable of replicating in Xf and that are stably maintained in the presence of antibiotics. These plasmids have been extremely useful for introducing genes into Xf and for in vitro complementation studies. Unfortunately, most of these plasmids are quickly lost from Xf in the absence of selective pressure, which limits the usefulness of these plasmids for studies en planta. Therefore, a major goal of this study is to develop a set of plasmid vectors that will allow researchers to perform complementation analysis en planta.

OBJECTIVES
1. Develop a stable plasmid vector for Xf
   a. Evaluate the potential of various plasmid addiction systems for ability to convert plasmids known to replicate in Xf into stable vectors.
   b. Evaluate how plasmid maintenance by Xf is affected by other genetic mechanisms known to affect plasmid stability, such as systems for multimer resolution and active partitioning systems.
2. Evaluate the stability of the newly development plasmid vectors when propagate in Xf en planta.
RESULTS
Develop a stable plasmid vector for Xf
A number of plasmid vectors have been developed that are capable of replicating in Xf (Qin and Hartung 2001, Vanamala et al. 2002, Guilhabert and Kirkpatrick 2003, Guilhabert et al. 2006). These plasmids have different origins of replication and belong to different incompatibility groups. However, in the absence of direct antibiotic selection, none of these plasmids are maintained in Xf. One set of plasmids that has been successfully introduced into Xf are derivatives of the IncQ broad host-range plasmid RSF1010. Three of the most useful RSF1010 derivatives are pXF004, pXF005 and pRL1342 (Guilhabert and Kirkpatrick 2003, P. Wolk, unpublished). Another plasmid that has been successfully introduced into Xf is pBBR1MCS-5 (Gabriel 2005). This plasmid contains the origin of replication from the broad host range plasmid pBBR1 from Bordetella bronchiseptica (Kovach et al. 1995). Significant to this proposed project is the fact that the only real problem with these vectors is that they are not maintained in Xf in the absence of antibiotic selection.

To circumvent this problem, we are evaluating whether stability can be achieved by introducing plasmid-addiction systems and plasmid partitioning elements into existing Xf vectors. A plasmid addiction system is a two-component stable toxin-unstable antitoxin system. Examples of these systems include the hok/sok system of plasmid R1 and the parDE system of plasmid RK2 (Zielenkiewicz and Ceglowski 2000). When a bacterium loses the plasmid harboring either of these addiction systems, the cured cells lose the ability to produce the unstable antitoxin and the lethal effect of the stable toxin quickly kills the bacterium. Thus, a plasmid addiction system guarantees that all living bacteria maintain the plasmid throughout infectious cycle. The plasmid partitioning system, parA, consists of a centromere-like region adjacent to two co-regulated genes that encode an ATPase and a centromere specific DNA-binding protein, which is required for faithful plasmid segregation at cell division (Gerdes et al. 2000). Addition of these systems to unstable plasmids has been demonstrated to increase plasmid stability in many Gram-negative bacteria (Zielenkiewicz and Ceglowski 2000, Gerdes et al. 2000).

The first set of plasmids we constructed contained both an RSF1010 replicon and an ori15A replicon. We found that the presence of the ori15A replicon made the pXF-derived vectors extremely unstable in E. coli. To overcome this problem, we generated a second set of pXF-derived plasmids that only carried the origin of replication from RSF1010 in combination with the hok/sok locus or other stability elements. The removal of ori15A greatly increased the stability of these plasmids in E. coli. Therefore, we decided to use this second set of plasmids in our Xf stability experiments. In addition to plasmids based on the pXF vectors, we have also generated a series of plasmids based on pRL1342, which carries a chloramphenicol resistance gene (P. Wolk, unpublished). Like pXF004 and pXF005, pRL1342 has the origin of replication from RSF1010 and is not stable in Xf in the absence of antibiotic selection. Since the pRL1342-derived series of plasmid vectors confer resistance to chloramphenicol, they might be particularly useful for genetic complementation analysis using Xf mutants that are resistant to kanamycin.

After each plasmid was constructed, we evaluated its long term inheritance properties. The individual plasmids, along with other representative control plasmids, were transferred into Xf by electroporation (Guilhabert and Kirkpatrick 2001). We then evaluated the effect of the addiction modules on plasmid stability in Xf using previously established methods (Guilhabert and Kirkpatrick 2003). As shown in Table 1, the parental plasmids pXF004 and pRL1342 were lost almost immediately. Thus, although plasmids containing hok/sok were not lost as quickly as the starting vectors, the increase in stability was not sufficient to make the series of vectors containing an RSF10101 replicon useful for complementation en planta.

Table 1. Successful plasmids in introducing into Xf.

<table>
<thead>
<tr>
<th>Replicon</th>
<th>Antibiotic marker</th>
<th>Addiction system</th>
<th>Partitioning system</th>
<th>Stability in Xf 10 generations</th>
<th>Stability in Xf 20 generations</th>
</tr>
</thead>
<tbody>
<tr>
<td>pXF004 a</td>
<td>RSF1010</td>
<td>Km</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAM18</td>
<td>RSF1010</td>
<td>Km</td>
<td>hok/sok, parDE</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pAM24</td>
<td>RSF1010</td>
<td>Km</td>
<td>hok/sok</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pRL1342 b</td>
<td>RSF1010</td>
<td>Cm</td>
<td>hok/sok</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pLLC005</td>
<td>RSF1010</td>
<td>Cm</td>
<td>hok/sok</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pAM59</td>
<td>RSF1010</td>
<td>Cm</td>
<td>hok/sok, parA</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pBBR1MCS-5 c</td>
<td>pBBR1</td>
<td>Gm</td>
<td>hok/sok, parA</td>
<td>in progress</td>
<td>in progress</td>
</tr>
<tr>
<td>pAM89</td>
<td>pBBR1</td>
<td>Gm</td>
<td>hok/sok</td>
<td>in progress</td>
<td>in progress</td>
</tr>
<tr>
<td>pAM90</td>
<td>pBBR1</td>
<td>Gm</td>
<td>parA</td>
<td>in progress</td>
<td>in progress</td>
</tr>
</tbody>
</table>

a Guilhabert and Kirkpatrick, 2003., b Peter C. Wolk (unpublished), c Kovach et al., 1995., d parA from pR1, e parA from Agrobacterium pTAR.
Another set of plasmids we constructed is based on plasmid pBBR1MCS-5 (Kovach et al. 1995). This plasmid was successfully used by Gabriel and his colleagues for complementation of the Xf tolC gene en planta (Gabriel 2005). Due to the severity of the tolC mutation on grapevine colonization, the presence of the wildtype tolC gene may be providing the selective pressure necessary for plasmid maintenance en planta. Since pBBR1MCS-5 is not stably maintained in many bacterial species in the absence of selective pressure, pBBR1MCS-5 in its current might not be as useful for complementation analysis of other Xf genes that have a less severe effect on Xf growth en planta. However, given the promising nature of the tolC studies, we decided to include pBBR1MCS-5 as one of the plasmids in our stability studies.

As shown in Table 1, we have successfully introduced pBBR1MCS-5 derivatives containing different stability elements into Xf and are currently examining their in vitro stability. We have also found that it is much easier to isolate pBBR1MCS-5 plasmid DNA from Xf than it is to isolate plasmid DNA containing RSF1010 origins of replication. This would suggest that pBBR1MCS-5-derived plasmids are present in Xf at a higher copy number than RSF1010–derived plasmids. It is our hope that the higher copy number of the pBBR1MCS-5 derivatives in Xf in combination with the increased stability provided by hok/sok will result in a plasmid that can be stably maintained in Xf in the absence of selective pressure.

**Development of integration vectors for complementation analysis en planta**

Another method commonly used for complementation in Gram negative bacteria is to use plasmid vectors that are capable of autonomous replication in E. coli, but are unable to replicate in host bacteria. In most integration vectors, the gene of interest and an antibiotic cassette are flanked by DNA sequences from a nonessential region of the bacterial chromosome. Recombination between the homologous regions of the plasmid and the bacterial chromosome results in the integration of the gene of interest and antibiotic resistance gene into the chromosome at the nonessential region. The antibiotic resistance cassette is included on these vectors to facilitate the identification of strains containing the integrated vector. Although it is not easy to resolate the introduced genetic markers from the bacteria following integration, insertion plasmids are extremely useful for certain types of genetic studies, particularly complementation analysis. One major advantage of using this type of vector is that once the gene of interest is integrated into the host bacterial chromosome, it will be maintained without antibiotics selection.

![Primer pairs](image1.png)

**Figure 2A.** Strategy of integration

<table>
<thead>
<tr>
<th>Lanes</th>
<th>Wild type</th>
<th>Integration strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 3, 5</td>
<td>Integration strain</td>
<td>Integration strain</td>
</tr>
<tr>
<td>2, 4, 6</td>
<td>Integration strain</td>
<td>Integration strain</td>
</tr>
</tbody>
</table>

The first step in developing integration vectors for use in Xf was to identify regions of the chromosome that could serve as targets for the integration event. One important characteristic of the target is that insertions into this location do not impact Xf physiology or its ability to cause PD. We have selected two places on Xf chromosome as potential targets of our integration vectors. The first integration target is PD0939, which encodes a phage-related protein. PD0939 was chosen because transposon insertions into this gene do not impact the development of PD in grapevine (Guilhabert and Kirkpatrick, personal communication). The integration vector that targets the PD0939 gene confers chloramphenicol resistance and is named pLLC021. The second target is the intergenic region between PD0702 and PD0703. Based on the genomic sequence of Xf-PD, both of these genes are predicted to contain frameshift mutations (Van Sluys et al. 2003). The integration vector that targets this intergenic region also confers chloramphenicol resistance and is named pLLC018. The relative orientation of PD0702 and PD0703 with respect to the targeted intergenic region is shown in Figure 2A.

The next step was to introduce our integration vectors into Xf and to select for chloramphenicol resistant transformants. Because these vectors are unable to replicate in Xf, the chloramphenicol resistant transformants must have arisen as the result of a recombination event(s) between the integration vector and the Xf chromosome. An example of the recombination events that led to one of these chloramphenicol resistant transformants is illustrated in Figure 2A. The PCR experiment, which confirmed that the recombination event had occurred at the appropriate chromosomal location for this transformant are shown in Figure 2B.
Finally, we examined the impact of insertions at PD0939 and within the intergenic region between PD0702 and PD0703 on the growth phenotypes of the two strains. These experiments indicated that the insertion containing strains have growth properties that are similar to a wildtype strain in both liquid culture and on solid medium. They also exhibit normal biofilm formation. We are currently evaluating the properties of the insertion strains en planta to make sure that these strains still exhibit the normal PD infectious cycle and have begun to examine the usefulness of both of these vectors for complementation analysis in Xf.

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FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce’s Disease and Glassy-winged Sharpshooter Board, and by the University of California Pierce’s Disease Grant Program.
GREENHOUSE RESPONSES OF VITIS VINIFERA ‘CHARDONNAY,’ AMBROSIA TRIFIDA VAR. TEXANA, AND IVA ANNUA WITH XYLELLA FASTIDIOSA ISOLATES FROM TEXAS HOST PLANTS

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ABSTRACT
Sixty isolates of Xylella fastidiosa (Xf) from species or cultivars of Vitaceae (33), Asteraceae (23), Platanaceae (2), Moraceae (1), and Sapindaceae (1) were twice inoculated (8May to 1Jun06, seven greenhouse experiments) into two adjacent internodes of own-rooted ‘Chardonnay’ grape. Each RCBD five-replication experiment had eight to twelve treatments that included at least one winegrape isolate and one SCP buffer check. Leaf scorch symptoms on 8Aug were compared with Xf-serology (DAS-ELISA OD and proportion OD>0.3) on petioles collected 8Aug to 1Sep06. Some grape isolates had consistently caused Pierce’s disease (PD) symptoms at 10 to 12 weeks after inoculation. A few ‘Chardonnay’ plants inoculated with certain Xf isolates from Vitis vinifera, Helianthus annuus, Iva annua, Ambrosia trifida var. texana, and Platanus occidentalis had mild PD symptoms and positive ELISA reactions. Some isolates did not cause symptoms. Evaluations will be repeated in late 2006. Twenty-one Xf isolates from Vitaceae (7), Asteraceae (12), Platanaceae (1), and Moraceae (1) were twice inoculated (10Jul to 20Jul06, one greenhouse experiments per host) into two adjacent internodes of A. trifida var. texana or I. annua grown from seed. Each RCBD six-replication experiment had twenty-three treatments that included six isolates from Vitis spp. and two SCP buffer checks. Symptoms were not detected. Two internode samples (inoculated zone, one internode above inoculation zone) collected 13,21,25,26Sep06 (9 to 10 weeks after inoculation) as plants senesced were assayed using Xf-serology (DAS-ELISA OD and proportion OD>0.3). One of six isolates from Vitis, one isolate from Platanas, and 12 isolates from spp. in Asteraceae colonized A. trifida var. texana. Three of six isolates from Vitis, one isolate from Morus, and 11 isolates from spp. in Asteraceae colonized I. annua.

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