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

Project Leaders:
Glenn M. Young
Dept. of Food Science and Technology
University of California
Davis, CA 95616

Michele Igo
Section of Microbiology
University of California
Davis, CA 95616

Cooperators:
Ayumi Matsumoto
Dept. of Food Science and Technology
University of California
Davis, CA 95616

Bruce Kirkpatrick
Dept. of Plant Pathology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted October 1, 2005 to September 30, 2006.

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 pBBR1MCS-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 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. 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 *ori*15A replicon We found that the presence of the *ori*15A 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 *ori*15A 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. In contrast, the new plasmids, which carried the *hok/sok* stability element, were still present after generation 10. This suggests that the presence of *hok/sok* is having an impact on plasmid maintenance. However, the new plasmid vectors were eventually lost in the absence of selective pressure by generation 20. 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*.

	Replicon	Antibiotic marker	Addiction system	Partitioning system	Stability in *Xf*	
					10 generations	20 generations
pXF004 [a]	RSF1010	Km	-	-	-	-
pAM18	RSF1010	Km	*hok/sok, parDE*	-	+	-
pAM24	RSF1010	Km	*hok/sok*	-	+	-
pRL1342 [b]	RSF1010	Cm	-	-	-	-
pLLC005	RSF1010	Cm	*hok/sok*	-	+	-
pAM59	RSF1010	Cm	*hok/sok*	*parA* [d]	+	-
pBBR1MCS-5 [c]	pBBR1	Gm	-	-	in progress	in progress
pAM89	pBBR1	Gm	*hok/sok*	*parA* [d]	in progress	in progress
pAM90	pBBR1	Gm	-	*parA* [e]	in progress	in progress

[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 reisolate 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.

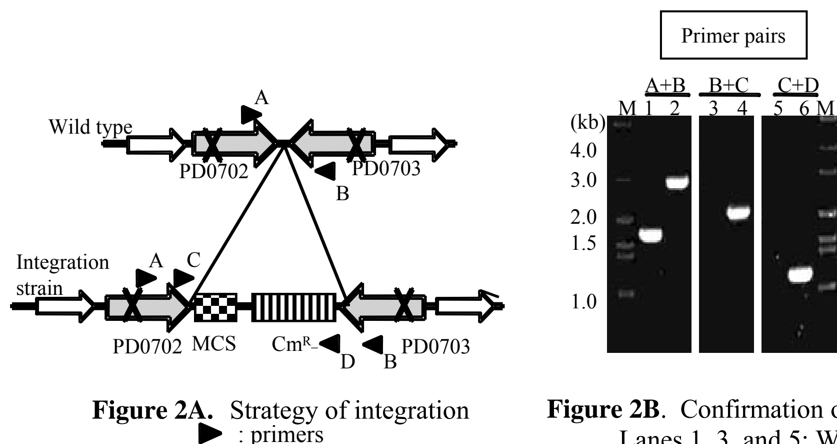

Figure 2A. Strategy of integration
▶ : primers

Figure 2B. Confirmation of integration
Lanes 1, 3, and 5: Wild type
Lanes 2, 4, and 6: Integration strain

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*.

REFERENCES

Feil, H. W. S. Feil, J. C. Detter, A. H. Purcell, and S. E. Lindow. 2003. Site-directed disruption of the fimA and fimF fimbrial Genes of *Xylella fastidiosa*. Phytopathology 93:675-682.

Gabriel, D. W. 2005. Role of type I secretion in Pierce's disease, p. 158-161. In M. Athar Tariq et al. (eds.), Proceedings of the 2005 Pierce's Disease Research Symposium. 5-7 December 2005, San Diego, CA.

Galen, J. E. et al. 1999. Optimization of plasmid maintenance in the attenuated live vector vaccine strain *Salmonella typhi* CVD 908-htrA. Infect. Immun. 67:6424-6433.

Gerdes, K., J. Moller-Jensen, and R. Bugge Jensen. 2000. Plasmid and chromosome partitioning: surprises from phylogeny. Mol. Microbiol. 37:455-466.

Guilhabert, M. R., L. M. Hoffman, D. A. Mills, and B. C. Kirkpatrick. 2001. Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. Mol. Plant-Microbe Interact. 14:701-706.

Guilhabert, M. R., and B. C. Kirkpatrick. 2003. Transformation of *Xylella fastidiosa* with broad host range RSF1010 derivative plasmids. Mol. Plant Pathol. 4:279-285.

Guilhabert, M. R., V. J. Stewart, and B. C. Kirkpatrick. 2006. Characterization of putative Rolling-Circle plasmids from the Gram-negative bacterium *Xylella fastidiosa* and their use as shuttle vectors. Plasmid 55:70-80.

Hernandez-Martinez, R., K. C. Dumenyo, and D. A. Cooksey. 2006. Site-directed mutagenesis of acvB gene in a Pierce's disease strain of *Xylella fastidiosa*. Phytopathology 96:S47.

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.

Kovach, M. E. et al. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes.

Gene. 166:175-176.

Meng Y, Y. Li, C. D. Galvani, G. Hao, J. N. Turner, T. J. Burr, and H. C. Hoch. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. J. Bacteriol. 187:5560-5567.

Qin, X., and J. S. Hartung. 2001. Construction of a shuttle vector and transformation of *Xylella fastidiosa* with plasmid DNA. Curr. Microbiol. 43:158-162.

Reddy, J. D., D. L. Hopkins, and D. W. Gabriel. 2004. Role of type I secretion in Pierce's disease.Phytopathology94:S87.

Roper, M. C., L. C. Greve, J. Labavitch, and B. C. Kirkpatrick. 2004. Construction and characterization of a polygalacturonase mutant of *Xylella fastidiosa*. Phytopathology 94:S89.

Saurugger, P., O. Hrabak, H. Schwab, and R. M. Lafferty. 1986. Mapping and cloning of the par-region of broad-host range plasmid RP4. J. Biotechnol. 4:333-343.

Van Sluys, M. A. et al. 2003. Comparative analysis of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. J. Bacteriol. 185:1018-1026.

Vanamala, A., R. Harakava, and D. W. Gabriel. 2002. Transformation of *Xylella fastidiosa* using replicative shuttle vector pUFR047. Phytopathology 92:S83.

Zielenkiewicz, U., and P. Ceglowski. 2001. Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems. Acta Biochimica Polonica 48:1003-1023.

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

Project Leaders:

Mark C. Black
Dept. of Plant Pathol. & Microbiology
Texas A&M University
Agric. Research and Extension Center
Uvalde, TX 78802
m-black@tamu.edu

James S. Kamas
Dept. of Horticultural Sciences
Texas A&M University
Fredericksburg, TX
j-kamas@tamu.edu

Alfred M. Sanchez
Dept. of Plant Pathol. & Microbiology
Texas A&M University
Agric. Research and Extension Center
Uvalde, TX 78802

Penny S. Adams
Department of Horticultural Sciences
Texas A&M University
Fredericksburg, TX

James L. Davis
Dept. of Plant Pathol. & Microbiology
Texas A&M University
Agric. Research and Extension Center
Uvalde, TX 78802

Reporting Period: The results reported here are from work conducted April 2006 to September 2006.

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.*

FUNDING AGENCIES

Funding for this project was provided by a cooperative agreement between the USDA Animal and Plant Health Inspection Service and Texas A&M University.