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

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ABSTRACT
Understanding the progression of Pierce's Disease (PD) has been limited by the lack of genetic and molecular tools that can be used to study the biology of *Xylella fastidiosa* (*Xf*). Although a number of potential plasmid vectors have been developed that are capable of replicating in *Xf*, none of these plasmids are stably maintained in *Xf* without antibiotic selection. To solve this problem, we have introduced two different types of stabilizing elements into the *Xf* plasmid vectors pXF004 and pXF005. These stabilizing elements include the plasmid addiction systems, *hok/sok* and *parDE*, and the active partitioning system, *parA*. Our preliminary studies indicate that the addition of the *hok/sok* addiction module to plasmid, pXF004, greatly increases its stability in *Xf*.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a fastidious, xylem-limited, Gram-negative bacterium, which is the causative agent of numerous economically important plant diseases (Hopkins and Purcell 2002). Diseases that are important to the California agricultural economy include PD of grapevine, almond leaf scorch, alfalfa dwarf, and oleander leaf scorch. 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 (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.

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. In recent years, a number of plasmid vectors have been developed that are capable of replicating in *Xf*. These plasmids have different origins of replication and belong to different incompatibility groups (Qin and Hartung 2001, Vanamala, A. *et al.* 2002, Guilhabert and Kirkpatrick 2003, Guilhabert *et al.* 2005) However, in the absence of antibiotic selection, none of these plasmids are stably maintained in *Xf*. Therefore, one extremely important tool that is needed to advance studies investigating *Xf* virulence is a plasmid that is stably maintained by *Xf* throughout the infectious cycle.

The goal of our project is to develop a plasmid that is stably maintained in *Xf* both *in vitro* and *en planta* in the absence of antibiotic selection. In our initial studies, we constructed these stable plasmids using the pXF plasmids, pXF004 and pXF005 (Guilhabert and Kirkpatrick 2003). These pXF plasmids contain the replicon from RSF1010 and confer resistance to kanamycin. They also are autonomously maintained with antibiotic selection and structurally unchanged by propagation in *Xf*. In fact, the only real problem with these vectors is that they are not maintained in *Xf* in 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 these existing *Xf* vectors. During the past year, we focused on plasmid addiction modules, which have been shown to dramatically increase plasmid stability in many Gram-negative bacteria (Engelbaerg-Kulka and Glaser 1999, Zielenkiewicz and Ceglowski 2001, Hayers 2003). 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 (Burkhardt *et al.* 1979, Saurugger *et al.* 1986, Gerdes 1988). When a bacterium loses the plasmid harboring either of these addiction systems, the cured cells loose 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. Recently, we have initiated studies to examine whether or not active partitioning systems enhance plasmid maintenance. Specifically, we plan to test the plasmid partitioning system, *parA*, which consist 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 this system to unstable plasmids has been demonstrated to increase plasmid stability in many Gram-negative bacteria (Zielenkiewicz and Ceglowski 2001).

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

During the past year, a series of 22 stability plasmids were constructed. This initial set of plasmids contains the *hok/sok* locus or other elements in combination with origins of replication from RSF1010 (derived from pXF vectors-Guilhabert and Kirkpatrick 2003) and *ori*15A (derived from pGEN vectors-Galen *et al.* 1997). The stability of each of these plasmids was then examined in *E. coli* to provide a preliminary evaluation of these vectors' long term inheritance properties. Significantly, twelve of these new plasmids display increased stability in *E. coli* relative to the pXF plasmid controls. Each of these plasmids, along with other representative control plasmids, is now being transferred into *Xf* using published protocols (Guilhabert and Kirkpatrick 2003). Once a plasmid has been introduced into *Xf*, we will then evaluate the effect of these addiction modules on plasmid stability in *Xf* using previously established methods (Guilhabert and Kirkpatrick 2003). The results for one series of constructs are shown in Table 1. The pXF plasmids were not stable in *Xf* after 1 passage (1-week-incubation without antibiotics). The instability we observed for the pXF plasmids is similar to that previously reported by Guilhabert and Kirkpatrick (2003). In contrast, plasmid pAM24, which is a derivative of pXF004 and carries the *hok/sok* module, is very stable in *Xf* after one generation (Table 1). Although our analysis of this plasmid is still at an earlier stage, we speculate that the addition of the *hok/sok* system to other *Xf* vectors will improve their stability in *Xf*.

Table 1. Series of stable plasmids based on RSF1010 plasmid.

	Antibiotic Marker	Addiction system	Partitioning system	GFP[c]	% of plasmid retention in *Xf* in generation 1
pXF004 [a]	Kan	-	-	-	40 [b]
pXF005 [a]	Kan	-	-	-	39
pAM24	Kan	*hok/sok*	-	*gfp*	~100
pAM18	Kan	*hok/sok, parDE*	-	-	In progress
pAM27	Kan	*hok/sok*	*parA*	*gfp*	In progress

[a] Plasmids were developed by Guilhabert and Kirkpatrick 2003
[b] Data from Guilhabert and Kirkpatrick 2003
[c] GFP= green fluorescent protein

Although our studies with the *hok/sok* system are promising, it is worth pointing out that the *hok/sok* system by itself is not capable of completely stabilizing plasmids in other Gram-negative bacteria under all conditions. Interestingly, placing more than one type of addiction system onto the same plasmid has been found to provide an additive effect on plasmid stability (Pecota *et al.* 1997). Based on this observation, we constructed the plasmid pAM18, which carries both the *hok/sok* system and the *parDE* system (Table 1). Another strategy for increasing plasmid stability is to introduce both a plasmid addiction system (*hok/sok*) and an active partitioning system (*parA*) in a plasmid (Galen *et al.* 1999). To examine whether or not these stabilizing elements will increase plasmid stability in *Xf*, we generated pAM27, which carries both the *hok/sok* system and the *parA* system (Table 1). Both pAM18 and pAM27 are stably maintained in *E. coli*. The next step will be to introduce these plasmids into *Xf* and then evaluate whether the presence of multiple stability elements on the same plasmid will result in further increases in plasmid stability in *Xf.*

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 (Table 2). Like pXF004 and pXF005, pRL1342 is not stable in *Xf* in the absence of antibiotic selection. However, we have recently generated derivatives of pRL1342 that carry either *hok/sok* alone or *hok/sok* in combination with *parA*. Since this series of plasmid vectors confer resistance to chloramphenicol, they will be particularly useful for genetic complementation analysis using *Xf* mutants that are resistant to kanamycin.

Table 2. Development of alternative RSF1010-based vectors

	Antibiotic Marker	Addiction system	Partitioning system	GFP [b]
pRL1342 [a]	Cam	-	-	-
pLLC005	Cam	*hok/sok*	-	*gfp*
pAM59	Cam	*hok/sok*	*parA*	*gfp*

[a] The plasmid was developed by Peter C. Wolk, based on pMMB66EH (Furste *et al.* 1986) with chloramphenicol resistance. DNA sequence is available at NCBI #AF403427
[b] GFP= green fluorescent protein

Finally, to facilitate future stability studies *en planta*, we have also included in the new stability plasmids a copy of *gfp*, which encodes a bacterial optimized green fluorescent protein (GFP) (Tables 1 and 2). Although this phenotypic marker does not aid in plasmid stability, it provides a convenient marker for tagging individual cells and provides an alternative tool for researchers to track the location of *Xf* during an infection. GFP has been used by others, such as the Lindow lab at UC-Berkeley, for tracking *Xf* during plant infections (Newman *et al.* 2003) and its inclusion in this new generation of stable *Xf* plasmids will expand the usefulness of this valuable molecular biology tool.

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