

PLASMID ADDICTION AS A NOVEL APPROACH TO DEVELOP A STABLE PLASMID VECTOR FOR *XYLELLA FASTIDIOSA*

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ABSTRACT

Xylella fastidiosa (*Xf*) is a Gram-negative, endophytic bacterium, which is the causative agent of Pierce's disease of grapevine (PD). Current approaches to understanding the progression of PD are limited by the lack of genetic and molecular tools that can be used to perform complementation analysis, an important step for establishing the importance of a particular gene in *Xf* virulence. To overcome this problem, we have developed integration vectors, which will allow researchers to introduce genes into two different nonessential regions of the *Xf* chromosome. The first set of vectors, the pAX1 series, contains an antibiotic resistant gene and a multiple cloning site (MCS) flanked by sequences homologous to the intergenic region between two divergently transcribed genes PD0702 and PD0703. Each vector in pAX1 series carries a different antibiotic resistance marker: pAX1Cm (chloramphenicol), pAX1Km (kanamycin), and pAX1Gm (gentamicin). We have introduced all three vectors into *Xf* and established that the antibiotic-containing cassette is integrated into the desired location in the *Xf* chromosome. We have also shown that the insertion of the antibiotic cassette between PD0702 and PD0703 does not alter the growth properties of *Xf in vitro*. Finally, we have cloned a wild-type copy of the *Xf* catalase gene into the MCS of pAX1Cm and introduced the resulting construct into a catalase-defective *Xf* mutant. The complemented strain showed a similar level of resistance to hydrogen peroxide as the wild type strain establishing the usefulness of our insertion vector for complementation analysis in *Xf*. Recently, we have constructed an additional set of vectors, the pAX2 series, which results in the insertion of the antibiotic resistant cassettes into the intergenic region between PD1160 and PD1161. Experiments are currently underway to test the usefulness of the pAX2 vectors for complementation analysis in *Xf*.

INTRODUCTION

The causative agent of Pierce's disease (PD) is the Gram-negative bacterium *Xylella fastidiosa* (*Xf*). *Xf* is highly specialized and is capable of multiplying in both the foregut of xylem-feeding insects, such as the glassy-winged sharpshooter (GWSS) and in the xylem system of susceptible host plants (Hopkins and Purcell 2002). 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. As with many bacterial pathogens, the construction of directed gene deletion mutants has been crucial for identifying possible roles for specific *Xf* genes in the development of PD. 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 the phenotypes of these mutants is that the disrupted gene is required for the normal development of PD, it is also possible that the gene disruption is affecting the expression of neighboring genes through its impact on operon structure or 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 for conclusively establishing that a specific gene is responsible for a specific physiological and virulence function is to perform complementation analysis. Thus, 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. Here, we report our construction of two series of integration vectors (the pAX1 series and pAX2 series). These vectors allow the insertion of a gene of interest and an antibiotic resistance cassette into specific nonessential regions of the *Xf* chromosome. Complementation using these vectors will provide researchers with a clearer interpretation of the precise role of specific genes in the PD infection cycle by eliminating issues concerning plasmid copy number, polar effects on operons, secondary mutations, and plasmid stability.

OBJECTIVES

Specific Objective 1: Develop a stable plasmid vector for *Xf*

- 1A. Evaluate the potential of various plasmid addiction systems for ability to convert plasmids known to replicate in *Xf* into stable vectors.
- 1B. 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.

Specific Objective 2: Evaluate the stability of the newly development plasmid vectors when propagate in *Xf en planta*.

RESULTS

To better understand how *Xf* survives in and interacts with its hosts, many research laboratories have been working to identify genes important for virulence and nutrient acquisition. However, rapid progress in this area is affected by the lack of genetic and molecular tools necessary to investigate the contribution of *Xf* genes to the infection process. One extremely important tool that is needed to advance these studies is molecular tools that will allow genes of interest to be stably maintained by *Xf* throughout the infectious cycle. The primary goal of this project was to develop this type of molecular tool. Our early studies focused on examining the impact of various stability elements on the maintenance of autonomously replicating plasmids in *Xf* in the absence of antibiotic selection. We found that addition of the plasmid addition module *hok/sok* resulted in a slight increase in plasmid stability *in vitro*. However, plasmids containing *hok/sok* were eventually lost from *Xf* in the absence of selective pressure, eliminating the usefulness of these plasmids for studies *en planta*. During the period under review, we have focused on developing a chromosomal based complementation system, which relies on vectors that allow the integration of genes into nonessential regions of the *Xf* chromosome. A major advantage of integration vectors is that the introduced genes are stably maintained as part of the bacterial chromosome in the absence of antibiotic selection. Here we report our progress on the development of these vectors.

Development of integration vectors for complementation analysis

In many Gram-negative bacteria, complementation analysis is performed using plasmid vectors that are capable of autonomous replication in *E. coli*, but are unable to replicate in the host bacteria. These integration vectors normally contain a multiple cloning site for inserting the gene of interest and an antibiotic cassette that are flanked by DNA sequences from a nonessential region of the bacterial chromosome. The antibiotic resistance cassette is included on these vectors to facilitate the identification of strains containing the integrated DNA fragment. 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. 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.

The first set of integration vectors we developed is the pAX1 series. The pAX1 plasmids contain an antibiotic resistant gene and a multiple cloning site (MCS) flanked by sequences homologous to the intergenic region between two divergently transcribed genes PD0702 and PD0703. Based on the genomic sequence of *Xf*-PD, these genes are divergently transcribed and both genes are predicted to contain multiple frameshift mutations (Van Sluys *et al.* 2003). Although they target the same region of the *Xf* chromosome, each vector in the pAX1 series carries a different antibiotic resistance marker: pAX1Cm (chloramphenicol), pAX1Km (kanamycin), and pAX1Gm (gentamicin). These vectors are presented in Figure 1.

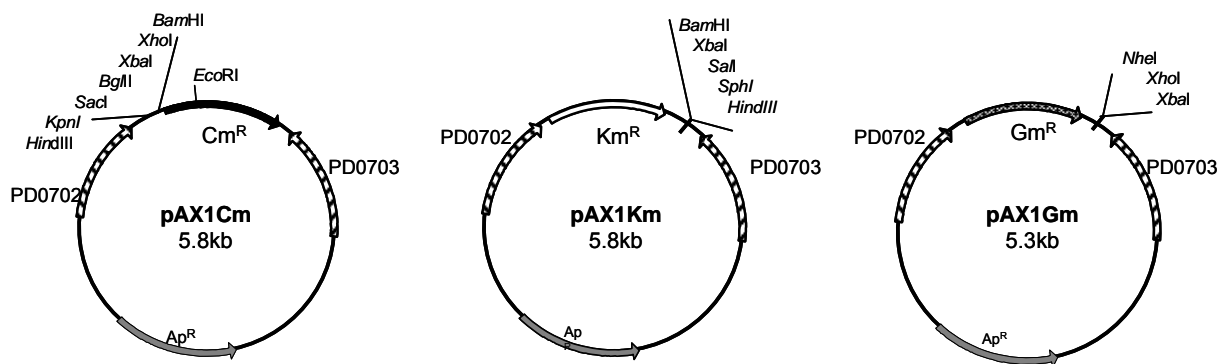


Figure 1. Restriction maps of pAX1 series of integration vectors

We have introduced all three pAX1 vectors into *Xf* and selected for antibiotic resistant transformants. Because these vectors are unable to replicate in *Xf*, the antibiotic resistant transformants must have arisen as the result of recombination between the integration vector and the *Xf* chromosome. We were able to confirm that the recombination event had occurred at the appropriate chromosomal location by PCR. We then examined the impact of the insertion in the PD702/703 intergenic region on the growth phenotypes of the resulting three strains *in vitro*. The properties of the three strains are presented in Table 1. 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. These strains 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.

Table 1. The integration strains exhibit a wild type phenotype.

Strain	Integration vector	Resistance marker	<i>In vitro</i>
Wildtype Temecula	---	---	Slow growth, biofilm
TAM22	pAX1Cm	Cm	Slow growth, biofilm
TAM91	pAX1Km	Km	Slow growth, biofilm
TAM105	pAX1Gm	Gm	Slow growth, biofilm

We also examined the usefulness of the pAX1 vectors for complementation analysis in *Xf*. For this analysis, we first generated a null mutation by gene disruption in the *cpeB* gene, which encodes catalase. The resulting catalase-defective *Xf* mutant ($\Delta cpeB$) exhibits greater sensitivity to hydrogen peroxide than the wild-type Temecula strain. We then cloned a wild-type copy of the *Xf* catalase gene into the MCS of pAX1Cm and introduced the resulting construct into the $\Delta cpeB$ mutant. As shown in Figure 2, the complemented strain showed a similar level of resistance to hydrogen peroxide as a wild type strain establishing the usefulness of our insertion vectors for complementation analysis in *Xf*.

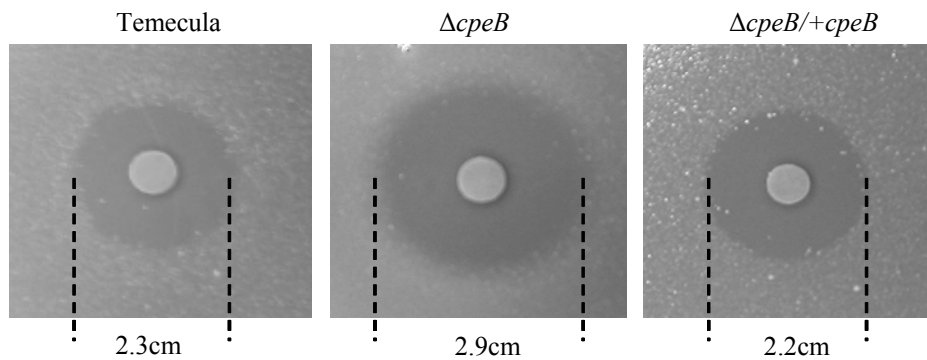


Figure 2. Complementation analysis of a catalase null mutation
P-values: Temecula vs. $\Delta cpeB$ ($P<0.01$); Temecula vs. $\Delta cpeB/+cpeB$ ($P>0.3$)
 $\Delta cpeB/+cpeB$ vs. $\Delta cpeB$ ($P<0.04$)

To facilitate complementation analysis in strains containing double mutations, we are also developing a new series of integration vectors that target a different region in the *Xf* chromosome. These plasmids (the pAX2 series) contain an antibiotic resistant gene and a multiple cloning site (MCS) flanked by sequences homologous to the intergenic region between PD1160 and PD1161. Based on the genomic sequence of *Xf-PD*, both of these genes are predicted to contain frameshift mutations (Van Sluys *et al.* 2003). Like the pAX1 series, each pAX2 vector carries a different antibiotic resistance marker: pAX2Cm (chloramphenicol), pAX2Km (kanamycin), and pAX2Gm (gentamicin). Experiments are currently underway to examine the *in vitro* properties of strains carrying an insertion in the PD1160/1161 intergenic region and to test the usefulness of this set of vectors for complementation analysis in *Xf*.

REFERENCES

- Hopkins, D. L., and A. H. Purcell. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. Plant Dis. 86:1056-1066.
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