Site Directed Gene Disruption of rpfA in Xylella fastidiosa: Non-Biofilm Producing Mutants as an Approach to Eradication of Pierce's Disease in Grapevine

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Site Directed Gene Disruption of rpfA in *Xylella fastidiosa*

By

Janice Diane Thomas

A Master’s Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

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Biology with Molecular Biology Concentration

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SITE DIRECTED \textit{rpfA} GENE DISRUPTION IN \textit{Xylella fastidiosa}: NON-BIOFILM PRODUCING MUTANTS AS AN APPROACH TO ERADICATION OF PIERCE'S DISEASE IN GRAPEVINE

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Abstract

*Xylella fastidiosa*, a gram-negative, rod shaped bacterium, causes disease in many economically important plants including citrus, coffee, and grapevine. *Xylella fastidiosa* is xylem limited and is dependent on insect vectors for transmission. Pierce’s Disease of *Vitis vinifera* grape, transmitted by an insect vector, poses a serious threat to California vineyards today. The glassy-winged sharp-shooter, *Homalodisca coagulata*, has caused millions of dollars of damage to the California wine industry in just a few years. There is evidence that *Xylella fastidiosa* may share the molecular mechanisms through which the bacterium establishes infection with a closely related species, *Xanthomonas campestris*. A cluster of genes designated as regulation of pathogenicity factors are responsible for extracellular polysaccharide production and have been found in the genome of *Xanthomonas campestris* and *Xylella fastidiosa*. Site-directed disruption of regulation of pathogenicity factor A, using homologous recombination in the *Xylella fastidiosa* strains Temecula and Napa, exhibited a decrease in extracellular polysaccharide that resulted in the inhibition of biofilm formation.
Introduction

*Xylella fastidiosa* (*Xf*) is a rod-shaped, gram-negative bacterium, and a xylem limited plant pathogen having a diameter of 0.25 to 0.50 μm, a length of 1.0 to 4.0 μm. *Xf* is transmitted by insects and is responsible for major economic and crop losses globally including citrus variegated chlorosis (CVC), Pierce’s Disease (PD) results in leaf scorch of almond, coffee, elm, oak, plum, mulberry, maple, oleander, periwinkle and grapevine (da Silva *et al.*, 2003). Due to vascular occlusion, the main affliction in PD, the plant tissue suffers from water and nutrient depletion, resulting in leaf scorch, dried fruit and plant death (Newman *et al.*, 2003).

Currently, plant vascular diseases are untreatable and tend to be devastating to many economically important crops. Pierce’s Disease is a disease that has a major impact on the California wine industry and the Brazilian citrus and coffee industries at a cost of $14 billion in the past ten years (Newman, *et al.*, 2004). In California, prior to 1990, several members of the Cicadellidae family of insects, the Blue Green Sharpshooter, the Green sharpshooter, and the Red-headed Sharpshooter transmitted PD of grapevine (Purcell *et al.*, 2002). Transmission of the disease by these leaf-hoppers was limited because the sharpshooter is weak and only able to fly more than a few feet at a time (Ceri *et al.*, 2005).

The Glassy-winged Sharpshooter (GWSS) (*Homalodisca coagulata*) is native to the southeastern United States. It is a large dark brown insect, (12mm) with transparent wings and the ability to fly several miles. It is able to survive temperatures as low as 6°C, lays its eggs from February through August, and is the prime vector of the bacterium *Xf* in grapevine, almond and oleander (Jefferson,
The Glassy-winged sharpshooter was first observed along the southern coast of California in 1990 and has been increasing in number ever since (Newman et al., 2002). It is responsible for transmitting PD in grapevine with the most dramatic losses occurring in the Napa Valley and in parts of the San Joaquin Valley (Jefferson 2006). It is believed that the GWSS entered the region as eggs that had been deposited on plant tissues and transported from other infected regions in the United States (Newman et al., 2002). It is feared that PD might be spread as far north as Mendocino County resulting in a major epidemic throughout much of the state (Dow et al., 2000).

Prior to 1971, PD was believed to be caused by a virus due to the inability to isolate the PD pathogen from the xylem of diseased plants. Once refinements in the medium were optimized, circular colonies, smooth, white and convex with a diameter up to 1.0 mm were observed within an incubation period of two weeks (Davis and Purcell, 1978). Due to this plant pathogen's slow growth and difficult isolation, detection can be hampered. Sequences of the 16S rRNA gene for 18 different strains of *Xf* have been determined, all showing homology in at least three different targeted segments. Polymerase Chain Reaction (PCR), using highly specific primers, has been used to target and amplify these segments. This method of identification is currently being used in the field and in the laboratory to positively identify *Xf*, the causative agent of PD (da Silva-Stenico et al., 2003).

*Xylella fastidiosa* is transmitted to plant-hosts during xylem feeding by infected
GWSS. The GWSS is a voracious eater, consuming many times its body weight in fluids per day (Harrison et al., 2004). The bacteria form a rudimentary biofilm inside the gut of the insect that allows them to be sloughed off in aggregates, allowing indefinite infection of other plants when the insect is feeding. The insect injects the bacteria directly into plant tissue while feeding through its specula, where they colonize by irreversibly adhering to the biotic surface of the cells that line the xylem. The *Xf* bacterium can also move internally from cell to cell (Marques et al., 2002).

As the bacteria multiply in the xylem, diffusible molecular signals passed individual bacteria, a process called “quorum-sensing”, providing information about the growing density of the colony (da Silva et al., 2003). Once the number of microbes reaches a level that begins to deplete the available water and nutrient supply, an extracellular substance composed of an expolysaccharide (EPS), DNA and proteins forms a matrix (da Silva et al., 2003). This polymeric substance is referred to as biofilm and is responsible for vascular occlusion that leads to water and nutrient stress in the plant-host (Osimo et al., 2004).

Because *Xf* is confined to an environment containing a limited number of different nutrients, its genome contains the biosynthetic pathways necessary for growth on a restricted diet. L-glutamine is known to be an important nutrient requirement in *Xf* media (Leite et al., 2004). L-glutamine is the most abundant amino acid in the xylem fluid of grapevine along with relatively low concentrations of glucose, < 50 μM (Leite et al., 2004). Carbohydrate metabolism in *Xf* is believed to involve the Entner-Doudoroff pathway, not the glycolytic pathway found in most
bacteria (Facincani et al, 2003). Genes necessary for gluconeogenesis are also absent. These factors may play a role in the slow division rate observed in Xf.

The majority of microorganisms live attached to a surface, together in large numbers rather than in a liquid phase or planktonic state as isolated individual cells. This bacterial community referred to as biofilm can consist of more than one species and can form layers, clumps and ridges from an extracellular polymeric substance (EPS), a matrix made of polysaccharides, DNA and proteins (Harrison et al., 2005). As more information becomes available it is becoming clear that communal life has significant advantages for microorganisms. Synergistic interactions are favored by physical proximity even between different species, including the transfer of genetic material, sharing of metabolic by-products, an increase in antibiotic resistance, shelter from changes in the environment and protection from the immune system in infected hosts (Harrison et al., 2005).

A biofilm matrix, considered to be a hydrogel, has been likened to a complex polymer hydrated with water, many times its dry weight. The hydrogel properties of a biofilm allow it to confer properties that enable it to resist shear and water fluctuations in its environment (Jefferson, 2006). As fluids pass over these slimy assemblages of microbes remaining attached to a surface, they often form streamer-like appendages that break free, spreading the microbial community downstream. The Center for Disease Control and Prevention reported that biofilms are responsible for up to 70% of human bacterial infections including kidney infections, tooth and gum disease, pneumonias and bacterial infection associated with medical implants, ventilators and catheters (Harrison et al., 2005). They function as a protective shield
against antibodies and phagocytes, and high concentrations of antibiotics that would otherwise be lethal in smaller doses to planktonic counterparts (Harrison et al., 2005).

The minimal biofilm elimination concentration assay for physiology and genetics (MBEC P&G) was developed by Marques et al. as a way to rapidly screen biofilms for their sensitivity to anti-microbials. The MBEC P&G is a 96 well microtiter plate that includes peg-lids that promote biofilm formation. This assay enables drug development laboratories to evaluate the effectiveness of an antibiotic on the free-floating pathogen and its effectiveness on the same organism in a biofilm (Marques et al., 2004). During the development of this product, it was discovered that remarkable heterogeneity exists inside the biofilm. The microbes living closest to the surface are exposed to greater concentrations of oxygen and nutrients compared to those living in the center or at the substratum level. This allows the bacteria to grow at a faster rate as they approach the surface of the biofilm. (Harrison et al., 2005) It is believed that this may play a role in the defense mechanisms of the biofilm community because many antibiotics are targeted to fast growing cells. Since the biofilm matrix carries a negative charge, it can bind to positively charged antimicrobials and prevent them from reaching the bacteria living in the interior of the colony. Genetic and physiological diversity, a consequence of these dynamics, insure that some cells will survive the most challenging conditions (Harrison et al., 2005).

The ability of bacteria to sense their surroundings enables them to adjust their metabolic processes for efficient use of the available substrates. Gene expression
resulting from environmental stress in bacteria growing within a biofilm results in phenotypic heterogeneity. One interpretation of these phenomena is specialization or division of labor that can be compared to cellular differentiation in multicellular organisms (Marques and Ceri, 2002). Evidence is emerging that this division of labor is regulated within biofilms through intercellular communication by small molecules expressed and released by bacteria (Camili and Bassler, 2006). In general, homoserine lactones found in gram-negative bacteria and peptides in gram-positive bacteria are released and when at a critical concentration, will induce specific gene expression (Marques and Ceri, 2002). Because gene expression is altered as a result of high-density bacterial population, these auto-inducing signals have been termed “quorum-sensing” signals. Quorum-sensing may have also evolved for bacterial community cooperation as a tool to sequester its own secreted proteases and the sub sequential amino acids degraded by them (Marques and Ceri, 2002).

The Xf genome is comprised of two plasmids and one circular chromosome, approximately 2.5 megabases, encoding for genes that are involved in metabolic processes and virulence (da Silva et al., 2001). Xf was the first plant pathogen to be completely sequenced. It has 22 genes encoding enzymes and regulatory proteins involved in biosynthesis of EPS similar to that of xanthan gum produced by several bacterial species but shared the highest similarity with Xanthomonas campestris (Xc), another vascular plant pathogen responsible for black rot disease in cruciferous plants (da Silva et al., 2001). The Xf genome revealed a 12 kb DNA fragment containing nine open reading frames (ORF’s) in an operon structure that was
arranged in an order identical to the *Xc* gum operon, thus deriving its name, the fastidium gum operon (da Silva *et al.*, 2001).

The virulence of *Xc* is dependent on synthesis of extracellular enzymes and EPS xanthan. Xanthan is a polymer consisting of mannose-1,4-β-glucuronic acid-1,2-β-mannose-1,3-α-cellbiose consisting of pentasaccharide repeats (Osirio *et al.*, 2004). Pyruvic acid moiety may be found in some of the terminal mannoses and acetyl groups may be found in the two mannoses as 6-0 linkages (Osirio *et al.*, 1993). All the genes related to the synthesis of the precursor molecules that act as substrates for enzymes encoded by the gum operon are present in the *Xf* genome except *Gum G, I and L*. Gum I adds a mannose and Gum L and G add pyruvyl and acetyl groups to this mannose (Dow *et al.*, 2000). The absence of these genes does not appear to interfere with the rate of EPS production in *Xf*. This data would indicate that the *Xf* xanthan polymer is not a pentamer as observed with the *Xc* xanthan but most likely a tetramer (Dow *et al.*, 2000).

Several genes involved in the regulation of xanthan biosynthesis have been identified in *Xf* including six sequences homologous to the *rpf* genes in *Xc*. No other putative EPS regulatory genes were found in the *Xf* genome (Dow *et al.*, 2000). The genes included in this cluster, *rpfA, rpfC, rpf, rpfE, rpfF and rpfG*, encode elements of a regulatory system that involve the diffusible signal factor (DSF) encoded by *rpfB*. The DSF signaling system controls formation and dispersal of *Xc* biofilms by regulating the *gum* gene cluster (Dow *et al.*, 2000).

*RpfA* encodes for the aconitase protein in *Xc* and in *Xf*. Aconitase is a 113 kd protein consisting of α-helices and β-pleated sheets with two iron atoms in the active
Aconitase plays a critical role in the Krebs cycle, catalyzing the conversion of citrate to isocitrate (Dow et al., 2000). Sequence analysis of aconitase expression genes in bacteria suggests that the protein may exist in two forms. Homologous to eukaryote aconitase, one form is found in the cytoplasm of the cell where the protein has two functions: enzymatic and iron-responsive binding (Wilson et al., 1998). In conditions of iron depletion, aconitase activity exhibits iron-level dependency. 2,2'-dipyridyl and diethylenetriaminepenta-acetic acid (0.32 mM, DTPA) (Dow et al., 2000) and ethylene-di-O-hydroxyphenol (range of 12.5-100μM), (EDDS), are metal chelators known to exhibit iron binding, and inhibit biofilm formation in Xc and Staphylococcus epidermidis when added to the culture media (Borland and Deighton, 1993). It has recently been established that Xf grown in PW media, supplemented with 1000 μg/ml ethylenediaminetetraacetic acid (EDTA), 38 mg/ml EDDS, or 1.0 mg/ml lactoferrin exhibits inhibition of biofilm formation up to 99% (Koh and Toney, 2006). This provides strong evidence that rpfA in Xf acts much like rpfA in Xc; iron homeostasis is in part dependent on aconitase (rpfA) (Crossman and Dow, 2004).

In Xc, iron restriction and or the loss of aconitase, results in the loss of the ability to sense and regulate intracellular iron levels via the rpf gene cluster. This leads to loss of transcription and activity of some extracellular enzymes resulting in the inhibition of the production of EPS (Arruda et al., 2001). This is evidence that supports the theory that aconitase may have a bifunctional role similar to the eukaryote counterpart: as a Krebs cycle enzyme and as a regulator. Because Xc and Xf both expresss another form of aconitase, the acnB gene that expresses aconitate
hydrotase or aconitase B, normal growth conditions can take place when \textit{rpfA} is knocked-out or when the aconitase protein is inhibited with chealators (Dow et al., 1998).

This thesis addresses the question as to whether gene disruption of \textit{rpfA} would affect biofilm formation in the causative agent of PD, \textit{Xf} Napa and Temecula strains isolated from grapevine. A plasmid specifically designed for site-directed recombination in \textit{Xf} was employed referred to as pSP-3 (Marques et al., 2003). pSP-3 was constructed using the \textit{colE1} plasmid pBluescript KS (3.6 kb) and the kanamycin resistant gene from transposon Tn5 (1.0 kb) under the control of the \textit{Xylella} rRNA operon promoter region (4.5 kb). This non-replicative vector (pSP-1) was not capable of generating colonies without addition of pXF1.3, an indigenous \textit{Xylella} plasmid containing a functional replication origin (1.3 kb). The final pSP-3 construct is a 5.8 kb plasmid that can successfully replicate and obtain gene disruption in \textit{X. fastidiosa} (da silva et al., 2002).

\textbf{Materials and Methods}

\textit{Bacterial Strains and Growth Conditions}

Bacterial strains used for this study are the \textit{Xf} strain Napa (American Type Culture Collection (ATCC) # 700963) isolated in Napa County, CA (USDA Permit # 65800) and \textit{X. fastidiosa} strain Temecula (ATCC# 700964) isolated in Riverside County, CA (USDA Permit # 71373) both causative agents of Pierce’s Disease in grapevine. All cultures were maintained in ATCC PWG medium (4% phytone peptone, 1% trypticase peptone,
0.4% MgSO₄, 1.2% KH₂PO₄, 0.2% phenol red, 0.1% hemin chloride, 8% gelrite, 0.8% L-glutamine, 0.3% bovine serum albumin at pH 7.2) supplemented with 50 µg mL⁻¹ kanamycin when necessary. Glutamic acid was used in place of L-glutamine. All cultures were maintained in aerobic growth conditions (no shaking) at 28°C. E. coli, DH5α, One Shot, Invitrogen, Inc. (Carlsbad, CA, USA) was used for all cloning procedures.

**Identification of X. fastidiosa using 16S rRNA**

*Amplification of the 16S rRNA gene*

0.2 µM oligonucleotides Xf-S19 (5' CGG CAG CAC ATT GGT AGT A 3'), Xf-S21 (5' GCA AAT TGG CAC TCA GTA TCG3'), Xf-A21 (5' CGA TAC TGA GTG CCA ATT TGC 3'), and Xf-A19 (5' CTC CTC GCG GTT AAG CTA C 3'), [Invitrogen OligoPerfect™ Designer (Carlsbad, CA, USA)] were used to amplify three segments of DNA found in the Xf 16S rRNA gene with 20 ng of DNA template, 200 µM deoxynucleoside triphosphates, 1.5 mM MgCl₂, 2.0 U Taq DNA polymerase, Invitrogen Inc. (Carlsbad, CA, USA) in a 50 µl total reaction volume (Figure 1). Polymerase chain reaction was initiated with three minutes of denaturation at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 30 sec of primer annealing at 55°C, and 2 min of extension at 72°C, followed by final extension at 72°C for 7 min (da Silva-Stenico et al., 2003). 1% Tris-Acetate-EDTA (TAE) agarose gel electrophoresis was used for DNA fragment size analysis followed by Ethidium Bromide staining for visualization.
Construction of the pSP-3(rpfA-639) plasmid

Truncation and amplification of rpfA

The pSP-3 suicide-vector used for site-directed recombination and rpfA gene disruption was kindly provided by Dr. M.V. Marques, university São Paulo, Brazil (Marques et al., 2003). A truncated rpfA gene segment was obtained by PCR using oligonucleotides Xf-acon-59 ($^{5'}$ CGC GTC TAG AAC TAT GCG CGA TTC ATT $^{3'}$) and Xf-acon-698 ($^{5'}$ TTT TGG GCC CAT CAT CGT GGT ATG GCT $^{3'}$) [Invitrogen OligoPerfect™ Designer (Carlsbad, CA, USA)]. These primers encode the restriction sites XhoI (Xf acon 59) and ApaI (Xf acon 698) and were used to amplify the first 639 bps of the Xf rpfA gene (da Silva et al., 2002). The PCR reactions were preformed in a final reaction volume of 50 μl using 3 μl of concentrated Xf cells resuspended in 50 μl 50mM Tris EDTA (TE) buffer pH 7.2, 0.5 μmol of each primer and 25 μl Go Taq® Green Master Mix 2X (Promega Corporation, Woods Hollow, WI, USA). The reactions were carried out in 30 cycles of 15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C, followed by 2 min at 72°C.

The amplified 639 bp truncated rpfA gene from both Napa and Temecula Xf strains were analyzed using 1% TAE agarose gel electrophoresis (Fig. 1). The DNA fragments were extracted from the agarose gel for purification with Pure Link™ Quick Gel Extraction Kit (Invitrogen™, Carlsbad, CA, USA). A final yield of the truncated rpfA639 bp PCR amplification product of 10 ng/μL was determined by measuring the OD of the extract at 260 nm.

Digestion of pSP-3 and rpfA639
The precipitated and dried pSP-3 vector was resuspended in 20 μl of TE buffer. 1 μl was used to transform *Escherichia coli* DH5α cells using heat shock (-4°C and 42°C). Plasmids were harvested using Pure Link™ Quick Plasmid Miniprep Kit (Invitrogen™ Carlsbad, CA, USA) at a final concentration of 1.0 μg/mL.

The pSP-3 plasmid and the *rpfA*639 DNA fragment were digested with 1 Unit of restriction enzymes XhoI and ApaI for one hour at 25°C (New England Biolabs, Boston, MA, USA) respectively in a total reaction volume of 50 μl. The digests were analyzed using 1% TAE agarose gel electrophoresis and purified using Pure Link™ Quick Gel Extraction Kit.

**Cloning of *rpfA*639**

The digested 5.8 kb pSP-3 vector (10 μg/mL) and the *rpfA*639 insert (25 μg/mL) were ligated with 1 Unit of T4 DNA Ligase (New England Biolabs, Boston, MA, USA) in a 50 μl final reaction volume, for 48 hours at 4°C followed by 1 hour at 25°C. The resulting 6.4 kb plasmid was analyzed with 1% TAE agarose gel electrophoresis. A yield of 100 μg/mL was determined by measuring the optical density (OD) of the DNA extract at 260 nm. The pSP-3(*rpfA*639) construct was purified using the Pure Link™ Quick Gel Extraction Kit with a final yield of 50 μl and stored in TE buffer at -20°C. The final yield of purified plasmid was 10 ng/μl.

**Transformation of *X. fastidiosa* with pSP-3(*rpfA*639)**

**Preparation of *X. fastidiosa* for electroporation**

Fifty ng/μl of the pSP-3(*rpfA*639) plasmid was transformed into *Xf* strains Napa and Temecula by electroporation. The cells were prepared by inoculating a single
colony from a freshly streaked PW agar plate into 2 mL of liquid PW medium (minus MgSO₄, plus L-histidine [0.1%, wt. vol.] by vortexing. The cell suspension was incubated at 28°C without agitation for 4 days. Three tenths of one mL of this culture was used to inoculate 30 mL of fresh PW medium and incubated under the same conditions for an additional 4 days (Monteiro et al., 2001).

The culture was transferred to a chilled 50 mL polypropylene tube and the cells were collected by centrifugation at 2,600 X g for 15 min at 4°C. The pelleted cells were washed twice in 30 mL chilled ultrapure (milli-Q: Millipore) water and washed once in chilled, filtered sterilized 10% glycerol. The final cell pellet was resuspended in 0.3 mL 10% glycerol and stored on ice (Monteiro et al., 2002).

**Electroporation of X. fastidiosa**

An 80µl aliquot of the final cell suspension was mixed with 10 ng of DNA in 5 µl of TE Buffer (10mM Tris-HCl [pH 8.0], 1mM EDTA) and transferred to cold 0.2-cm electroporation cuvettes and kept on ice for 1 minute. The cells were electroporated at 2.5 kV, 25 µF and 200 Ω to generate a pulse of approximately 6 msec (Monteiro et al., 2001). After electroporation, the cells were resuspended in 1 mL of PW (plus L-histidine [0.1% wt/vol]) and incubated at 28°C for 6 hours without agitation. 250µl of cell suspension was plated on PW agar supplemented with kanamycin (50µg/mL) for transformant selection, and incubated at 28°C for 14 days. The kanamycin resistant colonies were collected separately and inoculated into PW medium supplemented with kanamycin (50µg/mL) (Monteiro et al., 2001).
Analysis of the Transformants

PCR of the X. fastidiosa rpfA recombination site

Confirmation of recombination and integration of the plasmid pSP-3(rpfA639) into the rpfA gene in the X. fastidiosa transformants was carried out by PCR using genomic DNA of the putative disrupted clones and of strains Temecula and Napa. The reactions were carried out using oligonucleotides 16S-19 (5'-CGG CAG CAC ATT GGT AGT A) and XfAconFull1R (5'-TAA GCA GGC TTG CCA GTG TT'T') (Invitrogen OligoPerfect™ Designer (Carlsbad, CA, USA)). The PCR amplifications were performed in a total reaction volume of 50 µl using using 0.5 µmol of each primer and 25 µl Go Taq® Green Master Mix 2X (Promega Corporation, Woods Hollow, WI, USA). The reactions were carried out in 30 cycles of 15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C, followed by 2 min at 72°C (Promega corporation, Woods Hollow, WI, USA). 1% TAE agarose gel electrophoresis was used to analyze the PCR reaction (Figure 4). Pure Link™ Quick Gel Extraction Kit was used to purify the amplified DNA fragment. Measuring OD of the extraction at 260 nm determined a final yield of 50 ng/µl of DNA.

DNA sequencing of recombination site

Ten µl of 10mM Tris buffer containing 0.5µg of the recombination site DNA amplification was sent to Alpha BioLab Inc. (Sunnyvale, CA, USA) for DNA sequencing of the rpfA recombination site with Oligonucleotides, Xf-S19 (5'-CGG CAG CAC ATT GGT AGT A3') and Xf rpfA706 (5'-TTG CCG CTT CGG CTT CGA3') at a concentration of 10 µM [Invitrogen OligoPerfect™ Designer (Carlsbad,
Alpha Biolab Inc. prepared and sequenced the samples. A fluorescence-based capillary electrophoresis genetic analyzer, ABI 3730xl/3130xl [Applied Biosystems Inc. (Foster City, CA)] was used for the DNA sequencing.

**RT-PCR of rpfA**

Total RNA was isolated from Xf Temecula and Napa wild type and rpfA mutants using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) with a final reaction volume of 30 µl. A final yield 50 ng/µl was collected. RT-PCR was performed using Qiagen® OneStep RT-PCR Kit (Qiagen, Valencia, CA, USA) to verify complete knockout of the rpfA gene. The samples used were as follows; (1) negative control (no template), (2) Xf Temecula wild type (XftWt), Xf Napa (XfNwt), (3) Xf Temecula rpfA mutant 1-4 (XfTrpfA-/-), and Xf Napa rpfA mutant 1-4. 1.0 ng/µl was used in a total reaction volume of 50 µl. PCR was used to amplify the resulting cDNA with oligonucleotides Xf rpfA F (5'- CTGCAA GCC ATC CAA ACG TA 3') and Xf rpfA R (5'- TAA GCA GGC TTG CCA GTG TT 3') [Invitrogen OligoPerfect™ Designer (Carlsbad, CA, USA)] at a final concentration of 0.6 µM. The thermocycling conditions were set to 30 min at 50°C, 15 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C, followed by 10 min at 72°C. 1% TAE agarose gel electrophoresis was used to analyze the results of the RT-PCR reaction.
Biofilm quantitation of the X. fastidiosa rpfA (-/-) mutants

PVC microtiter plate assay

A volume of 30 mLs of PW media supplemented with 50µg/mL kanamycin, when appropriate, was inoculated with a single colony of XfTwt, XfNwt, XfT-/-, and XfN-/- grown on PW agar plates. The culture was incubated at 28°C without shaking until the optical density (OD) at 600nm reached 0.5 (approximately 1 X 10^6 cells/mL). The cell turbidity was measured using a microtiter plate reader, Spectra Max 384 Plus, Molecular Devices (Sunnyvale, CA, USA). After vortexing, 200 µl of culture was added to 96 well, PVC, microtiter plates, Beckton Dickinson (Franklin Lakes, NJ, USA). Each plate contained eight wells of PW media (without Xf as a negative control and eight wells of each Xf strain, mutant and wild type for a total of five experimental conditions. The plates were covered and incubated overnight at 28°C without shaking. Cell turbidity was monitored until OD_{600} reached at least 0.65 (approximately 1 X 10^8 cells/mL). Medium was removed from the wells and plated on PW agar. The wells were then washed with distilled water five times to remove sessile bacteria. The plates were then air-dried for 45 min after which 200 mL of a 1% crystal violet solution was added to each well. After staining, the plates were washed with distilled water five times. The quantitative analysis of biofilm was determined by adding 200 µl of 95% ethanol to the wells. 100 µl from each well was transferred to a new microtiter plate and level (OD_{595}) of crystal violet in the destaining solution was determined at 595 nm (McLandsborough et al., 2002). The microtiter assay was performed three times for all strains and mutants.
**MBEC microtiter plate assay**

Bacterial culture for *Xf* wild type strains and mutants were prepared as described previously for biofilm quantitation. After cells reached an OD$_{600}$ of 0.5, 150 µl of negative control and bacterial cultures were added to eight wells each in the 96 well microtiter MBEC™ Device, MBEC bioproducts (Calvary, Canada). The plate was covered with the MBEC peg lid and incubated overnight at 28°C without shaking.

The peg lid was removed from the plate and rinsed in a new plate containing 200 µl of 0.9% saline (X 3). 200 µl of fresh PW media was added to the wells of a new plate for recovery of the bacteria growing in the biofilm covered pegs. The peg lid was placed on the recovery plate and sonicated for approximately 5 minutes (Branson Ultrasonic cleaner model 3510). After the peg-lid was discarded, the recovery plate was covered and incubated overnight at 28°C without shaking. The OD$_{600}$ was measured and bacteria plated on PW agar to quantitate the amount of bacteria that had been growing in the biofilm.

**Electron microscopy of *Xf* wild type and rpfA mutants**

Sterile, microscope coverslips were placed in bacterial cultures containing *Xf* wild type or *rpfA* mutants and incubated at 28°C for 48 hours without shaking. The samples were fixed in 5% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.2 for one hour, followed by an increasing gradient of methanol (10% to 100%). The samples were then sputter-coated with 13nm of palladium and viewed using a Hitachi 230 scanning electron microscope at 8 kv (Purcell et al., 2002).
Results and Discussion

Identification of Xf

Agarose gel electrophoresis revealed three DNA fragments, approximately 603 bp, 745 bp, and 1348 bp (Figure 2). These PCR products were of the expected size. Amplification of the three segments found on the Xf 16S rRNA gene confirmed the identification of both the Xf Temecula and Napa strains.

Verification of construction of the rpfA(639) plasmid

Agarose gel electrophoresis revealed the PCR amplification of the truncated rpfA 639 DNA bp fragment with the incorporated ApaI and XhoI restriction sites to be approximately 650 bp (Figure 3). After digestion of the pSP-3 plasmid and the rpfA 639 bp fragment with ApaI and XhoI, gel electrophoresis revealed the approximate 600bp and 5800 bp fragments. After ligation of the rpfA639kb insert into the pSP-3 vector, a DNA fragment of approximately 6400 bp was observed (Figure 5). These results would indicate that the pSP-3rpfA639 suicide vector was successfully constructed (Figure 6).

Electroporation of Xf with pSP-3rpfA639 yield transformants

After 6 days of incubation, pSP-3rpfA(639) the plated, transformed colonies became visible on the PW agar. The Napa strain yielded 2 colonies and the Temecula strain yielded a total of 6 colonies.

Recombination was confirmed as early as the first passage in both Xf Napa and Temecula strains using PCR amplification of the recombination site. The primers were designed to amplify 0.9kb the newly incorporated 16S rRNA gene in the pSP-
3rpffA639 plasmid joined to the last 2000 bp of the disrupted rpfA gene. A segment of DNA approximately 3000 bp was observed using agarose gel electrophoresis in the recombinant mutants compared to no amplification of DNA in the Xf wild type strain (Figure 7). This data supports a pSP-3rpffA639 site-directed recombination event took place in both strains of Xf within hours of the transformation. The transformation of this construct was highly successful with an average of 600 transformants/μg DNA.

Total RNA was isolated from the mutants wild-type Xf to demonstrate that the recombinants were not expressing rpfA. Reverse transcription PCR was carried out to verify the absence of aconitase using the isolated RNA as a template. Agarose gel electrophoresis analysis showed the absence of rpfA mRNA transcription in the recombinant mutants, and the presence of rpfA mRNA transcription in the wild type strains by the absence and presence of the DNA fragment approximately 2700 bp in size respectively (Figure 8).

DNA sequencing of the recombination site from the 16S rRNA site to the first 60 bp of the second half of the disrupted rpfA gene, revealed a recombination site to be present at the predicted site (Figure 9).

**Biofilm Quantitation**

The crystal violet assay exhibited no significant difference in OD595 when comparing the de-staining solutions of both strains of Xf rpfA mutants to the negative control wells (Figure 10). Bacterial counts of the mutants ranging from 10^7 to 10^8 taken from the microtiter plate wells and plated on PW media, confirmed that the rpfA mutation was not a lethal gene mutation (Figure 11).
The MBEC microtiter plate biofilm quantitation confirmed the absence of biofilm formation in both strains of the \( Xf \ rpfA \) mutants and presence of biofilm formation of both strains of the wild type. The \( OD_{600} \) of the \( Xf \ rpfA \) mutants was unchanged after incubation overnight at 28°C without shaking without a significant difference to the \( OD_{600} \) of the control media (Figure 12).

\textit{Electron microscopy of Xf wild type and rpfA mutants}

Electron microscopy of slides containing \( Xf \) wild-type revealed many bacteria, grouped together (Figure 13). The Expolysaccharide matrix was present and water channels were observed. Slides containing the \( Xf \ rpfA(-) \) showed no expolysaccharide matrix and only a few isolated bacteria (Figure 14).

\textbf{Conclusions}

Although the Dutch microscopist Antonie van Leewenhoek first observed the resistant nature of biofilm growing on his dentures, it has only been during the past 15 to 20 years that the physiology of these bacterial communities have been examined. This new perspective of microbial communal living is beginning to have fundamental implications in medicine, industry and ecology.

\textit{Xylella fastidiosa rpfA} encodes the protein aconitase much like the very homologous plant pathogen \( Xc \). Regulation of pathogenicity factor A is part of the \( rpf \) gene cluster in \( Xc \) that is a regulator of pathogenicity factors that result in biofilm formation during stressful environmental conditions. Because of the homologous nature of the pathogenicity factors in \( Xc \) and \( Xf \), and the absence of any other pathogenic genes, it was suggested that the EPS pathways were very similar.
Evidence is emerging that aconitase in \( Xc \) and \( Xf \) both have an iron-related regulatory role due to the inability to produce EPS when iron is sequestered (Daniels et al., 1998). The lack of iron availability yields the same result as silencing \( rpfA \); inhibition of biofilm formation in both species (Koh and Toney, 2006). Iron homeostasis in \( Xc \) is in part, dependent on aconitase and that the loss of aconitase affects transcription and activity of some extracellular enzymes and reduces the amount of EPS produced by the organism (Daniels et al., 1998). The results observed in \( Xf \) closely mimic the behavior of \( Xc \) under the same conditions, thus providing greater evidence that \( rpfA \) in \( Xf \) may provide the same gene activity and controls as does \( rpfA \) in \( Xc \). This also suggests aconitase in \( Xf \) also has a bifunctional role: a Krebs cycle enzyme and a regulator.
Bibliography


Harrison, J., Turner, L., Marques, L., Ceri, H., *Biofilms A new understanding of these microbial communities is driving a revolution that may transform the science of microbiology*, *American Scientist*, 93 (2005) 456-462.


Figure 1. Illustration of 16S rRNA promoter PCR primers for *Xylella fastidiosa* identification.

![Diagram of PCR primers and their expected amplicon sizes](image)

Figure 2. 1% TAE Agarose Gel Electrophoresis of 16S rRNA gene segment amplification present in *Xylella fastidiosa* strains.

![Image of agarose gel electrophoresis with bands at 1348, 745, and 605 bp](image)
Figure 3. 1% TAE Agarose gel electrophoresis of *rpfA* truncation PCR amplification
Figure 4. 1% TAE Agarose gel electrophoresis of pSP-3rpfA(639) completed construct and pSP-3 plasmid without insert.
Figure 5. Construction of the pSP-3 rpfA(639) plasmid by insertion of the truncated rpfA639 gene into the Xylella fastidiosa specific pSP-3 vecto.
Figure 6. Illustration of the recombination site of pSP-3\textit{rpfA}(639) inserted within \textit{rpfA}.

![Diagram of recombination site](image)

Figure 7. 1% TAE Agarose gel electrophoresis of PCR amplification of recombination site (pSP-3\textit{rpfA}(639) integrated into \textit{rpfA} gene).

![Agarose gel electrophoresis](image)
Figure 8. 1% TAE Agarose gel electrophoresis of RT-PCR of rpfA comparing the Xylella fastidiosa wild-type (rpfA is present) and the rpfA mutants (rpfA is not present).

Figure 9. DNA sequencing of recombination site showing the 16S rRNA insert integrated into the rpfA gene at bp 700.

16S rRNA A21

*Recombination site

rpfA 640-700

tggcactcagttacctaaacgccttgctggggagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacggatggtgagtttttaatctgatcatcatttctctctgttgagctagctgcagcgcgaagacgg
Figure 10. Crystal violet microtiter 96 well plate biofilm assay results (side view). Crystal violet staining of the expolysaccharides indicates the presence of biofilm. The wells containing the *Xylella fastidiosa* wild-type are stained purple where the wells containing the *rpfA* mutants are colorless, indicating biofilm formation inhibition.

![Image of Crystal violet microtiter 96 well plate biofilm assay results (side view).](image)

Plated bacterial count

- Xf Temecula mut.: $0.68(\text{OD}_{600}) \times 10^8$
- Xf Nappa mut.: $0.61(\text{OD}_{600}) \times 10^7$
- Xf Tem wt: $0.74(\text{OD}_{600}) \times 10^9$
- Negative control: $0.00(\text{OD}_{600}) \times 10^0$

Figure 11. Crystal violet microtiter 96 well plate biofilm assay (top view).

![Image of Crystal violet microtiter 96 well plate biofilm assay (top view).](image)

Day 10 10 9 8 7 6 5 4 3 2 1 (-)

Xf Nappa wt Xf Nappa Mutant
Figure 12. MBEC microtiter 96 well plate assay results comparing formation of biofilm in *Xylella fastidiosa* wild-type and mutants. This experiment was done in triplicate with standard deviation values ranging ± 0.02.
Figure 13. Scanning electron microscopy of *Xylella fastidiosa* wild-type attached to glass slides, growing in PW media at 8.0 kv with a magnification of X 5k and at a 30 degree angle. Biofilm matrix is visible with bacterial colony.

Figure 14. Scanning electron microscopy of *Xylella fastidiosa* *rpfA* mutants under identical conditions. Biofilm matrix is absent with only a few isolated bacteria present.