AN OUTER MEMBRANE PROTEIN A FAMILY OUTER MEMBRANE PROTEIN IS REQUIRED FOR DISEASE SYMPTOM DEVELOPMENT AND COLONIZATION OF SUGARCANE BY *XANTHOMONAS ALBILINEANS*

By

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This work is dedicated to my family.

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LIST OF ABBREVIATIONS

bp	base pair
IM	inner membrane
MW	modified Wilbrink's
OM	outer membrane
OMP	outer membrane protein
ORF	open reading frame
SD	Shine-Dalgarno
SDS	sodium-dodecyl-sulfate
SLSD	Leaf Scald Disease
Tn*5*	prokaryotic transposon Tn*5*
Xa	*Xanthonomas albilineans*
Xcc	*Xanthomonas campestris* pv. *campestris*
Xf	*Xylella fastidiosa*

Abstract of Thesis Presented to the Graduate School
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AN OUTER MEMBRANE PROTEIN A FAMILY OUTER MEMBRANE PROTEIN IS REQUIRED FOR DISEASE SYMPTOM DEVELOPMENT AND COLONIZATION OF SUGARCANE BY XANTHOMONAS ALBILINEANS

By

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Xanthomonas albilineans (Xa) is a systemic, xylem-invading pathogen that causes sugarcane leaf scald. Xa produces albicidin, an antibiotic and phytotoxin which blocks chloroplast differentiation, causing the foliar symptoms of leaf scald, including long, thin chlorotic “pencil-line” streaks that are diagnostic of the disease. Albicidin is the only known pathogenicity factor in Xa. In an attempt to identify additional pathogenicity factors, 1,216 independent Tn*5* insertions in Xa strain XaFL07-1 were screened for reduced pathogenic symptoms and reduced capacity to multiply in stalks by inoculation onto sugarcane cultivar CP80-1743. Following such screening, 61 mutants were recovered and each insertion site determined by sequencing. Five (8.2%) of the Tn*5* insertions were found in *XaompA1* (XALc_0557), which is predicted to encode an OmpA family outer membrane protein. One mutant, M768, was able to consistently colonize stalk tissue but at severely reduced levels. Additional phenotypic studies showed that these mutants 1) produced albicidin, 2) were less motile (except M768), 3) were unable to grow in the presence of SDS (except M768) and 4) were slower growing

than the wild type Xa *in vitro.* The divergent phenotype of M768 may be due to a translational fusion of the Tn*5* insertion with an intact C-terminal domain of XaOmpA1.

Three different complementation constructs were created to verify the pleiotropic phenotypes of *ompA* mutants were due to Tn*5* insertional inactivation of *XaompA1*. One construct, designed to recreate the translational fusion identified in M768, restored SDS resistance and symptoms *in planta* to M1152, but did not restore motility. A second construct, carrying the entire *XaompA1* gene, fully complemented M468, including restoring SDS resistance, motility and symptoms *in planta*. A third construct, pRK-mopB, carrying an *ompA* homolog from *X.campestris* pv. *campestris* was unstable in Xa. However, in plate assays for SDS resistance and motility, where antibiotic selection could be used to maintain the plasmid, limited complementation was observed. Taken together, this work demonstrates that *ompA* is required for both disease symptom development and colonization of sugarcane by Xa.

CHAPTER 1
LITERATURE REVIEW

Introduction

The search for genes affecting pathogenicity has been accelerated by the decreased cost and increased efficiency of automated DNA sequencing. When the entire genomic DNA sequence of an organism is known, it becomes possible to apply reverse genetics to study the biological function of specific genes of potential interest, identified by homology searches and in some cases position in the genome. Investigators can create mutations in the gene of interest, study the corresponding phenotypes of the mutant, and complement the mutation to verify restoration of the wild-type phenotype. Reverse genetics is a much faster approach to understanding the function of a specific gene than forward genetics, the classic approach to functional genomics. With forward genetics, a mutagen is applied, mutants are screened for a specific phenotype, and the mutation is mapped within the chromosome. Regardless of the approach, the availability of sequence information has greatly facilitated biological research.

Pieretti, *et al.* (2009) published the complete genome sequence of *Xanthomonas albilineans*, the causal agent of sugarcane leaf scald. The only pathogenicity factor known prior to sequencing and annotation of the genome was albicidin, a phytotoxin and antibiotic produced only in this bacterial species. Albicidin is a moderately well characterized DNA gyrase inhibitor that blocks chloroplast differentiation and causes leaf scald, which includes pencil-line symptoms in sugarcane leaves that are diagnostic of the disease (Birch and Patil 1985 and 1987; Hashimi *et al.* 2007). At least 22 genes in three regions of the genome were found to be needed for *X. albilineans* to produce

albicidin (Vivien *et al.* 2005). As mutational studies of the albicidin genes progressed, it became apparent that albicidin was not the only pathogenicity factor contributing to symptom development and colonization of the sugarcane plant (Rott *et al.* 1996 and 2010). Understanding the genetics behind the virulence and pathogenicity of an organism is useful for understanding host-pathogen interactions and for developing better tools for disease control.

Sugarcane Leaf Scald Disease

Sugarcane Leaf Scald Disease (SLSD) is caused by *Xanthomonas albilineans*. *X. albilineans* is a Gram-negative, xylem-invading phytopathogen. It is a rod-shaped bacterium with a single polar flagellum. Colonies are shiny, slow-growing, non mucoid, honey yellow in color and often show dimorphism in size.

X. albilineans is quite distinct from other members of the genus, and is closest in phylogeny to *Xylella* (Figure 1-1; Pieretti *et al.* 2009). Like *Xylella*, and unlike all other pathogenic xanthomonads, it does not have the **h**ypersensitive **r**esponse and **p**athogenicity (*hrp*) secretion system. Furthermore, *X. albilineans* is the only known xanthomonad that does not produce xanthan gum, produces the unique phytotoxin albicidin and has a comparatively small genome of 3,768,695 bp (Pieretti *et al.* 2009).

The first reported sugarcane leaf scald disease (SLSD) outbreak was in Indonesia in the 1920s (Pan 1997; Ricaud and Ryan 1989). The pathogen was detected in the continental United States in 1967 (Koike 1968), and is present in at least 66 countries worldwide, including most of the major sugarcane producing countries (Table 1-1; Rott and Davis 2000; Champoiseau *et al.* 2006).

Table 1-1. The top 15 sugarcane producers, according to the United Nations Food and Agriculture Organization, as of 2009 (http://faostat.fao.org).

Country	Production (tons)	Xa present*
Brazil	671395000	Y
India	285029000	Y
China	116251272	Y
Thailand	66816400	Y
Pakistan	50045400	Y
Colombia	38500000	Y
Australia	31456900	Y
Argentina	29950000	Y
USA	27456000	Y
Indonesia	26500000	Y
Philippines	22932800	Y
South Africa	20500000	Y
Guatemala	18391700	Y
Egypt	17000000	N
Vietnam	15246400	Y

*Y=yes; N=no

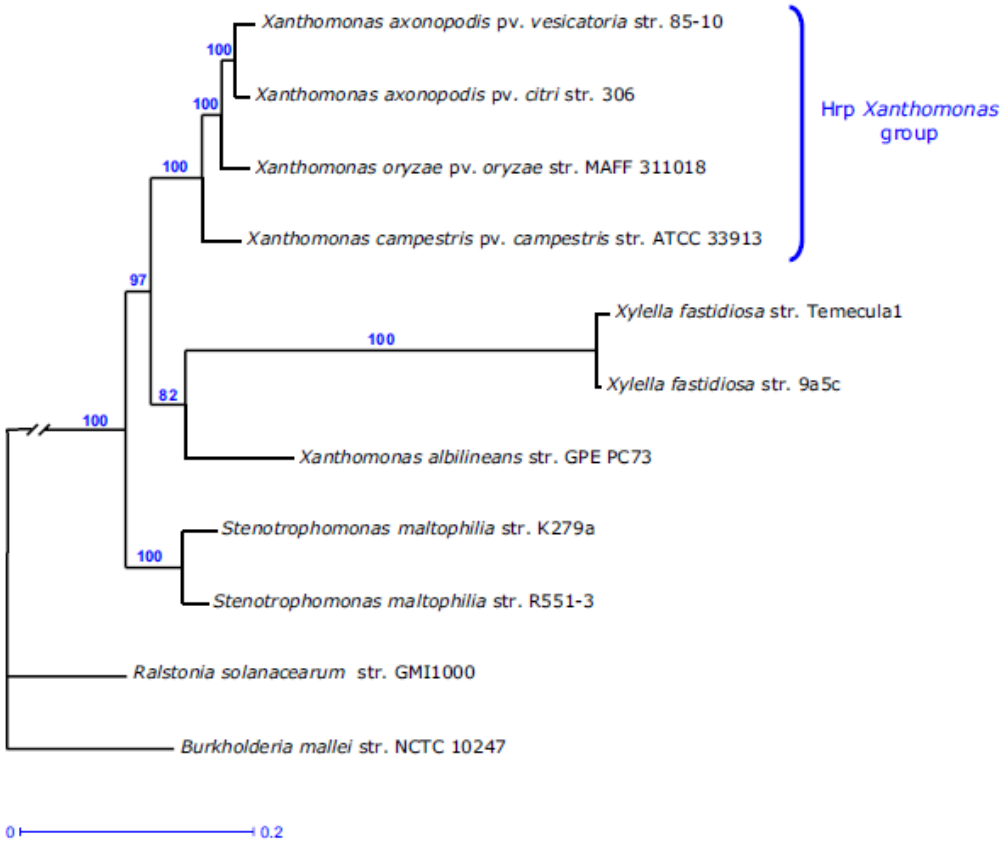


Figure 1-1. Phylogenetic tree based on the concatenated nucleotide sequences of seven housekeeping genes (*gyrB*, *atpD*, *dnaK*, *efp*, *groEL*, *glnA* and *recA*) (from Pieretti *et al*. 2009).

SLSD is described as having latent, chronic and acute phases. In latency, sugarcane is colonized by the pathogen but no symptoms are present. This phase can last for years, allowing the disease to spread in fields undetected. The chronic form is characterized by the classic pencil-line symptoms: white, sharply defined lines running parallel to the midvein for the entire length of the leaf blade (Figure 1-2). The pencil lines turn into a burnt reddish color and the leaf becomes necrotic from the apex inwards as the disease progresses. Vascular bundles in infected stalk tissues turn red (Figure 1-3). Side buds may proliferate earlier in infected plants. The infection can lead to death of the plant. The acute form is the most severe, where the plant wilts and dies often without expression of the symptoms associated with the chronic form (Rott and Davis 2000).

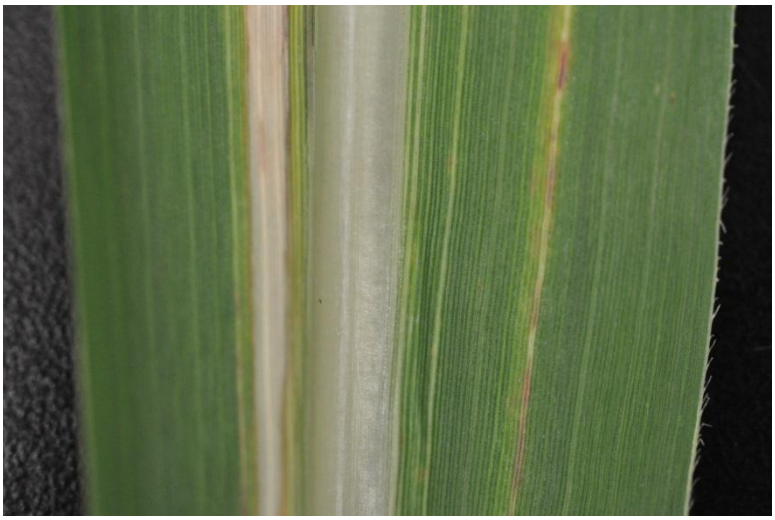


Figure 1-2. Symptoms of infection by *X. albilineans*. Arrow points to a pencil-line. Note necrotic tissue on other side of the midrib.

The host range of *X. albilineans* is limited to members of the Poaceae family. The major host affected by the pathogen is sugarcane, *Saccharum* spp. hybrids. Corn grown in proximity to infected sugarcane can become infected as well (Autrey *et al.* 1995; Birch 2001). Other natural hosts include *Brachiaria piligera*, *Imperata cylindrica*

Figure 1-3. Red vascular bundles in infected stalk.

(Cogongrass), *Panicum maximum* (Guineagrass), *Paspalum* spp., *Pennisetum purpureum* (Elephant grass) and *Rottboellia cochinchinensis* (Itchgrass) (Birch 2001). Disease symptoms have been reported on all of the aforementioned alternative hosts (Leyns *et al.* 1984). Because many of these alternative hosts grow in the same areas as sugarcane, and the disease may remain latent for extended periods of time, these grasses can serve as reservoirs for the pathogen. It should also be noted that some of these grasses are grown for grain, fodder, and biofuels, and therefore the reservoirs of this pathogen can be extensive.

X. albilineans is spread mechanically, via infected propagated material, aerially and through contaminated soil (Daugrois *et al.* 2003; Birch 2001). Disease control can be achieved by ensuring planting material is clean. Disinfection of tools can minimize the spread of SLSD; long-term control is achieved by breeding for resistance (Rott and Davis 2000).

The Tn*5* Transposon

Transposable elements are mobile genetic elements first discovered in maize by Barbara McClintock in 1948. Thought to be ancient in origin, transposable elements are found in both prokaryotic and eukaryotic cells, and are implicated as a driving force of evolution (Reznikoff 1993). They are currently defined as “specific DNA segments that can repeatedly insert into one or more sites in one or more genomes”, and include a wide array of elements from viral and nonviral retroposons to DNA transposons, to mobile genomic islands (such as pathogenicity islands), to transposable prophages (Roberts *et al.* 2008).

Sometimes referred to as jumping genes or molecular parasites, these segments of DNA are capable of moving from one place on a genome to another by nonhomologous (*recA* independent) recombination, which can result in mutations at both sites (Kleckner 1977). Transposition can be beneficial, neutral, deleterious or lethal in effect, depending on where the insertion occurs within a genome. Transposons fall into many classes based on the mechanism of transposition. Some copy themselves out of the donor site using the host’s replication machinery while others are directly excised. Likewise, some are pasted into random target sequence while in other types a copy is inserted into a target sequence. Transposons can also contain one or more accessory genes, including those encoding resistance to antibiotics (Roberts 2008).

The Tn*5* transposon is a bacterial transposon that inserts semi-randomly into chromosomes or plasmids, and has been widely used for gene discovery in bacterial genetics (Vizvaryova and Valkova 2004). It is a composite transposon, which is a transposable element that contains one or more antibiotic resistance genes flanked by insertion sequence (IS) elements (Roberts 2008). The IS elements of Tn*5* are IS50L

and IS50R; between the IS elements lie three antibiotic resistance genes. IS50R encodes a transposase (Tnp) and an inhibitor of transposition (Inh) (Reznikoff 2008). The IS elements themselves are each bracketed by 19-bp sequences called inside end (IE) and outside end (OE) sequences (Figure 1-4).

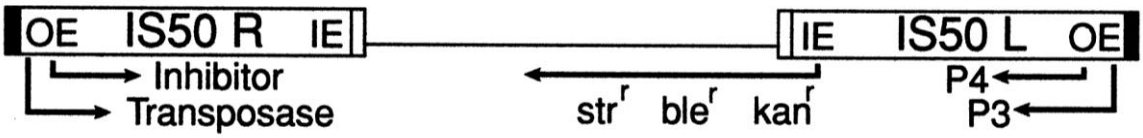


Figure 1-4. Map of the wild-type prokaryotic transposon Tn*5*. str^r, streptomycin resistance; ble^r, bleomycin resistance; kan^r, kanamycin resistance (from Naumann and Reznikoff 2000).

Transposons have proven to be useful tools for studying gene function for decades (Mills 1985). Early studies using various transposons to create random insertional knockouts in *Agrobacterium tumefaciens*, causal agent of crown gall disease, helped elucidate the role of the tumor inducing (Ti) plasmid in oncogenicity (Bevan 1982).

The development of an *in vitro* system for transposition of the Tn*5* transposon using an exogenously supplied and hyperactive transposase has significantly increased the frequency of transposition compared to that of the wild-type transposon, which has very low levels of transposition (Goryshin *et al.* 1998; Reznikoff 2008). Since this breakthrough, many commercial kits have been developed that exploit the efficiency of adding exogenous, hyperactive transposase to the system. The EZ-Tn*5* <KAN-2> insertion kit from Epicentre Biotechnologies, for example, has been used to generate libraries of random transposon insertion mutants in many genera of bacteria and has facilitated the search for genes involved in pathogenicity. From 10^6 to 10^9 mutants can

be obtained in a single reaction, and because the Tn*5* transposon inserts itself in a largely random manner one can obtain better coverage of a given genome if enough mutants are screened. For example, for an *X. albilineans* genome of 3.8Mb size, and assuming an average gene size of 1 kb, it would require 7.6×10^6 insertion events to obtain two Tn*5* insertions into each gene.

Outer Membrane Proteins

Gram-negative bacteria are characterized by the presence of an inner membrane (IM) comprising a lipid bilayer, an outer membrane (OM) and a layer of peptidoglycan in between (Figure 1-5; Dirienzo *et al.* 1978). The IM and OM are functionally and compositionally different. The OM of Gram-negative bacteria serves as an external barrier which protects bacteria from the surrounding environment (Koebnik *et al.* 2000), and contains a mosaic of lipopolysaccharides and proteins arranged in the fluid phospholipid bilayer (Osborn and Wu 1980). Outer membrane proteins (OMPs) are major components of the OM and account for approximately 50% of the total mass (Koebnik *et al.* 2000; Lin *et al.* 2002).

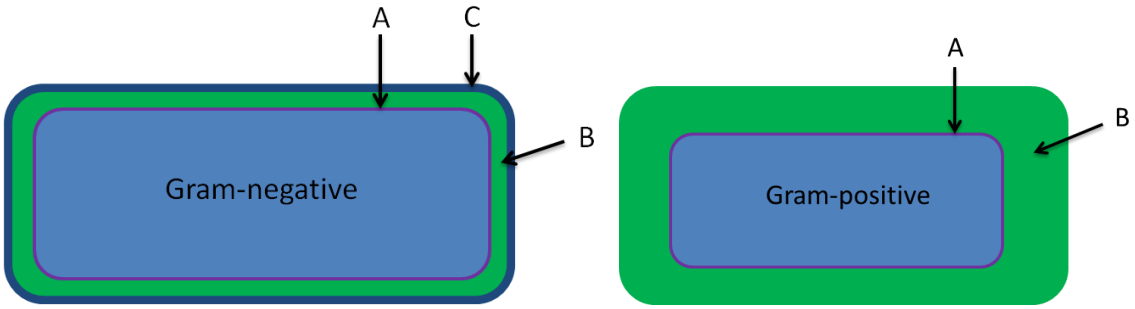


Figure 1-5. Comparison of Gram-negative and Gram-positive cellular walls. A, cytoplasmic membrane. B, peptidoglycan layer. C, outer membrane.

The proteins of the OM can be divided into a two basic categories: 1) integral membrane proteins, which are highly expressed and permanently bound to the OM, and

2) minor proteins that are expressed as needed. Most OMPs are characterized by β-barrel structures, as opposed to the inner membrane proteins which tend to have an α-helical structure (Figure 1-6; Koebnik *et al.* 2000).

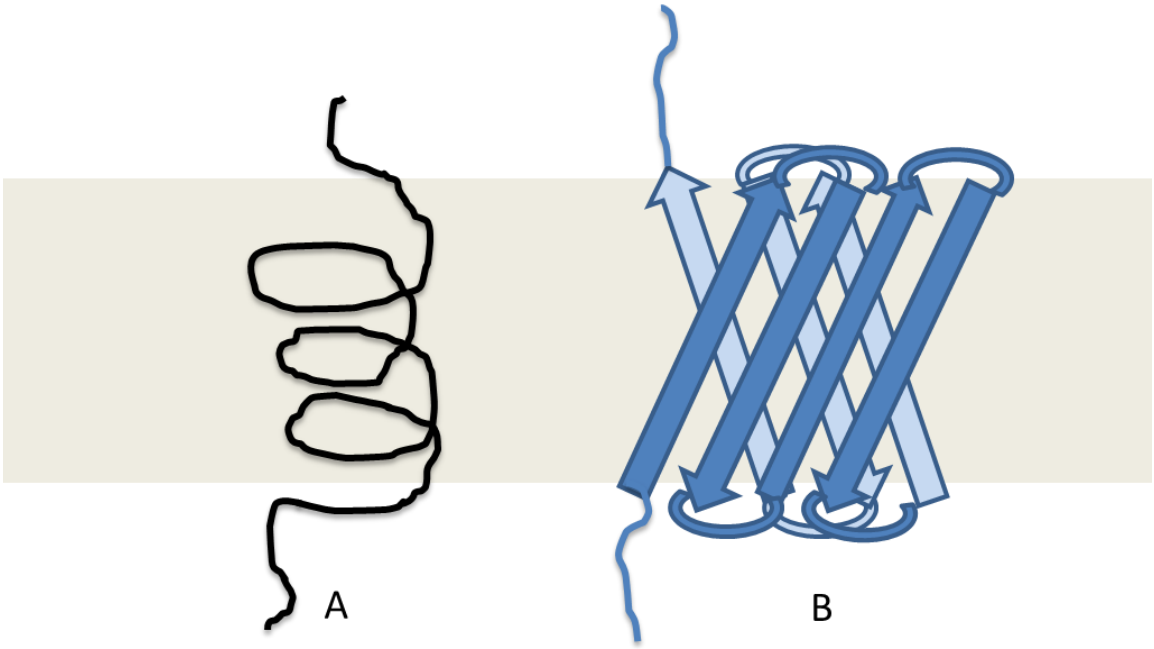


Figure 1-6. A, an α-helical secondary protein structure. B, a β-barrel tertiary protein structure. Gray shading represents the phospholipid bilayer.

OMPs serve many important roles in a variety of cellular processes. In addition to stabilizing the OM via linkages to the peptidoglycan layer, OMPs may have enzymatic activity, serve as receptors for bacteriophages, take part in signal transduction or play a role in horizontal gene transfer. A large subset serves as pores for cellular uptake of nutrients and other molecules. Some, such as OmpC, D and F, act as general porins, which allow for the nonspecific and passive diffusion of small hydrophilic solutes into the cell. Other porins are substrate-specific, such as LamB of *E. coli*, which allows the passage of maltose or malodextrins, and FhuA and FepA of *E. coli*, which are essential for uptake of large iron-siderophore complexes (Koebnik *et al.* 2000; Lin *et al.* 2002).

Other OMPs are involved in secretion and efflux of pathogenicity factors, antibiotics, detergents and other substances. While the general secretory pathway is sufficient for translocation of substances into extracellular space of Gram-positive bacteria, this system is only able to transport substances into the periplasm of Gram-negative bacteria because of the presence of the double membrane. Thus, systems involving multiple proteins evolved that coordinate the translocation of substances into the extracellular milieu, and in the case of the Type III Secretion System, directly into the host cell, in a one or multistep process. There are six known secretion systems in Gram-negative bacteria (Figure 1-7; Buttner and Bonas 2009; Tseng 2009). These secreted effectors perform a range of functions for the cell. Some examples of secreted molecules include phytotoxins (albicidin, *Xanthomonas albilineans*; Birch and Patil 1987; Bostock *et al.* 2006), adhesins involved in biofilm formation (XadA1 and XadA2, *Xylella fastidiosa*; Caserta *et al.* 2010) and nucleic acids (T-DNA, *Agrobacterium tumefaciens*; Matthysse and Stump 1976).

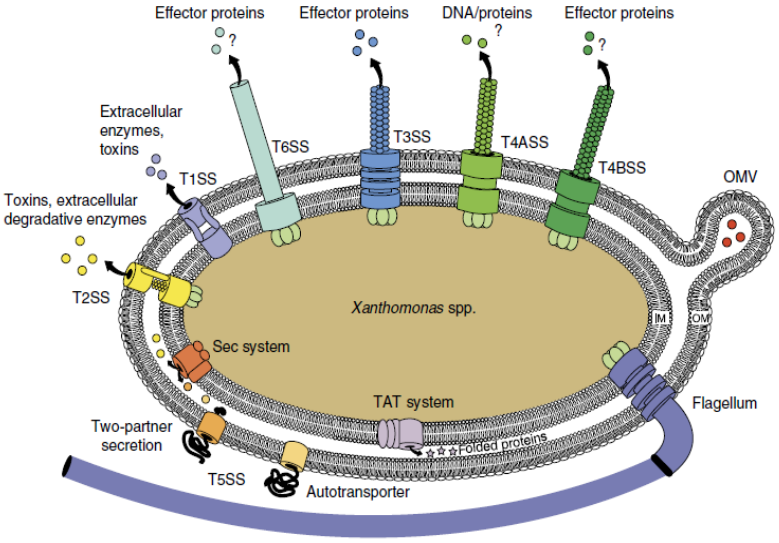


Figure 1-7. Drawing of the six known secretion systems from the genus *Xanthomonas* (from Buttner and Bonas 2009).

Outer Membrane Protein A (OmpA)

The role of OmpA in animal pathogens has been studied extensively. In several bacterial species, this protein has been shown to play a critical role in virulence. For example, in the enteric pathogen *Cronobacter sakazakii*, OmpA$^-$ mutants were 87% less invasive than the wild-type strain in INT 407, a human cell-line (Mohan Nair and Venkitanarayanan 2007). Similarly, OmpA$^-$ mutants of *Escherichia coli* were less able to adhere and invade C6 glioma cells (Wu *et al.* 2009). In another study, OmpA$^-$ mutants of *Escherichia coli* were less invasive in brain microvascular endothelial cells and less able to penetrate the blood-brain barrier than their wild-type counterparts (Wang and Kim 2002). OmpA$^-$ mutants of *Escherichia coli* have also been shown to be significantly decreased in biofilm formation (Barrios *et al.* 2005).

MopB of *Xylella fastidiosa* (Xf), a homolog of XaOmpA1, was associated with the ability of Xf to elicit chlorosis in *Chenopodium quinoa (*Bruening *et al*., 2001*)*. MopB was confirmed to be a major OMP of Xf and may be involved in xylem colonization, since purified protein adhered to xylem-rich balsa wood (Bruening *et al*. 2001). Several attempts to clone the gene with its native promoter in *E. coli* failed, but a construct containing the *mopB* open reading frame driven by an inducible bacteriophage T7 promoter was successfully created, and when expression was induced by IPTG, low levels of MopB were detected. A chimeric construct comprising the N-terminal region of Xf MopB fused to the C-terminal region of *E. coli* OmpA, with the the 5'UTR and leader peptide of OmpA of *E. coli* was also created. Bruening's group wanted to exploit the exposed elements of the MopB protein to find a high-affinity binding protein to inactivate MopB (Bruening *et al.* 2005). Ultimately, it was concluded that EF-Tu (elongation factor

temperature unstable), and not MopB, was responsible for the chlorosis inducing activity (Bruening *et al.* 2007).

Recently, the first evidence that a major OMP might be a pathogenicity factor in phytopathogenic bacteria was published (Chen *et al*. 2010). A knockout mutant of an OmpA homolog, MopB from *Xanthomonas campestris* pv*. campestris*, was created. MopB$^-$ mutants were no longer pathogenic *in planta*, and *in vitro* cells aggregated abnormally, were more sensitive to higher temperatures, SDS in the media, and alkaline pH, and were deficient in EPS production, adhesion and motility (Chen *et al.* 2010).

Objectives of this Study

The first objective of this study is to locate the precise insertion sites of the Tn*5* transposon in the five ompA mutants. Next, the phenotypes of the five mutants *in vitro* and *in planta* will be assessed and compared to the wild-type strain. Finally, the mutation must be complemented using the endogenous *XaompA1* gene to verify the ascertained phenotypes are due the insertional inactivation of the gene by the Tn*5* transposon.

CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains and Culture Media

Xanthomonas albilineans strain XaFL07-1, isolated in 2007 from sugarcane sampled in Canal Point, Florida, was used in all experiments. XaTn*5 ompA* mutants 227, 468, 573, 768 and 1152 were created by electroporation of XaFL07-1 with 20 ng of the transposase-Tn*5* DNA synaptic complex (Epicentre Biotechnologies, Madison, WI, U.S.A.; for detailed methodology see Rott *et al.* 2011). Bacteria were routinely cultured on Modified Wilbrinks (MW) medium (10 g sucrose, 5 g peptone, 0.50 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H2O$, 0.05 g Na_2SO_3, 15 g agar, 1 L deionized water at a pH of 6.8 to 7.0) at 28 to 30°C. Transposon Tn*5* insertion mutants were grown on MW agar supplemented with 20 mg/l kanamycin. All strains were stored at -80°C as turbid cell suspensions in sterile distilled water.

E. coli strains Mach1, TOP10 and DH5α were grown in Luria Broth (LB) (Sambrook *et al.* 1989) or PYGM (5 g peptone, 3 g yeast extract, 40 ml 50% glycerol, 15 g agar in 900 ml deionized water, pH 7.4, after autoclaving, 100 ml MOPS buffer was added; Defeyter *et al*. 1990) at 37°C. Antibiotics were used as needed at the following concentrations (in µg/ml): ampicillin 40, kanamycin 50, tetracycline 10, chloramphenicol 25 and gentamycin 3. Chemically competent *E. coli* was transformed using 10 to 150 ng DNA in 50µl cells. After adding DNA, cells were incubated for 10 minutes on ice, heat shocked at 42°C for 30 seconds and 250 µl room temperature SOC medium (Sambrook *et al.* 1989) was immediately added. The mixture was then incubated at 37°C for one hour and plated on appropriate selective media.

Electrocompetent *X. albilineans* Cells

Liquid cultures of XaTn*5 ompA* mutants in a total volume of 25 ml MW medium were grown at 28°C for two to three days (to OD_{600} = 0.4 to 0.7) shaking at 125rpm in a rotary waterbath shaker. Cultures were chilled in ice water for 15 to 30 minutes and centrifuged at 4°C at 3200 x g for 15 minutes. Cells were washed first in 50 ml and then in 25 ml ice cold distilled water and centrifuged at 4°C at 3200 x g for 15 minutes. Pellets were resuspended in 1 ml sterile distilled water and stored as 50 µl aliquots at -80°C.

Electroporation of *X. albilineans*

Electrocompetent *X. albilineans* cells were thawed on ice. Plasmid DNA or ligation mixes (1 to 5 µl or approximately 50 to 150 ng) were added to pre-chilled cuvettes with 1 mm gaps and put on ice. 40 µl competent cells were pipetted into the cuvettes and incubated on ice for 30 seconds. Cells were electroporated at 1800kV using an Eppendorf 2510 electroporator (Westbury, NY, USA). Typically, time constants were between 5.6 and 6.2. Occasionally time constants below 3 were obtained apparently due to arcing of the current; these electroporations were repeated to obtain a higher time constant. Immediately after electroporating, 900 µl MW medium was added and cells were transferred to 14 ml Falcon tubes and allowed to recover at 28°C for three to four hours. Transformed cells were plated on MW medium with appropriate antibiotics (gentamycin 3mg/l for pUFR047 constructs and tetracycline 10mg/l for pRK415 constructs).

Construction of Complementing Plasmid pLF004

An 1156 bp fragment comprising the full length XALc_0557 predicted open reading frame, annotated as XaompA1 and including the native Shine-Dalgarno region,

was amplified by polymerase chain reaction (PCR) from strain XaFL07-1. Accuprime Taq High Fidelity polymerase (Invitrogen), 2X Failsafe Buffer D (Epicentre), primer ompAF1 (5' CGG ATC CTG CCT CTA TCG TTT TAT CTC 3') with an added *Bam*HI site and ompA R1 (5' AAA GCT TCA CTT GTT CTC GAC GTT CAG 3') with an added *Hin*dIII site were used. PCR products were cloned into TOPO2.1 (Invitrogen) and transformed into *E. coli* Mach 1 cells. Plasmid DNA was extracted from 30 colonies and sequenced.

Because no single PCR product cloned was a perfect DNA sequence match to XALc_0557, one clone carrying an *Age*I-*Sac*I segment containing an error was corrected by swapping the fragment with an error free *Age*I-*Sac*I section from another XALc_0557 plasmid clone, and transformed into *E. coli* Mach 1. After the sequence was verified, this construct was given the name pLF003. The plasmid was digested sequentially with *Bam*HI and *Hin*dIII and the resulting fragment was gel purified and ligated into a stable, broad host range, repW shuttle vector pUFR047 (DeFeyter *et al.* 1990) also digested with *Bam*HI and *Hin*dIII. Ligation mix was transformed into *E. coli* Mach 1 and transformants were selected on LB agar medium with ampicillin. The resulting plasmid, pLF004, was digested with *Bgl*II to verify the profile.

Construction of Complementing Plasmid pCT47.3

A 351 bp fragment comprising the C-Terminal end of XaOmpA1 from XALc_0557 was amplified by PCR from XaFL07-1. Accuprime Taq High Fidelity polymerase (Invitrogen), 2X Failsafe Buffer D (Epicentre), primer CTermF (5' TG AAT TCA GGA GTC TAG ATG CTG AAG GGT GTC AAC TTT GAC TTC 3') with an added *Eco*RI site and Shine-Dalgarno and CTermR (5' TTA AGC TTA TCA CTT GTT CTC GAC GTT CAG CT 3') with an added *Hin*dIII site were used. The construct was digested

sequentially, first with *Eco*RI and then with *Hin*dIII and the resulting fragment was gel purified and ligated into pUFR047 digested with *Eco*RI and *Hin*dIII. Ligation mix was directly transformed into electrocompetent XaTn*5*ompA M1152. Ten colonies were obtained. All colonies were screened using several primer sets to verify the presence of the gene within the vector. Seven of the ten colonies were PCR positive. Four of the seven colonies were inoculated in 10 ml liquid cultures in MW medium, grown for three days and plasmids were extracted and sequenced. Only one plasmid extracted from colony 3 had a sequence that was 100% identical to the expected XALc_0557 sequence, and this construct was given the name pCT47.3 (for C-terminus in pUFR047 colony 3).

Recovery of Empty Vector from pRK-mopB

Plasmid pRK-mopB was kindly provided by Yi-Hsiung Tseng of Institute of Microbiology, Immunology and Molecular Medicine, Tzu Chi University, Hualien 907, Taiwan, ROC. In order to compare the phenotypes of XaTn*5 ompA* mutants transformed with this plasmid to the wild-type, the wild-type must contain the empty vector. The mutants must also contain the empty vector. Otherwise, effects of the vector itself might be confused with effects of the gene. To release the empty vector, pRK-mopB was digested with *Hin*dIII and *Xba*I. DNA was digested next with *Age*I to further cut the insert DNA, reducing the chance of contamination of the vector with the insert. The 10666 bp band representing the empty vector was gel purified and blunted using DNA polymerase I, Large (Klenow) fragment. The DNA was then purified using QIAEXII gel extraction kit following the protocol for desalting and concentrating DNA solutions (Qiagen, Valencia, CA, U.S.A.). DNA was ligated to itself and transformed into *E. coli* TOP10 chemically competent cells. Plasmid DNA from four blue colonies was cut with

*Hin*dIII (which should not cut if the site was eliminated via Klenow end filling) and in a separate reaction *Eco*RI (which should linearize the plasmid). All four showed the correct restriction profiles and consequently plasmid extracts from two colonies were sent to be sequenced. The plasmid extracted from colony 2 had the predicted sequence. This plasmid was given the name pRK415K.2.

Methylation of pLF004 and pRK-mopB

E. coli DH5α containing pMetXa, a plasmid containing the methylase gene cluster from *X. albilineans* strain GPE PC73 (Champoiseau *et al.* 2006), was inoculated into 1ml LB broth supplemented with 25 µg/ml chloramphenicol. Cells were cultured at 37°C for 16 hours. A 50 µl aliquot was inoculated in 10 ml LB supplemented with 25 µg/ml chloramphenicol and incubated at 37°C for 2 hours. Cells were transferred to a 40 ml Oakridge tube and pelleted at room temperature. The cell pellet was resuspended in 700 µl cold 0.1M $CaCl_2$ solution and 200 µl aliquots of cells were transferred to four sterile 16 x 100 mm glass tubes and placed on ice. Approximately 250 ng plasmid DNA was added to the cells and incubated on ice for 20 minutes. Tubes were transferred to 42°C for one minute and incubated on ice for an additional 10 minutes. A volume of 1.8 ml LB medium was added to each tube and cells were incubated without shaking at 37°C for 2 hours. Aliquots of 100 µl were plated on PYGM agar supplemented with gentamycin and chloramphenicol. Methlyation was verified by comparing the *Hin*cII restriction profile of methylated and unmethylated pLF004 or pRK-mopB extracts.

Sodium-Dodecyl-Sulfate (SDS) Tolerance Assay

MW agar medium was prepared as described above, autoclaved, and filtered SDS (10%) was added to a final concentration of 25ppm. Two to three day old cultures of Xa

wild type and mutant cultures were streaked onto these plates, incubated at 30˚C for four days, and relative growth rates were compared.

Motility Assay

Sucrose peptone agar (SPA) medium (20 g sucrose, 5 g peptone, 1 L deionized water, pH 6.8-7.0; Hayward 1960) was prepared with 0.25% (wt/vol) agar. Media was allowed to solidify for one day. Two to three day old cultures were stab inoculated into the center of the Petri dishes and incubated (agar side down) at 28°C for 6 to 10 days.

Albicidin Assay

Albicidin assays were performed as described by Rott *et al.* (1996) with some modifications. Basically, three day old cultures of Xa were suspended in sterile distilled water, standardized to OD_{600} = 0.450 ± 0.010, diluted with sterile distilled water to 10^{-6} and 40 µl aliquots were spread onto at least 3 plates of SPA medium. Plates were incubated at 30°C until colonies were ~1 mm in diameter. Colonies were then overlaid with 4ml of a mix containing 2 ml of 1.5% Noble (Difco) agar (wt/vol) and a 2 ml suspension of *E. coli* DH5α (10^7 CFU/ml sterile distilled water) and incubated at 37°C for 24 to 48 hours. Albicidin production was quantified by measuring the width of the *E. coli* growth inhibition ring (GIR): (D – d)/2 where D = diameter of the *E. coli* growth inhibition ring and d = diameter of the *X. albilineans* colony.

Assessment of Growth Rates

Two day old cultures were transferred from solid media to sterile distilled water, and suspensions were standardized to a starting OD_{600} = 0.300 ± 0.010. Aliquots of 5 µl were immediately transferred into 25 ml MW medium in 250 ml Nephelo flasks. Cultures were incubated at 28°C and OD_{600} was measured in four hour intervals for 12 days.

Inoculation of Sugarcane

Suspensions of Xa strains to be tested for pathogenicity were standardized to OD_{600} = 0.300 ± 0.025 in sterile distilled water. Sugarcane cultivar CP80-1743 with at least 3 developed stalk nodes was inoculated by the decapitation method as described by Rott *et al.* (1997) in greenhouse conditions. Briefly, using sterile pruning shears, the plant was pruned below the third dewlap and 300 to 600 µl cell suspension was immediately added to the exposed leaf whorl. Each strain was inoculated into at least five plants per assay.

Symptom Assessments

One month post inoculation, visual observations of qualitatively assessed leaf symptoms were recorded for at least three emerging leaves per plant. The symptoms were scored as follows: 0 = no symptoms, 1 = one to five pencil-lines, 2 = six to ten pencil-lines, 3 = more than 10 pencil-lines, 4 = leaf chlorosis or less than 10% necrosis, 5 = 10-50% leaf necrosis, and 6 = more than 50% necrosis.

Assessment of Leaf Colonization

One month post inoculation, leaves were sampled using scissors sterilized with 95% ethanol. Leaves were cut with a scalpel into ~2 inch sections and weighed. Leaf tissue was sterilized by submersion in 95% ethanol and flaming. Sterilized leaf tissue was then chopped into small pieces using a sterile scalpel and forceps in plastic Petri dishes and 1 ml TBS buffer was pipetted onto the chopped leaf fragments. After 2 hours incubation at room temperature, homogenates were serially diluted and plated in triplicate on WCNCB medium (= MW medium supplemented with 25 mg/l cephalexin, 30 mg/l novobiocin, 50 mg/l cycloheximide and 12.5 mg/l benomyl; Rott *el at.* 2011) and

WKNCB medium (= MW medium supplemented with 20 mg/l kanamycin, 30 mg/l novobiocin, 50 mg/l cycloheximide and 12.5 mg/l benomyl; Rott *et al.* 2011). Plates were incubated for three to five days at 30°C.

Assessment of Stalk Colonization

Two months post inoculation, leaves were removed and stalks were cut at the soil level by sterile pruning shears. The rind of the stalk was cleaned with 95% ethanol and paper towels. It was sprayed again with 95% ethanol and flame sterilized. Using sterilized pruning shears, the stalk was cut in between nodes and the cut section was pressed onto WCNCB and WKNCB for Tn*5* mutants or WGNCB for Tn*5* mutants carrying plasmid constructs with gentamycin resistance (= MW medium supplemented with 3 mg/l gentamycin, 30 mg/l novobiocin, 50 mg/l cycloheximide and 12.5 mg/l benomyl) or WTNCB for plasmid constructs with tetracycline resistance (= MW medium supplemented with 10 mg/l tetracycline, 30 mg/l novobiocin, 50 mg/l cycloheximide and 12.5 mg/l benomyl). Stalk colonization was assessed in 10 locations: I_{-4} (representing four internodes below the point of inoculation) through I_{+5} (representing five internodes above the point of inoculation). Stalk colonization was quantified with the following scoring system: 0 = no bacterial colony in the stalk imprint, 1 = 1 to 10 colonies in the stalk imprint, 2 = more than 10 colonies or confluent growth of bacteria in less than 25% of the stalk imprint, 3 = confluent growth of bacteria in 25 to 75% of the stalk imprint, 4 = confluent growth of bacteria in more than 75% of the stalk imprint.

Measurement of Stalk Elongation

Two months post inoculation, the elongation of harvested stalks was assessed. The length of the stalk was measured with a tape measure from the bottom of node “0” to the top of node “+8” (I_0 to I_{+8}).

DNA Sequence Analysis

ORFs were predicted using pDRAW32 by AcaClone Software (http://www.acaclone.com). Tn*5* insertions within the Xa genome were located using the iANT (integrated ANnotation Tool) platform at INRA Toulouse (France). Conserved OmpA domains were identified using BLASTp, and homologues of XaompA1 were identified using the BLASTn algorithms from NCBI (http://blast.ncbi.nlm.nih.gov/).

CHAPTER 3
RESULTS

Transposon Mutagenesis

A total of 1216 independently derived EZ-Tn*5* KAN-2 Tn*5* insertions in strain XaFL07-1 were screened in sugarcane for reduced pathogenicity (reduced ability to cause disease symptoms and/or reduced ability to colonize the sugarcane stalk). Sequence analysis of the regions flanking the Tn*5* insertion sites in first 10 mutants obtained indicated that the transposon integrated randomly into the genome. Of the 1216 Tn*5* mutants screened in sugarcane, 61 mutants affected pathogenicity. These mutants were analyzed to determine the precise location of the Tn*5* insertion site (Rott *et al.* 2011).

Genomic DNA from the mutants was digested with *Eco*RI, which is not found in the Tn*5*. The mixture of fragments was cloned into a TA vector, transformed into *E. coli* and transformants containing the Tn*5* were selected for with kanamycin. Primers that read off of the ends of the Tn*5* were used for sequencing (Figure 3-1).

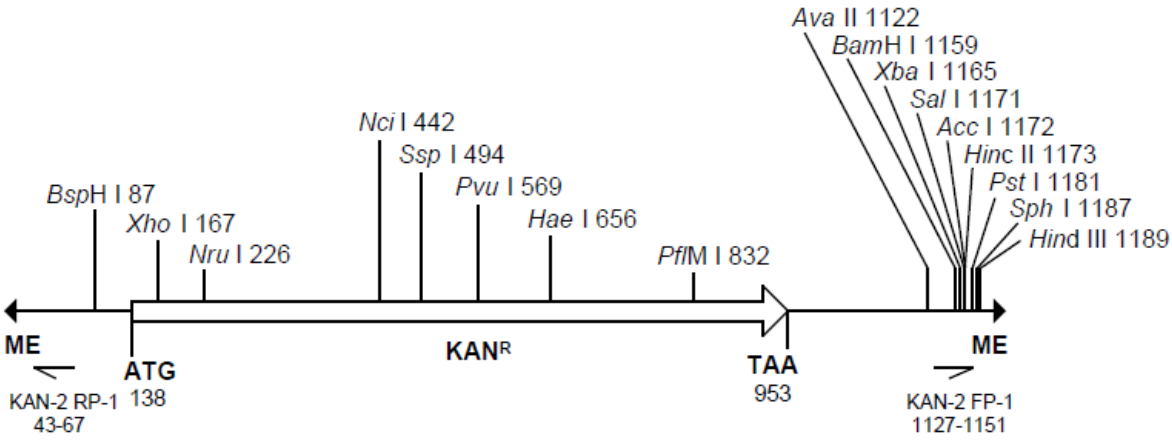


Figure 3-1. Map of the EZ-Tn*5* KAN-2 transposon, showing location of sequencing primers KAN-2-FP-1 and KAN-2-RP-1.

Surprisingly, a total of five insertions were found within XaompA1 (Figure 3-2), and these became the focus of the remainder of this study.

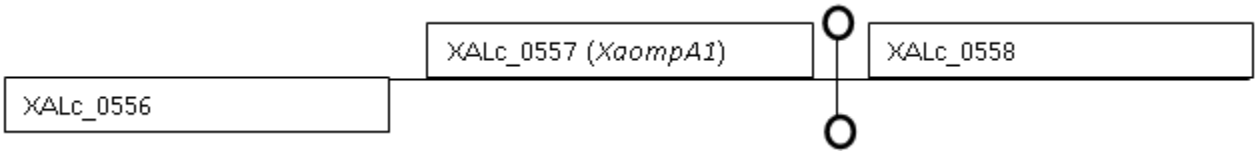
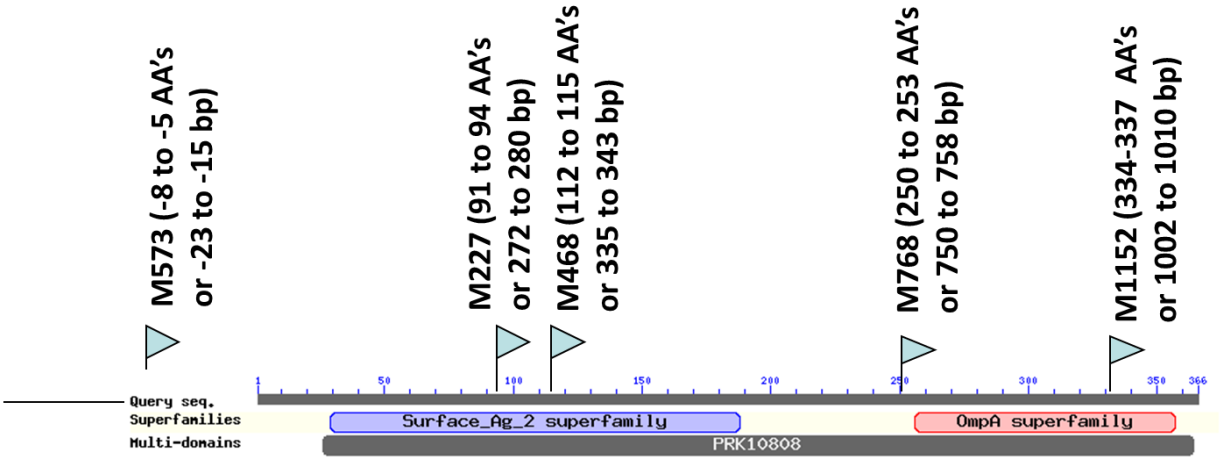


Figure 3-2. Schematic of the location of *XaompA1* and adjacent genes. Not drawn to scale. Locus tags indicating genes predicted to be transcribed left to right are shown above the horizontal line; the gene indicated below the horizontal line is predicted to be transcribed right to left. Predicted rho independent transcriptional terminator regions are indicated by lollipops in both directions.

Again, surprisingly, all insertions were in the same direction. In M573, the Tn*5* was inserted in the promoter region upstream of the Shine-Dalgarno sequence. M227 and M468 had insertions in the Surface Antigen 2 (SAG-2) domain. In M1152 the transposon was inserted in the OmpA domain. Lastly, in M768 the transposon inserted in between the two domains (Figure 3-3).



promoter region

Figure 3-3. Location and directionality of Tn5 insertions within and adjacent to XaompA1 (XALc_0557). The Sag and OmpA domain regions are indicated by blue and pink, respectively.

Phenotypes of XaTn5 ompA Mutants: *in planta*

All *ompA* mutants were significantly reduced in ability to cause symptoms of SLSD (Figure 3-4). Whereas the typical disease severity of wild-type Xa was above 5 (on a scale of 6), none of the XaTn*5 ompA* mutants tested exhibited disease symptoms anywhere near the severity of wild-type Xa (Table 3-1). The highest disease severity obtained was with XaTn*5 ompA* M768 with a disease severity of 0.4 in one experiment. Sugarcane inoculated with any of the *ompA* mutants was usually completely asymptomatic up to two months post inoculation, when stalks were harvested to measure the extent of stalk colonization.

Figure 3-4. Symptoms two months post inoculation. A, M227. B, M468. C, M573. D, M768. E, M1152. F, XaWT. G, H_2O.

All *ompA* mutants were significantly reduced in ability to colonize sugarcane stalks (Table 3-1). Across multiple independent plant inoculation assays, the wild-type strain exhibited a minimum extent of stalk colonization (ESC) of 82% and maximum ESC of 99%. By contrast, in one experiment M227 colonized 14% and M573 colonized 6% of the stalk tissue assayed, but neither strain was detected again in stalk tissue in independent plant inoculation assays. The most consistent colonization was seen with M768, which colonized 5% of the stalk in one assay and 60% in a separate assay. M468 and M1152 were never recovered from sampled stalk tissue.

Table 3-1. Summary of the pathogenicity data of the Tn*5* mutants and wild-type strains. Disease severity values are on a 0-6 scale and extent of stalk colonization values are on a 0-100 scale.

Strain	Disease severity on leaves	Extent of stalk colonization
XaFL07-1	4.6	90
M227	0.12	4.7
M468	0.02	0
M573	0	3
M768	0.2	32.5
M1152	0.08	0

The mechanical damage inflicted by inoculation of sugarcane using the decapitation method resulted in stunted growth of stalk tissue around the point of inoculation; nonetheless, the plants quickly recovered and normal growth patterns returned. The elongation of sugarcane stalk tissue inoculated with the wild-type strain was not only impeded to an extent surpassing the effects of the injury but the diameter of the stalk contracts as well. This stunting and girdling was not observed for any XaTn*5 ompA* mutant (Figure 3-5).

Figure 3-5. Comparison of stalk elongation and girdling around inoculation zone. Lines highlight the elongation from node '0' to node '5'. A, H_2O. B, XaWT. C, M468.

Leaf colonization was assayed for all *ompA* mutants at least twice; bacteria were recovered at least once for all XaTn*5* mutants (Table 3-2).

Table 3-2. Assessment of leaf colonization of Tn*5* mutants and the wild-type strain.

	Isolation 1 (01-09)	Isolation 2 (10-09)	Isolation 3 (06-10)	Isolation 4 (12-10)
WT	+	+	+	+
227	+	+	-	-
468	+	ND	+	+
573	+	ND	-	-
768	ND	ND	+	+
1152	ND	ND	+	-

+ = Xa recovered; - = Xa not recovered, ND = not determined

Phenotypes of XaTn*5* ompA Mutants: *in vitro*

Despite the lack of symptoms in the plant, albicidin production was detected in all three XaTn*5* *ompA* mutants assayed. M227, M468 and M573 all showed visible growth inhibition rings on media overlain with albicidin-sensitive *E. coli* DH*5*α (Figure 3-6). There was no reason to expect *ompA* was involved in albicidin production.

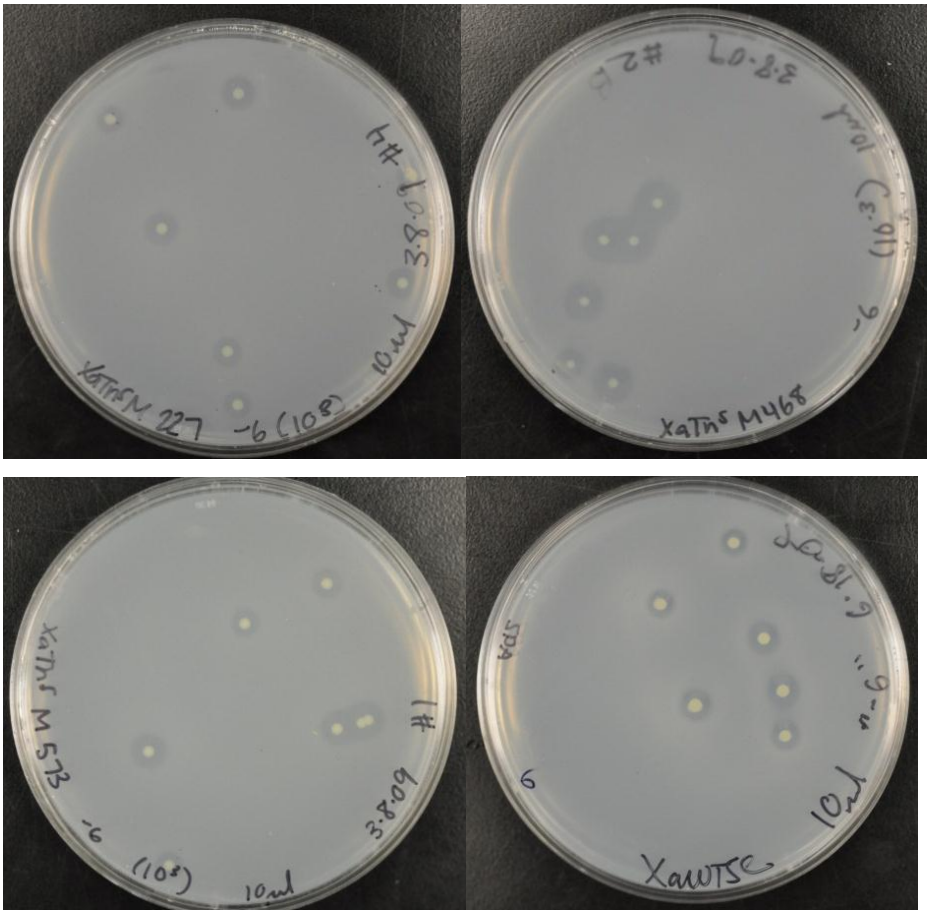


Figure 3-6. Albicidin production by XaTn*5* ompA mutants and wild-type strain, as evidenced by visible clearing of media surrounding single colonies of Xa.

All mutants tested showed significantly reduced growth rates in liquid media. Mutants reached the exponential growth stage later and overall cell yield was lower than the wild-type strain (Figure 3-7; data shown are from two experiments).

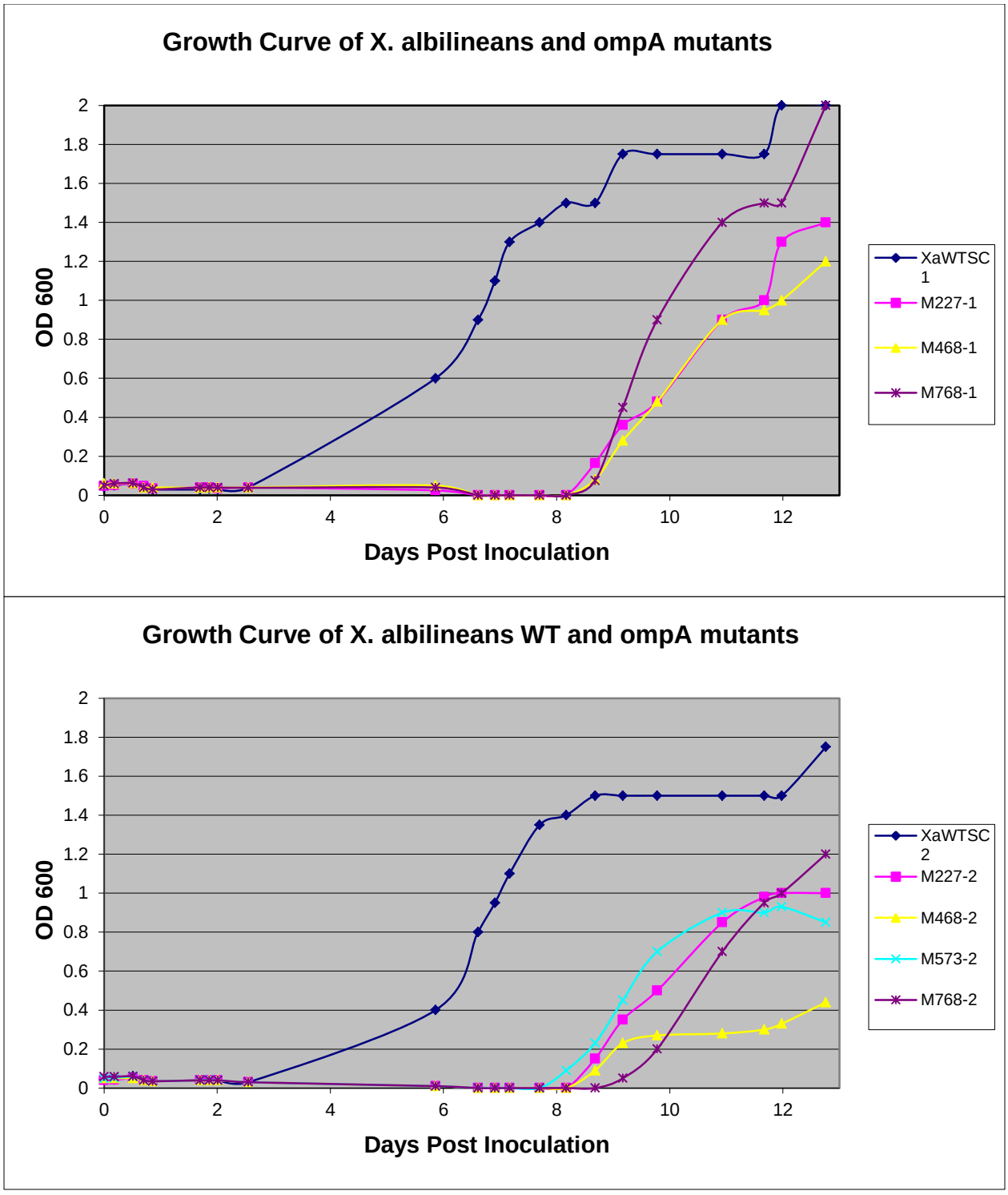


Figure 3-7. Growth curves of Xa strains in liquid MW in two separate experiments.

XaTn*5* *ompA* mutants were variably affected in an assay designed to assess swimming motility. M768, notably, was more motile than the wild-type strain. All other mutants displayed some degree of attenuated motility (Table 3-3 and Figure 3-8).

Table 3-3. Motility assay for Tn*5* mutants and the wild-type strain.

	Diameter of bacterial growth (DBG) in mm in plate #					Mean	SD
Strain	1	2	3	4	5	DBG	DBG
XaFL07-1 WT	21	23	26	26	19	23	3
M227	10	6	12	9	9	9.2	2
M468	17	15	10	10	8	12	4
M573	10	10	10	11	8	9.8	1
M768	29	30	36	29	28	30.4	3
M1152	19	15	12	11	10	13.4	4

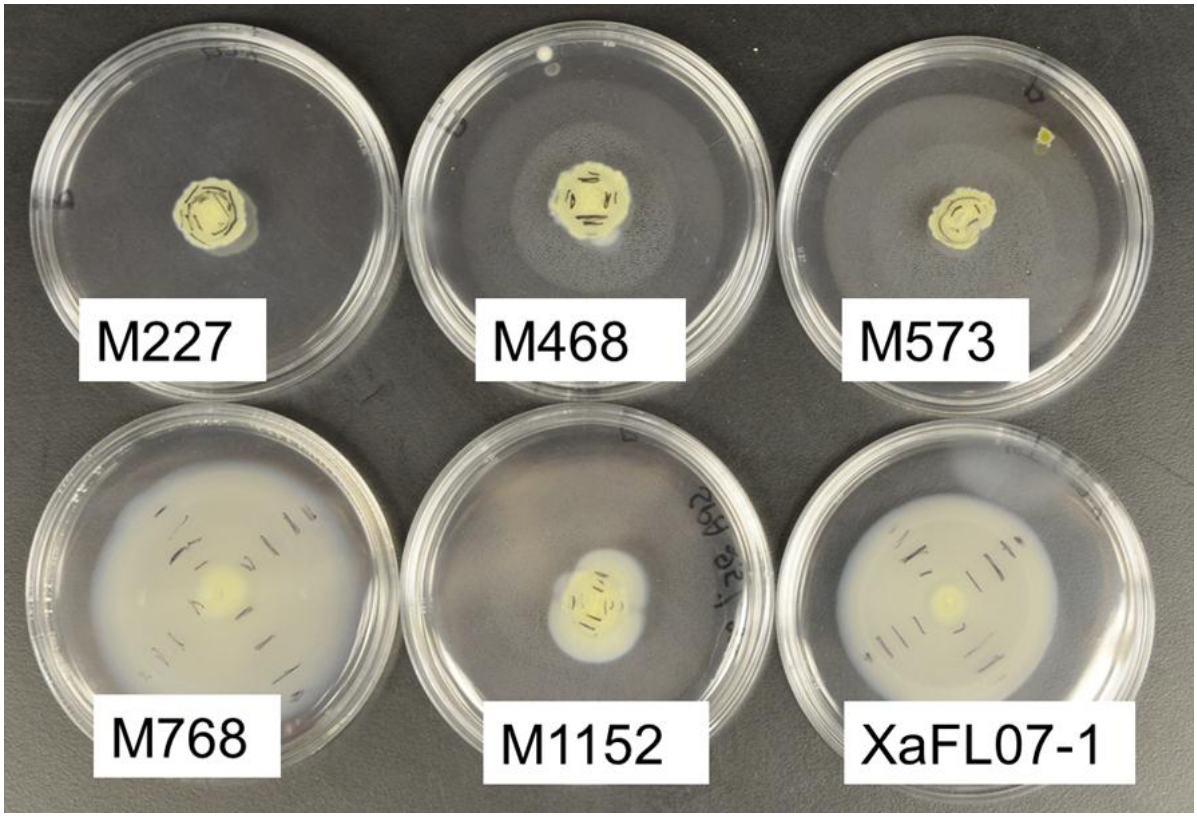


Figure 3-8. Growth of Xa strains ten days post inoculation in motility medium.

Sodium-dodecyl-sulfate (SDS) is a detergent that has been used to detect perturbations in bacterial membranes. The wild-type strain was able to grow in media

supplemented with 25ppm SDS, but was unable to grow at the next highest concentration tested, 100ppm. All XaTn*5 ompA* mutants, except M768, were unable to grow in media supplemented with SDS at 25ppm (Figure 3-9).

Figure 3-9. Growth of Xa strains on SDS medium three days post inoculation.

Because of the unexpected growth of M768 on medium containing SDS, cells were tested by PCR to verify that this strain was not a revertant and the transposon was still intact. Three different primer sets were used: 1/ Kan2FP + ompAR1, 2/ Kan2RP + ompAF2, and 3/ ompAF2 + ompAR1 (Figure 3-10). Using M768 as PCR substrate, primer set 1 should amplify a 437bp band, set 2 a 1379bp band and set 3 a 2884bp band. The wild-type strain should display a 1663bp amplicon with primer set 3. Colony touch PCR was performed directly off the plates containing SDS as well as from media without SDS. The predicted amplicon was obtained for all primer sets (Figure 3-11).

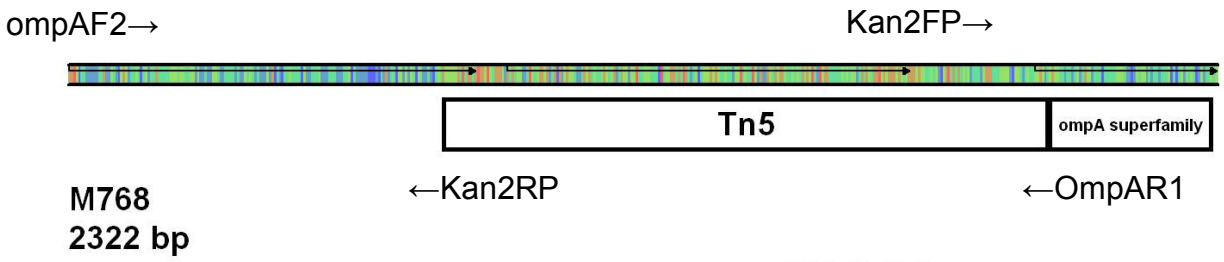


Figure 3-10. Location of primers in the context of M768.

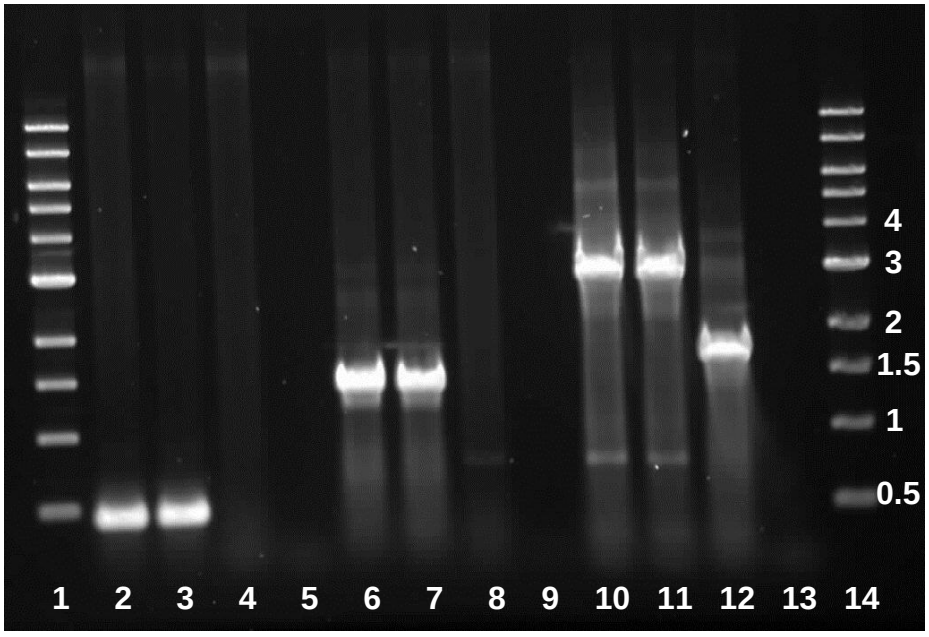


Figure 3-11. PCR to check for reversion of M768. Lanes 1 and 14, 1kb ladder (New England Biolabs); Lanes 2-5 = primer set 1; Lanes 6-9 = primer set 2; Lanes 10-13 = primer set 3; Lane 2, 6 and 10: M768 from SDS media; Lane 3, 7 and 11: M768 from plain media; Lane 4, 8 and 12: XaWT; Lane 5, 9 and 13: H_20

The inconsistent phenotype observed with M768 (motility comparable to the wild-type strain, SDS resistance, and more consistent stalk colonization) was unexpected. A closer look at the Tn*5* transposon sequence showed a weak predicted Shine-Dalgarno (SD) beginning at the 1196th base and an ATG start site 6 bp immediately downstream, which could initiate an ORF and form a translational fusion with an existing ORF if the EZ-Tn*5* KAN-2 transposon inserted in frame. Importantly, no known transcriptional

terminator is known to be downstream from the *kan2* gene on the transposon, which could allow formation of an operon driven by the Kan-2 promoter and including the translational fusion (Figure 3-12). All *ompA* mutants were checked for possible transcriptional/translational fusions with the Tn*5* transposon.

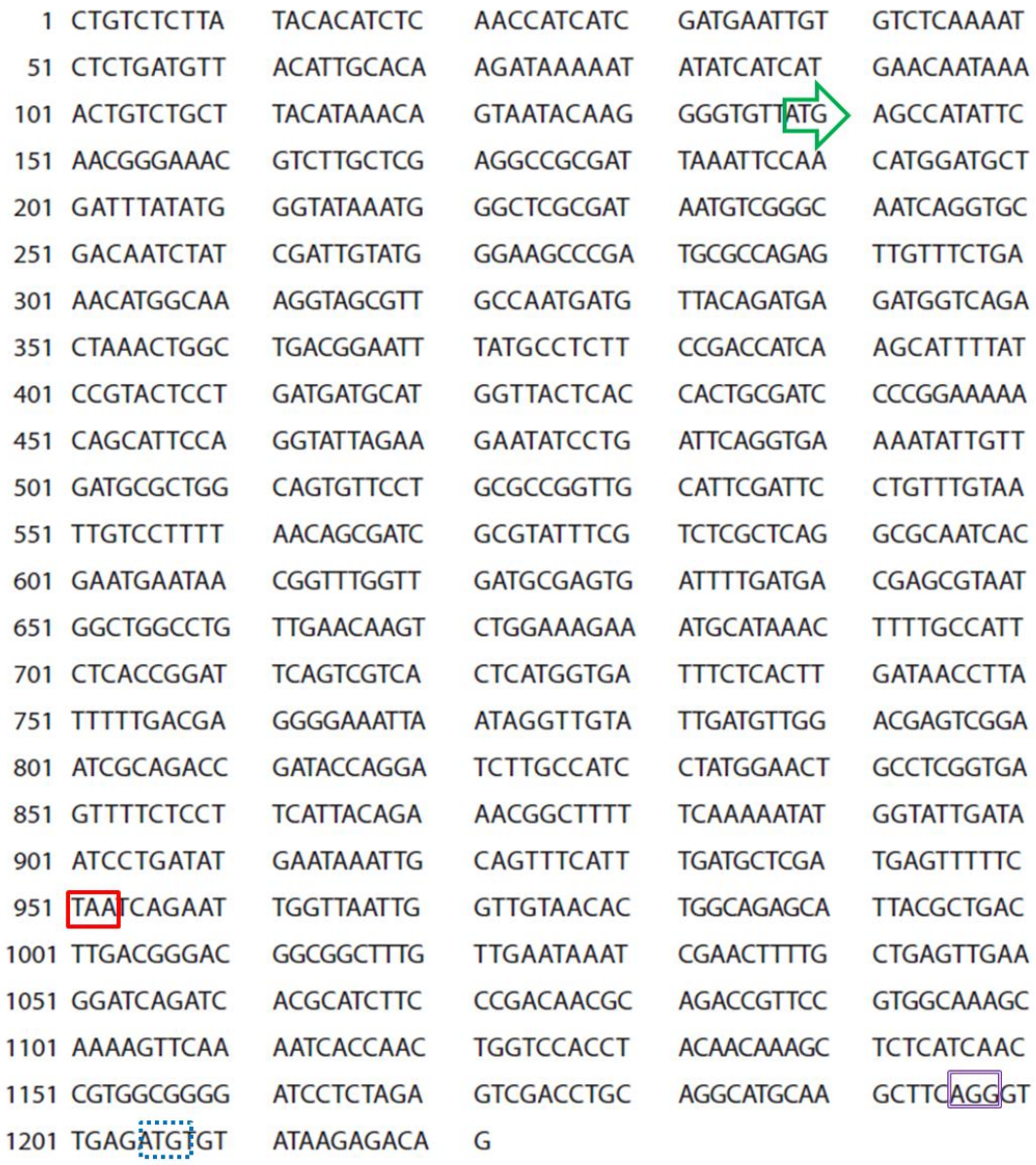


Figure 3-12. The complete DNA sequence of Tn*5* Transposon. The start codon and stop codon of the *kan-2* gene are indicated by the green arrow and red box, respectively. The putative SD and methionine start site are indicated by the purple box and blue box, respectively.

When the sequence of M768 was analyzed in detail, the transcriptional and translational fusion of the entire *ompA* domain of XaompA1 was predicted. Three separate ORFs were predicted and the *ompA* domain was functionally and cleanly separated from the SAG-2 domain with the M768 insertion (Figure 3-13). No translational (in frame) fusions were created by insertions in the four other *ompA* mutants obtained in this study.

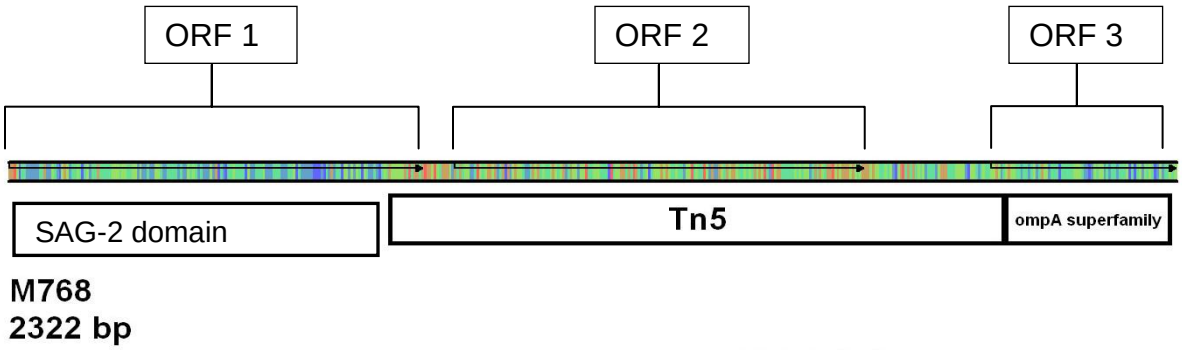


Figure 3-13. Depiction of the translational fusion created by the Tn*5* insertion in M768. - The arrows pointing to the right within the colored bar represent ORFs.

Construction and Assessment of Complementing Clone pLF004

Several unsuccessful attempts were made to clone the full-length (1101 bp) *XaompA1* gene with its native promoter, but all clones contained sequence errors in different locations. Similarly, cloning of *XaompA1* without its native promoter was initially unsuccessful. A DNA fragment swapping strategy was then employed in which error-free fragments from different constructs were used to construct the full-length XaompA1 gene with 100% sequence identity to the native gene in vector TOPO2.1. Once this construct, pLF003, was made, the gene was subcloned into broad host range vector pUFR047, resulting in pLF004, and transformed into *E. coli*. The DNA was digested with *Bgl*II and the correct profile was observed (Figure 3-14). This construct was moved by electroporation into M227. One colony was obtained.

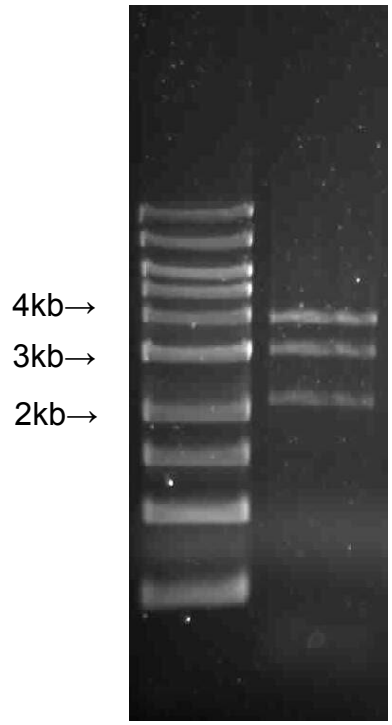


Figure 3-14. Verification of profile of pLF004. Lane 1, 1kb ladder (New England Biolabs). Lane 2,- *Bgl*II profile of pLF004 extracted from *E. coli*. Expected profile = 4113bp, 3129bp and 2514bp.

The plasmid, pLF004, was then extracted from M227 and digested with *Bgl*II to check for rearrangement. The expected profile was not observed.

X. albilineans is known to carry a restriction modification (RM) system (Philippe Rott, unpublished data). RM systems can interfere with transformations using double stranded DNA plasmids. Therefore a plasmid carrying the methylation gene cluster (as a 10599 bp insert) cloned in pCNS (pSU18 derived; Bartolome *et al*. 1991) was used in *E. coli* in an attempt to methylate pLF004 prior to extraction and use in electroporation. The (methylated) pLF004 plasmid DNA extracted from *E. coli* carrying pMetXa was transformed by electroporation into M468. Many colonies (thousands) were obtained. Potential complementation was first checked by plating on media amended with SDS. Resistance to SDS was restored (Figure 3-15).

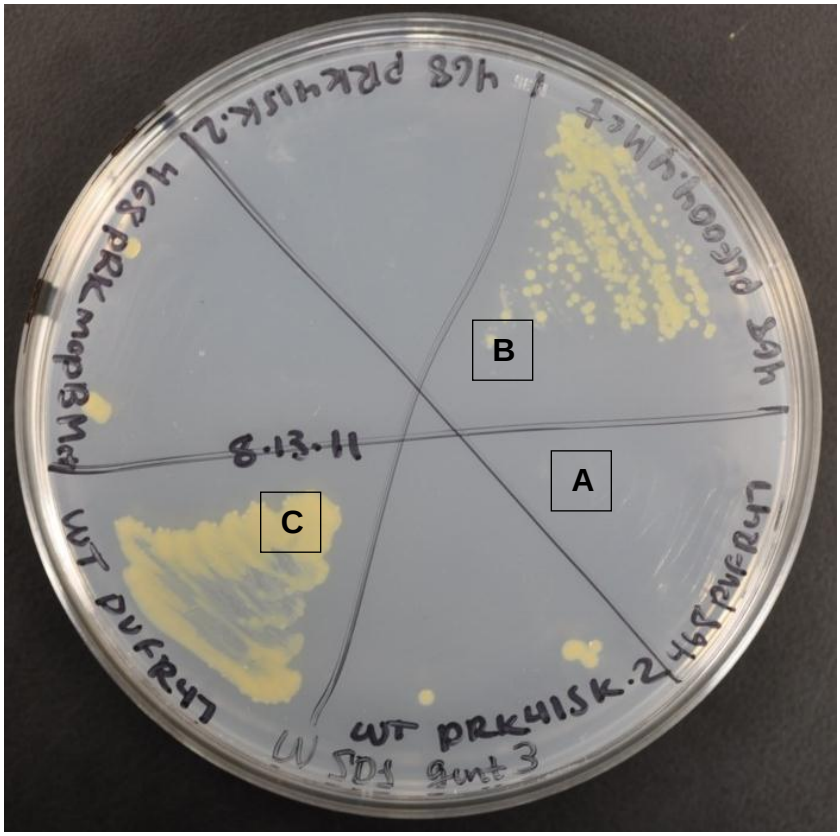


Figure 3-15. Growth of strains on Wilbrink's medium amended with SDS and 3µg/ml gentamycin. A, M468 + pUFR047; B, M468 + pLF004; C, XaWT + pUFR047.

Complementation appeared to be confirmed when motility was also restored for M468 containing pLF004 in two independent assays (Table 3-4).

Table 3-4. Motility assay of M468 containing complementation construct pLF004.

	Diameter of bacterial growth (DBG) in mm in plate #									Mean	SD
Strain	1	2	3	4	5	6	7	8	9	DBG	DBG
WT/pUFR047	43	37	45	40	39	38	44	42		41.0	3
M468/pUFR047	32	32	35	30	30	30	32	35	30	31.8	2
M468/pLF004.4Met	37	39	39	39	36	39	36	38	34	37.4	2

Finally, complementation was confirmed *in planta*. Symptoms in sugarcane plants inoculated with M468 containing pLF004, although not as severe as the wild-type strain, were restored. M468/pLF004 exhibited diagnostic pencil-lines and necrosis typical of SLSD (Figure 3-16). These results confirmed that the Tn5 insertion in M468 was

responsible for the mutant phenotypes, including loss of pathogenicity (Figure 3-4), loss of motility (Figure 3-8), and sensitivity to SDS (Figure 3-9).

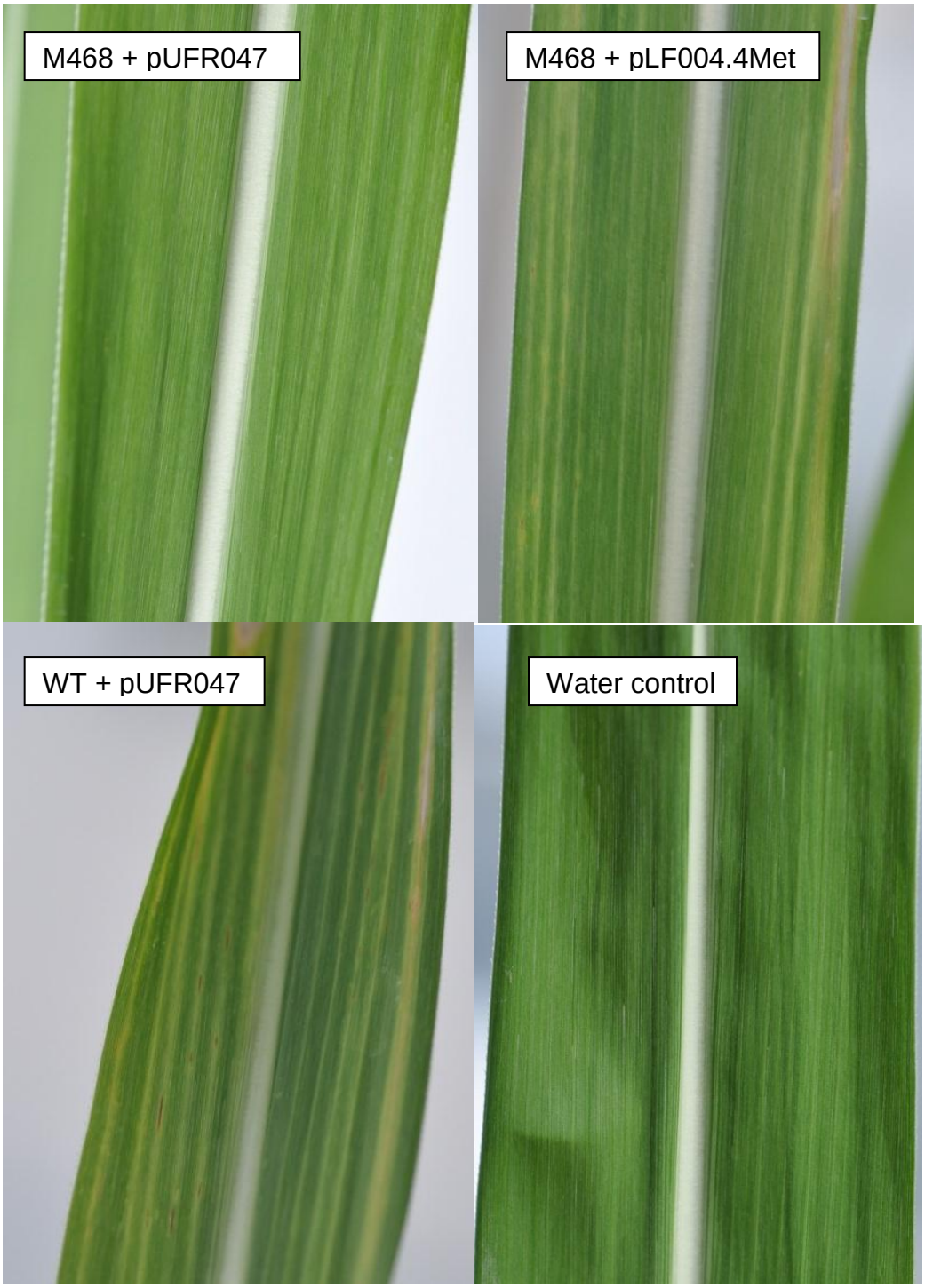


Figure 3-16. Symptoms in sugarcane inoculated with M468 containing complementation construct pLF004 (two weeks post inoculation).

Assessment of Complementing Clone pCT47.3

Plasmid pCT47.3 was constructed in an attempt to recreate the aberrant phenotype of M768. To this end, the translational fusion created by the Tn*5* insertion just upstream of the *ompA* domain of *XaompA1* was duplicated by cloning the 3' end of *XaompA1* from the wild-type strain using primers with an added SD and methionine start site, into pUFR047. The methionine start site of the forward primer is followed by the first codon of the *ompA* domain of *XaompA1*. The weak SD of the Tn*5* was replaced by the native SD from *XaompA1*, however. When transformed into M1152, pCT47.3 restored resistance to SDS (Figure 3-17), as well as modest levels of pathogenicity (Figure 3-18). However, motility was not restored (Table 3-5).

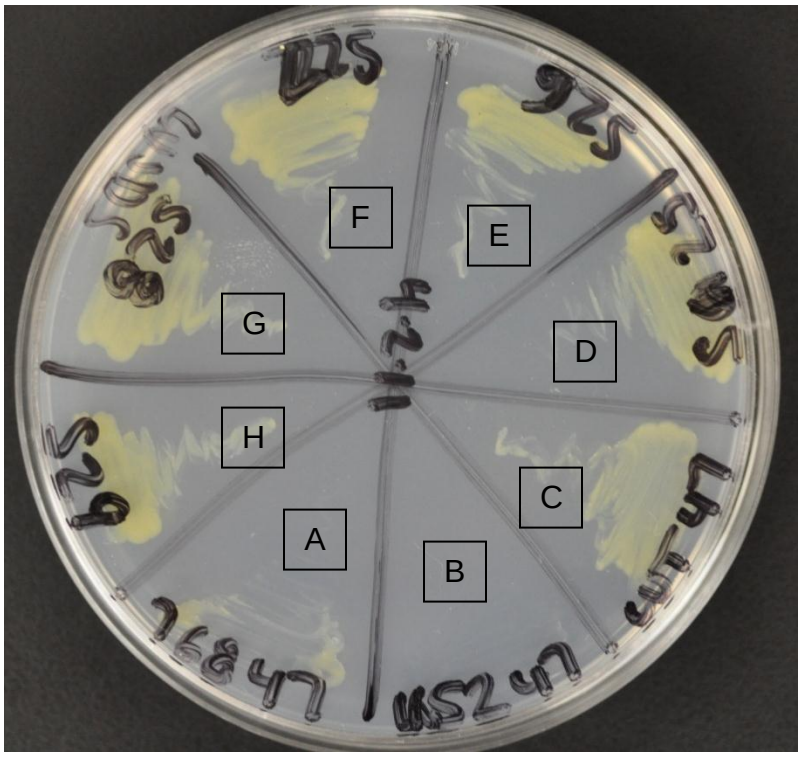


Figure 3-17. Growth of strains on Wilbrink's medium amended with SDS. A, M768 + pUFR047; B, M1152 + pUFR047; C, XaWT + pUFR047; D, M1152 + pCT47.3 col. S25; E, M1152 + pCT47.3 col. S26; F, M1152 + pCT47.3 col. S27; F, M1152 + pCT47.3 col. S28; G, M1152 + pCT47.3 col. S29.

Table 3-5. Motility assay of M1152 containing complementation construct pCT47.3.

	Diameter of bacterial growth (DBG) in mm in plate #									Mean	SD
Strain	1	2	3	4	5	6	7	8	9	DBG	DBG
WT/pUFR047	42	50	45	c*	51	51	45	44		46.9	4
M1152/pUFR047	33	34	31	28	33	30	30	32		31.4	2
M1152/pCT47.3 S6	28	26	26	31	24	24	29	22	22	25.8	3
M1152/pCT47.3 S21	32	25	20	23	21	22	22	23	21	23.2	4

* contaminated

Symptoms are not as severe as the wild-type strain or M468 containing the complementing construct pLF004 (Figure 3-18).

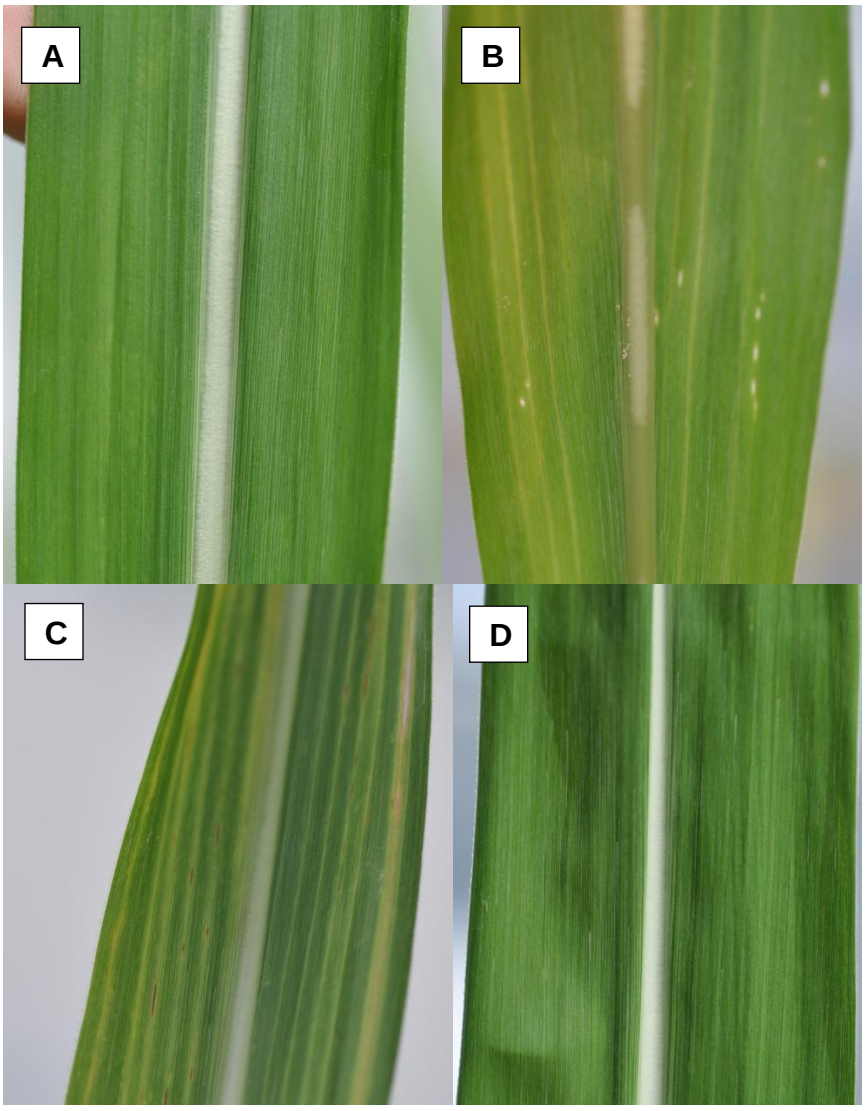


Figure 3-18. Symptoms in sugarcane inoculated with M1152 containing complementation construct pCT47.3 (two weeks post inoculation). A, M1152 + pUFR047; B, M1152 + pCT47.3 S21; C, XaWT + pUFR047; D, H_2O.

Assessment of Complementing Clone pRK-mopB

The plasmid pRK-mopB (kindly provided by Yi-Hsiung Tseng of Tzu Chi University, Taiwan, ROC) contains *mopB* cloned from *Xanthomonas campestris* pv. *campestris* in vector pRK415 (Chen *et al.* 2010). This gene is 83% identical at the nucleotide level to *XaompA1*. When this (methylated) plasmid was electroporated into M468, resistance to SDS in solid media was restored, provided antibiotic selection pressure (ie., tetracycline in the medium) for the plasmid was maintained (Figure 3-19).

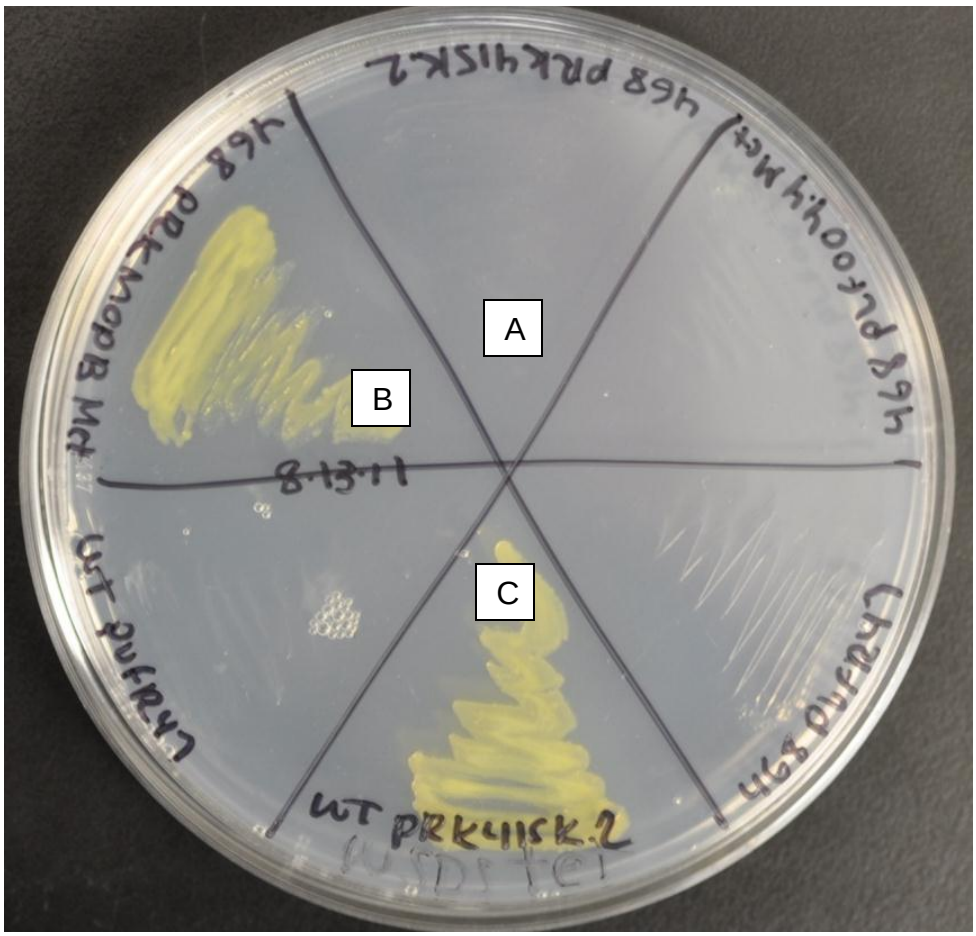


Figure 3-19. Growth of strains in MW medium amended with SDS and 10µg/ml tetracycline. A, M468 + pRK415K; B, M468 + pRK-mopBMet colony 1; C, XaWT + pRK415K.

However, when selection pressure was dropped in motility assays, inconsistent results were obtained. In the first assay, three colonies that originally were positive by

PCR for the insert (colony 1, 3 and 9) and one colony that was negative by PCR (colony 2) were tested. The PCR positive colonies were more motile than the PCR negative colony, but the standard deviations were too large to be statistically significant (Table 3-6).

Table 3-6. Motility assay of M468 containing complementation construct pRK-mopBMet (non-selective media).

	Diameter of bacterial growth (DBG)						Mean	SD
Strain	1	2	3	4	5	6	DBG	DBG
WT	45	63	60	55	51	43	52.8	8
M468	48	50	44	43	46	32	43.8	6
M468/pRK-mopBMet c.1 (PCR +)	62	37	36	44	40	46	44.2	10
M468/pRK-mopBMet c. 2 (PCR -)	34	47	32	37	38		37.6	6
M468/pRK-mopBMet c. 3 (PCR +)	41	52	50	43	50	54	48.3	5
M468/pRK-mopBMet c. 9 (PCR +)	55	51	51	49	48	50	50.7	2

Bacteria from the edge of every plate (23 plates) were transferred to MW agar with 10µg/ml tetracycline to check for loss of the plasmid pRK-mopB. There was no growth for any strain, indicating that this plasmid was lost in Xa in the absence of tetracycline selection.

A second motility assay was performed with and without antibiotic selection. In these assays there was no evidence of complementation of this phenotype (Tables 3-7 and 3-8).

Table 3-7. Motility assay of M468 containing complementation construct pRK-mopBMet (selective media).

	Diameter of bacterial growth (DBG) in mm plate #									Mean	SD
Strain	1	2	3	4	5	6	7	8	9	DBG	DBG
WT/pRK415k.2 c.l	26	29	27	28	28	29	26	28		27.6	1
M468/pRK-415K.2 c. 1	23	24	22	21	20	22	19	20	20	21.2	2
M468/pRK-mopBMet c. 1	21	19	22	22	20	25	20	21		21.3	2

Table 3-8. Motility assay of M468 containing complementation construct pRK-mopBMet (nonselective media).

	Diameter of bacterial growth (DBG) in mm									Mean	SD
Strain	1	2	3	4	5	6	7	8	9	DBG	DBG
WT/pRK415K.2 col.1	38	32	34	37	34	35	41	31		35.3	3
M468/pRK415K.2 col.1	35	34	42	34	36	33	41	37	36	36.4	3
M468/pRK-mopBMet col.1	37	37	37	32	37	41	41	35	34	36.8	3

Despite the apparent instability of this plasmid, plants were inoculated to assess the phenotype in sugarcane. As expected, plants inoculated with M468 containing pRK-mopBMet remained completely asymptomatic and no bacteria were recovered from stalk tissue.

CHAPTER 4
DISCUSSION

In the present study, five independent Tn*5* insertions out of 61 tested that affected Xa pathogenicity and growth were found within gene *XaompA1*. Complementation was achieved with two of the Tn*5* mutants tested. These results demonstrate that *XaompA1* is required for full pathogenicity of Xa in sugarcane. Interestingly, *XaompA*- strains were unable to colonize or cause the characteristic pencil-line streaks and chlorosis in leaf tissue, despite their ability to produce albicidin when grown on agar plates. Albicidin was previously identified as the only known pathogenicity factor in Xa (Birch and Patil 1985 and 1987; Hashimi *et al*. 2007). Furthermore, it was also shown that some albicidin-deficient mutants are still able to cause severe leaf symptoms in sugarcane, demonstrating that undescribed mechanisms are involved in pathogenicity of Xa (Rott *et al*., 2011). Although albicidin production involves at least 22 genes in three regions of the genome (Vivien *et al*. 2005 and 2007), *XaompA1* is not among them. Since *XaompA1* affects both symptoms and growth *in planta*, while albicidin affects symptoms but not growth, *XaompA1* represents a new and independent Xa pathogenicity factor.

XaompA1 may play at least an indirect role in the efficiency of albicidin export and secretion from Xa cells *in planta*. Even when leaf populations of *Xaompa1*- mutants were determined to be similar to the wild-type strain, the leaves of these plants remained asymptomatic. It is also possible that *XaompA1* may play a regulatory role or is part of a signal transduction pathway that regulates albicidin synthesis or other molecules in the plant. For example, outer membrane protein FecA serves as a signal receiver and transmitter (Braun *et al.* 2006). The fact that *Xaompa1*- mutants are sensitive to SDS strongly indicates that the integrity of the outer membrane is affected

in these mutants, and this in turn may cause missassembly or loss of function of the albicidin efflux pump (Pieretti *et al*. 2009). The sensitivity to SDS of the mutants also strongly indicates that the barrier function of the outer membrane has been compromised, which in several plant/bacterial systems (Kingsley *et al*. 1993, Balsanelli *et al*. 2010) has been shown to allow host defense compounds, including phytoalexins or reactive oxygen species, to be much more effective against these mutants.

Interruption of a gene can cause polar effects downstream if the given gene is within an operon. The 3' end of *XaompA1* is predicted to be followed by a rho-independent terminator; the closest gene upstream is read in the opposite direction (Figure 3-1). Therefore, no polar effects of the Tn*5* insertions on downstream genes were predicted.

Evidence linking small non-coding RNAs to regulation of *ompA* in *Vibrio cholerae* (Song *et al.* 2008) opens up a different area that might be investigated in the Xa pathosystem. The findings of Song *et al.* (2008) show that *vrrA*, the gene that encodes the regulatory sRNA, represses *ompA* translation and is correlated with increased vesicle formation. This is especially intriguing because the *XaompA1* mutants show hyperproduction of outer membrane vesicles (Rott, unpublished data).

This research confirms and extends recent work done by Chen *et al*. (2010) in *Xanthomonas campestris* pv. *campestris* (Xcc), the causal agent of black rot of crucifers. In that study, it was shown that *mopB*, a gene with close homology to *XaompA1*, is required for disease symptom development in cabbage. It was also shown that Xcc *mopB* mutants were less motile and more sensitive to SDS than the wild-type strain, consistent with what was found for *XaompA1*. In cross-complementation studies,

the Xcc *mopB* gene restored resistance to SDS in the *XaompA1* mutants, as long as selection pressure was maintained. In the absence of selection pressure, no restoration of motility was observed, and even in the presence of selection, only partial and inconsistent restoration of motility was observed. These data were statistically insignificant. Because of the instability of this plasmid in Xa, complementation *in planta* could not be assessed. The outer membrane proteins *XaompA1* and Xcc *mopB* appear to represent critical pathogenicity factors in both Xa and Xcc. It seems surprising that mutations affecting these genes in other plant pathogenic bacteria were not previously reported. Both of these xanthomonads are systemic xylem invaders, and it is possible that these proteins are more critical for xylem invaders than for mesophilic xanthomonads.

The decreased motility *in vitro* might explain the lack of colonization in the sugarcane stalk, because the mutant strains were inoculated into the leaf tissue and not into the stalk. However, mutants of the *rpf* quorum sensing system in Xa, which are highly affected in motility *in vitro*, are still able to spread in the sugarcane stalk like the wild-type strain (Rott *et al*. unpublished data). Alternatively, the xylem of leaf tissue might be easier to traverse for the *XaompA1* mutants, possibly due to structural differences or differences in tonicity.

The difficulty in cloning *XaompA1* in *E. coli* might be due to toxicity of the hydrophobic domains on the host cell (Laage and Langosch 2000). Alternatively, if *XaompA1* functions as a porin, the full-length gene might allow solutes into the cell that are not tolerated by *E. coli*. Other reports of difficulty or inability to clone an OMP in *E. coli* abound (*omp1* of *Fusobacterium nucleatum*, Bolstad and Jensen 1993; *ompU* of

Vibrio cholerae, Sperandio *et al.* 1996; *ompH* of *Pasteurella multocida*, Lee *et al*. 2004; *mopB* of *Xylella fastidiosa,* Bruening *et al.* 2005 and 2007*).* Notably, low copy vectors were used in both the work reported here and by Chen *et al*. (2010).

The predicted XaOmpA1 protein appears to play a role in disease symptom development and colonization of the sugarcane stalk tissue The localization of outer membrane proteins on the outer surface of the cell makes them vulnerable to proteins or other molecules that can bind or otherwise deactivate the protein. Because this gene is well conserved among all xanthomonads, the applicability of such research is potentially wide. The Xanthomonadaceae family contains many economically important plant pathogens, including *Xanthomonas citri* (citrus canker) and *Xylella fastidiosa* (citrus variegated chlorosis and pierce's disease). The high degree of conservation and critical role of pathogenesis makes XaOmpA1 a potential target for the development of new control methods such as the development of transgenic plants producing molecules inhibiting XaOmpA1, not only in Xa but also in other bacterial plant pathogens that possess homologues of this protein.

LIST OF REFERENCES

Autrey, L. J. C., Saumtally, S., and Dookun, A. 1995. Studies on variation in the leaf cald pathogen, *Xanthomonas albilineans*. Proc. Int. Soc. Sugar Cane Technol. 21:485-497.

Balsanelli, E., Serrato, R. V., de Baura, V. A., Sassaki, G., Yates, M. G., Rigo, L. U., Pedrosa, F. O., de Souza, E. M., and Monteiro, R.2010. *Herbaspirillum seropedicae rfbB* and *rfbC* genes are required for maize colonization. Env. Microbiol. 12(8):2233-2244.

Bartolome, B., Jubete, Y., Martinez, E., and de la Cruz, F. 1991. Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene 102(1):75-78.

Bevan, M. W., and Chilton, M.-D. 1982. T-DNA of the *Agrobacterium* Ti and Ri plasmids. Annu. Rev. Genet. 16:357-384.

Birch, R. G., and Patil, S. S. 1985. Preliminary characterization of an antibiotic produced by *Xanthomonas albilineans* which inhibits DNA synthesis in *Escherichia coli*. J. Gen. Microbiol. 131:1069-1075.

Birch, R. G., and Patil, S. S. 1987. Evidence that an albicidin-like phytotoxin induces chlorosis in sugarcane leaf scald disease by blocking plastid DNA replication. Physiol. Mol. Plant Pathol. 30:207-214.

Birch, R. G. 2001. *Xanthomonas albilineans* and the antipathogenesis approach to disease control. Mol. Plant Pathol. 2:1-11.

Bolstad, A. I. and Jensen, H. B. 1993. Complete sequence of *omp1*, the structural gene encoding the 40-kDa outer membrane protein of *Fusobacterium nucleatum* strain Fev1. Gene 132:107-112.

Bostock, J. M., Huang, G., Hashimi, S. M., Zhang, L., and Birch, R. G. 2006. A DHA14 drug efflux gene from *Xanthomonas albilineans* confers high-level albicidin antibiotic resistance in *Escherichia coli*. J. Appl. Microbiol. 101:151-160.

Braun, V., Mahren, S., and Sauter, A. 2006. Gene regulation by transmembrane signaling. BioMetals. 19:103-113.

Bruening, G., and E. L. Civerolo. 2005. Exploiting *Xylella fastidiosa* proteins for Pierce's disease control, pp. 221-224. In M.A. Tariq, S. Oswalt, P. Blincoe, A. Ba, T. Lorick and T. Esser [eds.], Pierce's Disease Control Program Symposium. California Department of Food and Agriculture, San Diego, CA.

Bruening, G., E. L. Civerolo, and P. A. Feldstein. 2007. Exploiting *Xylella fastidiosa* proteins for Pierce's disease control, pp. 173-176, Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA, T. Esser, editor, San Diego, CA.

Buttner, D., and Bonas, U. 2009. Regulation and secretion of *Xanthomonas* virulence factors. FEMS Microbiol. Rev. 34(2):107-133.

Caserta, R., Takita, M. A., Targon, M. L., Rosselli-Murai, L. K., de Souza, A. P., Peroni, L., Stach-Machado, D. R., Andrade, A., Labate, C. A., Kitajima, E. W., Machado, M. A., and de Souza, A. A. 2010. Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. Appl. Env. Microbiol. 76:4250-4259.

Champoiseau, P., Daugrois, J.-H., Girard, J.-C., Royer, M., and Rott, P. 2006. Variation in albicidin biosynthesis genes and in pathogenicity of *Xanthomonas albilineans*, the Sugarcane Leaf Scald Pathogen. Phytopathology 96:33-45.

Champoiseau, P., Daugrois, J.-H., Pieretti, I., Cociancich, S., Royer, M., and Rott, P. 2006. High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe. Phytopathology 96:1081-1091.

Chen, Y.-Y., Wu, C.-H., Lin, J.-W., Weng, S.-F., and Tseng, Y.-H. 2010. Mutation of the gene encoding a major outer-membrane protein in *Xanthomonas campestris* pv. *campestris* causes pleiotropic effects, including loss of pathogenicity. Microbiology 156:2842-2854.

Daugrois, J.-H., Dumont, V., Champoiseau, P., Costet, L., Boisne-Noc, R., and Rott, P. 2003. Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*. Eur. J. Plant Pathol. 109:445-458.

Defeyter, R., C. I. Kado, and D. W. Gabriel. 1990. Small, stable shuttle vectors for use in *Xanthomonas*. Gene 88:65-72.

Dirienzo, J. M., Nakamura, K., and Inouye, M. 1978. The outer membrane proteins of Gram-negative bacteria : biosynthesis, assembly, and functions. Ann. Rev. Biochem. 47:481-532.

Gonzalez Barrios, A. F., Zuo, R., Ren, D., and Wood, T. K. 2005. Hha, YbaJ, and OmpA regulate *Escherichia coli* K12 biofilm formation and conjugation plasmids abolish motility. Biotechnol. Bioeng. 93:188-200.

Goryshin, I. Y., and Reznikoff , W. S. 1998. Tn*5 in vitro* transposition. J. Biol. Chem. 273:7367-7374.

Hashimi, S. M., Wall, M. K., Smith, A. B., Maxwell, A., and Birch, R. G. 2007. The phytotoxin albicidin is a novel inhibitor of DNA gyrase. Antimicrob. Agents Chemother. 51:181-187.

Hayward, A. C. 1960. A method for characterizing *Pseudomonas solanacearum*. Nature 186:405-406.

Kleckner, N. 1977. Translocatable elements in procaryotes. Cell 11:11-23.

Koebnik, R., Locher, K. P., and Van Gelder, P. 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol. Microbiol. 37:239-253.

Koike, H. 1968. Leaf scald of sugarcane in continental United States – a first report. Plant Dis. Rep. 52:646-649.

Laage, R., and Langosch, D. Strategies for prokaryotic expression of eukaryotic membrane proteins. 2001. Traffic 2:99-104.

Lee, J., Kang, S., Park, S. I., Woo, H.-J., and Kwon, W. 2004. Molecular cloning and characterization of the gene for outer membrane protein H in a *Pasteurella multocida* (D:4) isolate from pigs with atrophic rhinitis symptoms in Korea. J. Microbiol. Biotechnol. 14(6):1343-1349.

Leyns, F., De Cleene, M., Swings, J.-G., and De Ley, J. The host range of the genus *Xanthomonas*. Bot. Rev. 50(3):308-356.

Lin, J., Huang, S., and Zhang, Q. 2002. Outer membrane proteins: key players for bacterial adaptation to host niches. Microb. Infect. 4:325-331.

Matthysse, A. G., and Stump, A. J. 1976. The presence of *Agrobacterium tumefaciens* plasmid DNA in crown gall tumour cells. J. Gen. Microbiol. 95:9-16.

McClintock, B. 1948. Mutable loci in maize. Carnegie Inst. Wash. Year Book, Leo Baeck Institute. 47:155.

Mills, D. 1985. Transposon mutagenesis and its potential for studying virulence genes in plant pathogens. Annu. Rev. Phytopath. 23:297-320.

Mohan Nair, M. K., and Venkitanarayanan, K. 2007. Role of bacterial OmpA and host cytoskeleton in the invasion of human intestinal epithelial cells by *Enterobacter sakazakii*. Pediatr. Res. 62:664-669.

Osborn, M. J., and Wu, H. C. P. 1980. Proteins of the outer membrane of Gram-negative bacteria. Annu. Rev. Microbiol. 34:369-422.

Pan, Y.-B., Grisham, M. P., and Burner, D. M. 1997. A polymerase chain reaction protocol for the detection of *Xanthomonas albilineans*, the causal agent of sugarcane leaf scald disease. Plant Dis. 81:189-194.

pDRAW32 by AcaClone Software (http://www.acaclone.com)

Pieretti, I., Royer, M., Barbe, V., Carrere, S., Koebnik, R., Cociancich, S., Couloux, A., Darrasse, A., Gouzy, J., Jacques, M.-A., Lauber, E., Manceau, C., Mangenot, S., Poussier, S., Segurens, B., Szurek, B., Verdier, V., Arlat, M., and Rott, P. 2009. The complete genome of *Xanthomonas albilineans* provides new insights into the reductive genome evolution of the xylem-limited *Xanthomonadaceae*. BMC Genomics 10:616.

Ricaud, C., and Ryan, C. C. 1989. Leaf Scald. Pages 39-58 in: Diseases of Sugarcane, Major Diseases. Ricaud, C., Egan, B. T., Gillaspie Jr., A. G., and Hughes, C. G. Elsevier, Amsterdam, The Netherlands.

Reznikoff, W. S. 1993. The Tn*5* transposon. Annu. Rev. Microbiol. 47:945-963.

Reznikoff, W. S. 2008. Transposon Tn*5*. Annu. Rev. Genet. 42:269-286.

Roberts, A. P., Chandler, M., Courvalin, P., Guedon, G., Mullany, P., Pembroke, T., Rood, J. I., Smith, C. J., Summers, A. O., Tsuda, M., and Berg, D. 2008. Revised nomenclature for transposable genetic elements. Plasmid 60:167-173.

Rott, P. C., Costet, L., Davis, M. J., Frutos, R., and Gabriel, D. W.1996. At least two separate gene clusters are involved in albicidin production by *Xanthomonas albilineans*. J. Bacteriol. 178:4590-4596.

Rott, P., and Davis, M. J. 2000. Leaf Scald. Pages 38-44 in: A Guide to Sugarcane Diseases. P. Rott, R. A. Bailey, J. C. Comstock, B. J. Croft, and A. S. Saumtally, eds. La Librairie du Cirad, Montpellier, France.

Rott, P., Fleites, L., Marlow, G., Royer, M., and Gabriel, D. W. 2011. Identification of new candidate pathogenicity factors in the xylem-invading pathogen *Xanthomonas albilineans* by transposon mutagenesis. Mol. Plant-Microbe Interact. 24:594-605.

Sambrook, J. E., E. F. Fritsh, and T. A. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Song, T., Mika, F., Lindmark, B., Liu, Z., Schild, S., Bishop, A., Zhu, J., Camilli, A., Johansson, J., Vogel, J. and Wai, S. N. 2008. A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. Mol. Microbiol. 70(1):100-111.

Sperandio, V., Bailey, C., Giron, J. A., DiRita, V. J., Silveira, W. D., Vettore, A. L., and Kaper, J. B. 1996. Cloning and characterization of the gene encoding the OmpU outer membrane protein of *Vibrio cholerae*. Infect. Immun. 64(12):5406-5409.

Tseng, T.-T., Tyler, B. M., and Setubal, J. C. 2009. Protein secretion systems in bacterial-host associations, and their description in the gene ontology. BMC Microbiol. 9(Suppl 1):S2.

Vieira, J., and Messing, J. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene 100:189-194.

Vivien, E., Megissier, S., Pieretti, I., Cociancich, S., Frutos, R., Gabriel, D. W., Rott, P.C., and Royer, M. 2005. *Xanthomonas albilineans* HtpG is required for biosynthesis of the antibiotic and phytotoxin albicidin. FEMS Microbiol. Lett. 251:81-89.

Vivien, E., Pitorre, D., Cociancich, S., Pieretti, I.,Gabriel, D. W., Rott, P. C., and Royer, M. 2007. Heterologous production of albicidin: a promising approach to overproducing and characterizing this potent inhibitor of DNA gyrase. Antimicrob. Agents Chemother. 51(4):1549-1552.

Vizvaryova, M., Valkoa, D. 2004. Transposons – the useful genetic tools. Biologia, Bratislava. 59(3):309-318.

Wang, Y., and Kim, K. S. 2002. Role of OmpA and IbeB in *Escherichia coli* K1 invasion of brain microvascular endothelial cells *in vitro* and *in vivo*. Pediatr. Res. 51:559-563.

Wu, H.-H., Yang, Y.-Y., Hsieh, W.-S., Lee, C.-H., Leu, S.-J., and Chen, M.-R. 2009. OmpA is the critical component for *Escherichia coli* invasion-induced astrocyte activation. J. Neuropathol. Exp. Neurol. 68:677-690.

BIOGRAPHICAL SKETCH

Laura Ashley Fleites was born in Miami, Florida in 1983. She attended the School for Advanced Studies, a high school full-time dual enrollment program, which allowed her to enter the University of Florida as a junior. She obtained her Bachelor of Science in advertising with a minor in psychology in 2004. Shortly before graduating, she finally realized that advertising was not for her.

Her first job after graduating was at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry. She initially thought of this job as temporary until she could decide what direction to go in, but unbeknownst to her, she already found it. When the citrus she maintained exhibited strange, corky lesions, she brought a leaf to her boss, Dr. Ru Nguyen, who said “oh, that’s citrus scab, it’s a fungus…” and she was immediately intrigued. She told a coworker that she wanted to work in plant pathology, but was discouraged when the coworker said a degree in science would be necessary, and the bachelors of “science” in advertising probably wouldn’t suffice. Perhaps Dr. Xiao-an Sun could see the genuine interest and excitement in her when they discussed a position that opened up in the citrus diagnostic lab, because he hired her on the spot. She was on her way.

In 2008 she left DPI and joined Dr. Dean Gabriel’s lab as a technician, and in 2009 she began her studies as a master’s student in Dr. Gabriel’s lab. While there she had the great opportunity to attend meetings of the American Phytopathological Society, was involved in the Plant Pathology Graduate Student Association, helped produce the departmental newsletter, and made lifelong friends.