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Paratransgenic control of Pierce's disease

Arinder Arora

Arinder K. Arora
Candidate

Biology
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Dr. Eric S. Loker, Chairperson

Dr. Ravi V. Durvasula

Dr. Coenraad M. Adema

Dr. Cristina D. Takacs-Vesbach

PARATRANSGENIC CONTROL OF PIERCE'S DISEASE

by

ARINDER K. ARORA

M.S., Cell Molecular and Developmental Biology, University of California, 2010

DISSERTATION

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Dedication

To my first teacher, my mom.

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Abstract of Dissertation

Pierce's Disease (PD) is a deadly grapevine disease, which is caused by a Gram negative bacterium, *Xylella fastidiosa* and transmitted by leafhoppers commonly known as sharpshooters, including the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*). We developed paratransgenic control of PD using a commensal bacterium *Pantoea agglomerans* E325. The GWSSs acquired enhanced green fluorescent (EGFP)-expressing *P. agglomerans* from an artificial feeding system and the bacteria persisted within the insect foregut for two weeks. *P. agglomerans was* selected as the paratransgenic control agent based on its persistence within the sharpshooter foregut, a niche also inhabited by *X. fastidiosa*. *P. agglomerans* lines secreting two antimicrobial peptides (AMPs), melittin and scorpine-like molecule (SLM), were generated to accomplish transmission blockage. These AMPs were selected after testing their toxicity to *P. agglomerans* and *X. fastidiosa*. Our experiments indicated that both melittin and SLM are more toxic to *X. fastidiosa* compared to *P. agglomerans*. Paratransgenic GWSSs, which have acquired an AMP-expressing *P. agglomerans* prior to *X. fastidiosa*

acquisition, displayed a lower competence to acquire the pathogen in comparison to control insects. These paratransgenic sharpshooters failed to transmit *X. fastidiosa* to naive grapevines. This is the first instance, wherein paratransgenesis has been demonstrated to block PD transmission. We established melittin and SLM expression within the paratransgenic sharpshooters via Western blot and confirmed that transmission blockage was as a direct result of AMPs expressed by *P. agglomerans* within the insect gut.

Subsequently, we also engineered calcium-alginate based microparticles to reduce environmental contamination during field application of genetically modified bacteria. The insects were able to acquire the bacteria from plants painted with *P. agglomerans* - containing micropaticles. Amongst the tested microparticles (engineered using 1%, 2% and 3% alginate) the ones engineered using 1% alginate resulted in the highest *P. agglomerans* acquisition (51.8%) by the sharpshooters. These microparticles will be a means to take the technology from a lab to the field.

Active molecules, which target specifically the pathogen only, have an advantage that they won't affect the natural flora and fauna. Keeping this in mind we have engineered antibodies that bind to *X. fastidiosa* surface protein mopB using ribosomal display methodology. These antibodies bind specifically to *X. fastidiosa* surface protein. In the future we can express these antibodies individually or as chimeras in combination with AMPs via *P. agglomerans*. Expression of two active molecules will also decrease the rate of resistance development and will increase the useful life span of this technology in the field.

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Chapter 1: Introduction

Arthropod transmitted pathogens and parasites take a huge toll on economics as well as on human health by causing diseases in humans, livestock and plants. Malaria killed 627,000 people in 2012 and caused losses of USD 12 billion (CDC: http://www.cdc.gov/malaria/malaria_worldwide/impact.html). Ten million individuals get infected with Chagas disease and a whopping $ 7 billion are lost to this disease every year (Lee *et al.,* 2013). Nagana, a disease of livestock, kills 3 million cattle every year (Enyaru *et al.,* 2014). Vector borne plant diseases destroy the agricultural crops and the damages caused by these diseases run into billions of dollars. According to an estimate by Hodges and Spreen (2012) citrus greening caused a loss of $1.709 billion over a period of 5 years (from 2006/07-2010/11). These examples show that vector borne diseases are a big threat to human population as well as to the global food security. Spread of these diseases is kept under check by suppressing vector populations using chemical insecticides. Though chemical insecticides give a quick kill, they cause environmental contamination and their prolonged use results in resistance development in the target insects (Pimentel, 2005). Biological control using natural enemies is environmentally friendly, but does not always curb insect populations sufficiently to impact disease transmission. Ineffectiveness of biological control and concerns over insecticide use demand development of novel methods and strategies to control disease spread. Paratransgenesis is one such strategy that can be used to control different vector borne diseases.

Paratransgenesis, a "Trojan Horse" strategy, intends to block disease transmission by delivering anti-pathogen molecules within the vector-gut using genetically modified

symbionts or commensal bacteria. In this approach, a symbiont is genetically modified to produce anti-pathogen molecules after its isolation from the vector gut. The anti-pathogen molecules-expressing symbiont is then reintroduced into the vector gut. The
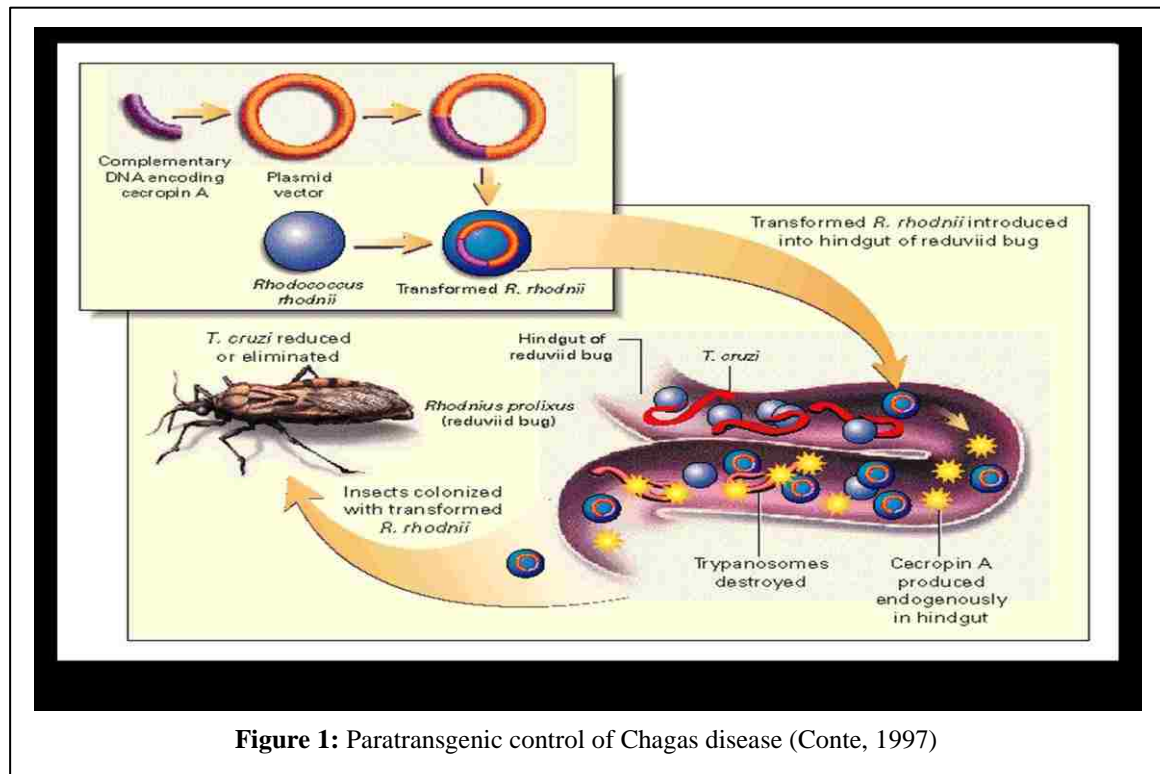
Figure 1: Paratransgenic control of Chagas disease (Conte, 1997)

paratransgenic vectors carrying genetically modified symbiont lose their ability to transmit the disease owing to presence of pathogen interfering molecules.

Durvasula *et al.* (1997) pioneered the paratransgenesis approach and demonstrated its applicability in blocking Chagas disease (Figure 1). Chagas disease is a human ailment caused by the parasite, *Trypanosoma cruzi*, an infection transmitted by bugs of the family Triatominae. *Rhodnius prolixus* is one of the important Triatomine bugs that carries *T. cruzi* in its hindgut and transmits the parasite to human. Durvasuala *et al.* (1997) identified a *R. prolixus* bacterial symbiont *Rhodococcus rhodnii* as a paratransgenic control agent. They isolated and genetically modified the symbiont to produce an antimicrobial peptide (AMP) - cecropin A. By introducing the genetically

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modified *R. rhodnii* into the insect gut they generated paratransgenic *R. prolixus*. They

reported that thus generated paratransgenic vectors were refractory to *T. cruzi* acquisition

and 5 out of 7 paratransgenic insects were free of *T. cruzi*.

In 2012, paratransgenic mosquitoes were generated using genetically modified

Pantoea agglomerans, which inhabits the mosquito midgut. Wang *et al*. (2012)

engineered eight *P. agglomerans* lines each expressing one of these eight molecules - (i)

two copies of the 12-aa SM1 peptide [(SM1)2], a molecule that binds to mosquito midgut

and blocks ookinete invasion; (ii) a mutant phospholipase mPLA2, which inhibits

ookinete invasion into hemolymph; (iii) pbs21scFv-Shiva1 -a single chain monoclonal

antibody (scFv) targeting the *Plasmodium berghei* major ookinete surface and lytic

peptide Shiva1 chimera; (iv) a chitinase propeptide (Pro) that inhibits the chitinase

enzyme; (v) Shiva1 - a synthetic antiparasitic peptide; (vi) scorpine, an antimicrobial

peptide from *Pandinus imperator* (vii) (EPIP)4 four copies of the *Plasmodium* enolase–

plasminogen interaction peptide that inhibits mosquito midgut invasion by preventing

plasminogen binding to the ookinete surface; and (viii) a fusion peptide composed of Pro

and EPIP (Pro:EPIP). They reported > 85% decrease in *P. falciparum* carriage by these

paratransgenic mosquitoes. These studies show that symbiotic/commensal bacteria can be

used to deliver antiparasitic/antipathogenic molecules within an insect gut to decease

parasite carriage by a vector.

Paratransgenesis has an advantage that the arthropod vectors themselves are not

genetically modified, which is a very tedious process in comparison to genetically

altering a bacterium. However, not every microbial symbiont can be used as a

paratransgenic control agent and a symbiotic bacterium has to meet certain requirements

before it can be used as a paratransgenic control agent. Beard *et al.* (2002) listed these criteria:

1. An appropriate symbiotic association must exist between the bacteria and the vector.

2. Only culturable and genetically alterable symbionts can be used for paratransgenic control of a disease.

3. Genetically modified symbionts should remain stable long enough to deliver anti-pathogenic molecules within the insect gut.

4. Fitness of the genetically modified symbionts should not be compromised.

5. Normal functions of the symbiont should not be affected by introduction of new gene.

6. Anti-pathogen molecules released from the genetically modified symbionts should interact effectively with the target pathogen to interfere with its life cycle.

7. A method must exist or should be developed for field application of genetically modified symbionts.

We developed a paratransgenesis strategy to control Pierce's Disease (PD) of grapevine. PD is a devastating disease of grapes and is a major threat to the $3.2 billion grape industry of California (Hoemmen *et al.*, 2013).

Figure 2: *Homalodisca vitripennis*

PD is caused by a Gram negative, rod shaped, nonflagellate and xylem-limited bacterium - *Xylella fastidiosa*. *X. fastidiosa* is transmitted by several species of xylem feeding leafhoppers commonly known as sharpshooters namely - the blue green sharpshooter

(*Graphocephala atropunctata*), the green sharpshooter (*Draeculacephala minerva*), the black winged sharpshooter (*Oncometopia nigricans*), the red sharpshooter (*Xyphon fulgida*) and the glassy-winged sharpshooter (*Homalodisca vitripennis*) (Fig. 2) (Janes and Obradovic, 2012). In addition to infecting grapevines, *X. fastidiosa* has been reported to infect more than 150 plant species, including economically important plants such as citrus, coffee, almond and alfalfa species. (Hopkins, 1989; Janes and Obradovic, 2012).

Xylella fastidiosa

PD was first documented by Newton Pierce in late 19th century (Pierce, 1892). For almost a century, PD was misdiagnosed as a disease caused first by a virus and later by a mycoplasma-like organism (MLO), until the bacterium was first cultured by Davis *et al.* (1978). *X. fastidiosa* inhabits the xylem vessels of the grapevines and other plants. Xylem vessels are groups of small chambers, which are joined by pit membrane. These conduits carry water and minerals from the plant roots to the leaves. After entering into xylem vessels *X. fastidiosa* multiplies and forms biofilms. The bacteria moves basipetally as well as acropetally colonizing other parts of infected plants. Pit membranes, which join the vessels, are made up of pectins and other

Figure 3: Plant showing PD symptoms

polysaccharides and *X. fastidiosa* digests the membranes using enzymes such as polygalatouronase (PG) to enter into new vessels (Roper *et al.*, 2007).

Xylella biofilms interfere with water movement and result in water-stress like symptoms such as chlorosis, followed by tip necrosis (Fig. 3). Later the leaves become brown and fall down leaving the petioles behind, also known as match stick symptoms. The infected plant remains stunted and produces shriveled berries. The wood matures irregularly and the plant dies within 1-5 years after the infection (Janes and Obradovic, 2012).

Though *X. fastidiosa* colonizes the xylem vessels, it rarely clogs all the vessels. Newman *et al.* (2003) reported that even in highly symptomatic grape plants only 10-15 % of xylem vessels were completely clogged and amongst different tissues, petioles had the highest percent of clogged vessels.

Biofilm formation, quorum sensing, movement of bacteria and ability of *X. fastidiosa* to break down the pit membrane have been implicated in disease development.

X. fastidiosa produces diffusible signaling factor (DSF), a quorum sensing molecule, which mediates cell to cell signaling and plays a role in biofilm formation. DSF is encoded by a gene *RpfF*. *X. fastidiosa RpfF* mutants lack the ability to form a biofilm. However, these mutants were found to be hypervirulent in grape plants (Newman *et al.,* 2004). This hypervirulence was attributed to both increased bacterial movement as well as higher bacterial multiplication in the xylem vessels of the plant as compared to wild type *X. fastidiosa* (Chatterjee *et al.,* 2008).

Within the *RpfF* gene cluster, another gene *RpfC* is also present and its product acts as a *RpfF* regulator. *RpfC* mutants hyperexpressed rpfF and in turn overproduce DSF. These mutants were deficient in movement as compared to wild type *X. fastidiosa* in the xylem vessels of grape plant and multiply at a lower rate as compared to wild type

X. fastidiosa. Subsequently, *RpfC* mutants displayed a lower virulence in comparison to the wild type *X. fastidiosa* as well as the *RpfF* mutants (Chatterjee *et al.,* 2008).

Type IV pili located on the pole of *X. fastidiosa* cells are involved in twitching motility and migration. The mutants lacking type IV pili have been reported to be deficient in twitching motility (Meng *et al.,* 2005). Basipetal movement (against the flow of water) of these mutants in grape plants was significantly retarded compared to the wild type, and the type IV pilli mutants showed hypovirulence.

Additionally, hemogluttin proteins hxfA and hxfB appear to have a role in attachment and biofilms formation. The mutants lacking hxfA and hxfB expression were found to be hypervirulent because of their reduced binding to the vessel walls, which in turn caused an increased and faster spread of the bacteria inside the xylem vessels of the plant (Guilhabert and Kirckpatrick, 2005).

As mentioned earlier *X. fastidiosa* produces polyglactouronase (PG) to break down pit membrane. Roper *et al.* (2007) generated *X. fastidiosa* mutants that lacked PG expression. When inoculated in grapevines these mutants failed to cross pit membranes and stayed in the vessel, in which they were inoculated, and failed to produce any PD symptoms.

From the above examples it can be inferred that pathogenicity/virulence is an interplay between attachment, biofilm formation and movement of bacteria within the xylem vessels. Strong attachment to the xylem vessels restricts the movement of the cells, which interferes with systemic infections. Mutants that attach strongly to the walls in general are less virulent. On the other hand the mutants which are hypermotile and bind loosely to the xylem walls are hypervirulent.

Biology of *H. vitripennis*

      H. vitripennis is the most important vector amongst the sharpshooter-vectors that carry *X. fastidiosa*. Similar to other sharpshooters *H. vitripennis* transmits *X. fastidiosa* in semi-persistent manner, though the exact mechanism of transmission is not known. *H. vitripennis* acquires *X. fastidiosa* from the xylem vessels of an infected plant during feeding. *X. fastidiosa* adheres and multiplies in the precibarium and cibarial region of the insect foregut where it forms biofims. Both adult and nymphs are capable of acquiring and transmitting *X. fastidiosa.* Once infected adult sharpshooters can harbor *X. fastidiosa* in their foregut for their entire life, whereas the nymphs lose the bacteria along with the cuticular lining of their foregut during molting. The nymphs have to reacquire the bacteria to become infective again. Both adults and nymphs transmit *X. fastidiosa* to naive plants without any latent period (Almeida and Purcell, 2003).

      H. vitripennis has a transmission efficiency of ~20%. This is low compared to other sharpshooters such as *Graphocephala atropunctata*, which transmits *X. fastidiosa* with an efficiency of >90% (Almeida and Purcell, 2003; Hill and Purcell, 1995). However, the GWSS makes up for this deficiency with high fecundity, long-range mobility and ability to feed on hard wood (Redak *et al.*, 2004). Feeding on hard wood allows *H. vitripennis* to inoculate the bacterium away from the green portion of the plant, which usually gets pruned during winter. These infections become chronic and persist in the plants over winter. Sharpshooters such as *G. atropunctata* feed on the green part of the plants only, which usually gets pruned during winter. This pruning helps the plant to get rid of the infections. Thus sharpshooter feeding preferences play a major role in

deciding whether the infections will persist into next summer or not (Almeida and Purcell, 2003; Redak *et al.*, 2004).

X. fastidiosa binds to and multiplies in the foregut of the insects. This is the only niche other than plant xylem where bacteria survive. Binding to the foregut cuticle plays an important role in survival and transmission of *X. fastidiosa*. Killiny and Almeida (2009) demonstrated that *X. fastidiosa* binds to different carbohydrates with different affinities. They tested different *X. fastidiosa* mutants and reported that mutants lacking hemagglutnin-like proteins hxfA and hxfB expression lost their ability to bind to extracts of *G. atropunctata* foregut. In their transmission experiment *hxfA* and hxfB mutants were acquired at low rates in comparison to wild type *X. fastidiosa*, owing to their decreased initial adhesion to insect foregut. The poor early colonization also diminished transmission of *hxfA* and *hxfB* mutants compared to wild type *X. fastidiosa*.

Different molecules that interfere with *X. fastidiosa* binding to the sharpshooter foregut can be used to block the disease transmission. Killiney *et al.* (2012) conducted competition essays using different carbohydrates and *X. fastidiosa* surface protein binding antibodies to block disease transmission by interfering with *X. fastidiosa* attachment to the insect foregut. *X. fastidiosa* treated with GlcNAc, chitobiose (GlcNAc)$_2$ and chitotriose (GlcNAc)$_3$ and antibodies specific to afimbrial adhesins (XadA1 and XadA2) and Hxfs (hemagglutunins) were transmitted at lower rates compared to controls. This emphasized the importance of *X. fastidiosa* binding to the insect foregut in transmission. This is in line with our hypothesis that we can block transmission by targeting *X. fastidiosa*-GWSS interactions within the sharpshooter foregut utilizing a genetically modified symbiont, which produces anti-*Xylella* molecules.

<u>Disease Control</u>

Different methods are used or under development to save the grapevines from the menace of PD. A majority of grape growers rely on imidcloprid, a systemic insecticide, to keep *H. vitripennis* populations low. However, recent reports show that different insect species are developing resistance against imidacloprid (Zhao *et al.*, 1995; Grafius and Bishop, 1996; Prabhaker *et al.*, 1997; vanToor *et al.*, 2008; Gorman *et al.*, 2008; Matsumara *et al.*, 2008; Wen *et al.*, 2009), raising a red flag on over-reliance on a single chemical. Additionally, researchers have tested an egg parasitoid, a mymarid wasp, for its efficacy to control GWSS population in Southern California and it resulted in 30% egg parasitism only (Pilkington *et al.*, 2005).

Transgenic grape varieties, which either interfere with *X. fastidiosa*'s ability to cross xylem vessels by digesting pit membrane or produce antimicrobial peptides-scFv chimeras, have also been developed. *X. fastidiosa* secretes an enzyme polyglactouronase (PG) to digest pit membrane to further its spread within xylem vessels of a plant. Grape vines expressing pear polygalactouronase-inhibiting protein (PGIP) were developed by Aguero *et al*. (2005). These PGIP expressing grapevines interfered with the activity of PG produced by *X. fastidiosa* and exhibited resistance to *X. fastidiosa* infections. Dandekar *et al.* (2012) employed a different approach and developed transgenic grape plants that expressed cecropin A-anti mopB-antibody chimeras. Cecropin A, an antimicrobial peptide (AMP), was the active molecule against *X. fastidiosa* in that chimera, whereas anti-mopB antibody provided specificity. These transgenic lines harbored lower *X. fastidiosa* CFUs and were less symptomatic in comparison to the

control line. These lines are still being tested under field conditions before they can be released.

Insecticides, despite the concerns, are the main weapon against the vector. Until now biocontrol has not offered reliable control of *H. vitripennis*. Transgenic grape lines require uprooting and replacement of whole vineyards and this is not a cost effective
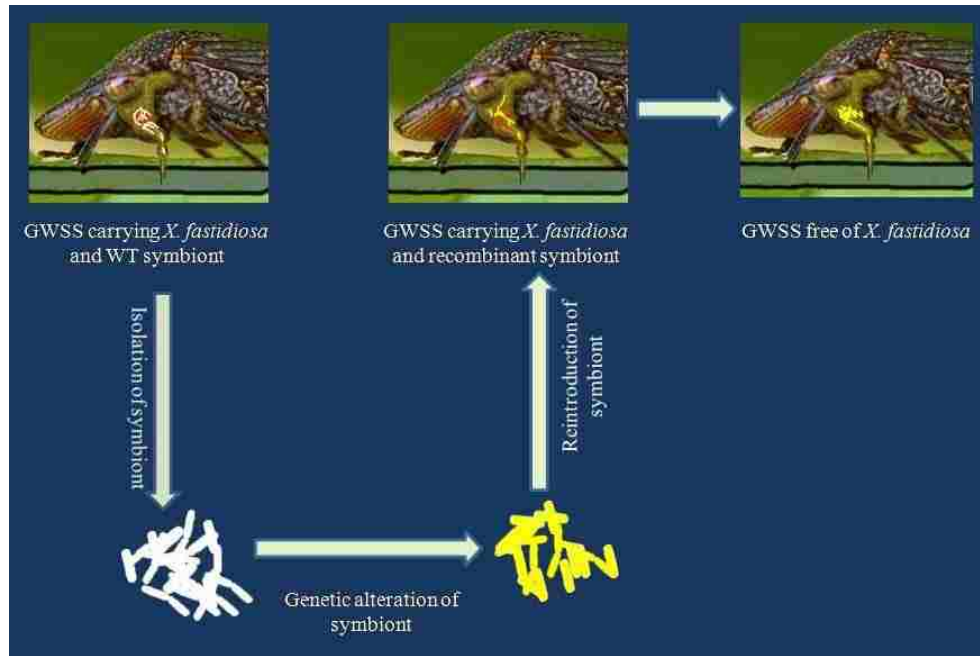
Figure 4. Paratransgenic control of Pierce's disease

measure. Therefore it is crucial to develop new strategies, which can be used to control PD and other diseases caused by *X. fastidiosa* in other plant species. This dissertation focuses on blocking transmission of *X. fastidiosa* by GWSS utilizing paratransgenesis (Figure 4) with the following specific aims:

(i) Identification of *P. agglomerans* as a paratransgenic control agent for PD

(ii) Development of a viable and safe field release strategy

(iii) Achieving paratransgenic control of PD (Proof of concept)

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iv) Engineering of *X. fastidiosa* surface specific antibodies that can be used to control PD by paratransgenesis of *P. agglomerans*

The paratransgenesis approach was first developed about two decades ago and has not been applied in the field yet due to concerns over environmental contamination by the released recombinant bacteria. The second chapter of this dissertation is focused on the identification of *P. agglomerans* as a paratransgneic control agent for PD as well as development of a method for field dispersal of genetically engineered *P. agglomerans* lines. It describes binding and persistence of *P. agglomerans* inside the insect forgut, a niche also colonized by *X. fastidiosa*. Further, the second chapter includes engineering of calcium-alginate microparticles, which can be employed for field release of genetically engineered *P. agglomerans*. We have submitted a manuscript describing the development of Calcium alginate microparticles, which has been accepted and published in *BMC Biotechnology* (Arora *et al*., 2015).

Chapter three is the heart of the dissertation and focuses on development of paratransgenic *H. vitripennis* refractory to *X. fastidiosa*. After identification of *P. agglomerans* as a paratransgenic control agent, the next step was to identify and express anti-*Xylella* molecules via *P. agglomerans* and utilize those genetically modified *P. agglomerans* lines to block disease transmission. The third chapter identifies two antimicrobial peptides (AMPs), scorpine-like molecule (SLM) and melittin, as active molecules against *X. fastidiosa*. The *Escherichia coli* hemolysin secretion system was used to engineer *P. agglomerans* lines that can produce and secrete the active AMPs. This chapter also includes generation of paratrangenic sharpshooters that were refractory to *X. fastidiosa* acquisition and did not transmit the pathogen.

We have used two AMPs as active molecules to block *X. fastidiosa* transmission. However, AMPs are not specific and can kill a number of different microbes and utilization of these molecules in the field may result in development of resistance. Identification and engineering of other anti-*Xylella* molecules, which can be expressed in tandem with these AMPs will reduce the risk of resistance development. Single chain antibodies (scFv) are such molecules that are specific and can be used in tandem with AMPs. The focus of the fourth chapter is engineering of scFvs that bind to mopB, a surface protein of *X. fastidiosa*. A manuscript illustrating engineering anti-mopB antibodies has been published in *Applied and Environmental Microbiology* (Azizi *et al.*, 2012). In future, we can engineer *P. agglomerans* lines that will express these scFvs individually or in tandem with AMPs to block PD transmission.

Chapter five is the summary of all the chapters. It brings together all the aspects of PD paratransgenic control and conclude the dissertation. It also talks about the future application of PD paratransgenesis.

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Chapter 2: A Delivery System for Field Application of Paratransgenic Control.

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Chapter 2: A Delivery System for Field Application of Paratransgenic Control

<u>**Abstract**</u>

Background

  As an alternative to chemical pesticides, paratransgenesis relies on transformation of symbiotic bacteria of an arthropod vector to deliver molecules that disrupt pathogen transmission. For over a decade paratransgenesis has remained a laboratory-based endeavor owing to regulatory concerns regarding introduction of transformed microorganisms into the environment. To facilitate field application of paratransgenic strategies, risk mitigation approaches that address environmental contamination and gene spread must be developed.

Results

  Using biopolymer manipulation, we introduce a novel microencapsulation platform for containment and targeted delivery of engineered bacteria to the gut of a disease-transmitting arthropod. We demonstrate the first proof of principle of targeted delivery of EPA-approved *Pantoea agglomerans* E325 in a paratransgenic system to control spread of Pierce's Disease by glassy-winged sharpshooters, (GWSS, *Homalodisca vitripennis*) under simulated field conditions. Engineered microcapsules may address regulatory concerns regarding containment of recombinant bacteria and environmental spread of foreign genetic material and may represent an important step in translating paratransgenic science beyond the lab and into the field.

Conclusions

We present, for the first time, a microencapsulation strategy to deliver recombinant bacteria to an insect and demonstrate targeted release of bacteria into the physiologically relevant region of the insect gut. This is a first step toward addressing concerns related to field application of recombinant bacteria. Engineered microparticles may decrease environmental contamination, horizontal gene transfer and competition with native species by acting as a barrier between recombinant bacteria and the environment.

Keywords

Paratransgenesis, Microencapsulation, Pierce's Disease, *Xylella fastidiosa*, *Homalodisca vitripennis*, *Pantoea agglomerans*, Calcium-alginate.

<u>Background</u>

Arthropod-borne diseases remain a major threat to global health and exact a huge toll on agriculture and food security. These diseases are largely controlled through use of insecticides that reduce insect populations. Environmental applications of these chemicals may be associated with toxic residues and emergence of target insect resistance. Alternatives to insecticide-based control include paratransgenic manipulation of insects with genetically engineered bacteria that deliver transmission-blocking molecules to disrupt pathogens within the arthropod vector [1–7]. Though several paratransgenic insect systems are under development, field use of this strategy has not yet been realized, largely due to lack of delivery methods that target transformed bacteria to the arthropod while minimizing collateral spread of foreign DNA. The United States Environmental

Protection Agency (EPA) has issued guidelines for introduction of genetically engineered (GE) species that state that GE organisms must: (a) Be contained to specific environments of introduction, (b) Not out-compete native species for resources, (c) Minimize foreign gene contamination (horizontal gene transfer) into native gene pools [8].

Here we report a novel strategy for delivery of genetically-engineered bacteria in a paratransgenic system that targets the glassy-winged sharpshooter (*Homalodisca vitripennis*), a pest of grapes and citrus that spreads the pathogen, *Xylella fastidiosa* [9–12]. Using simple and inexpensive materials for bioencapsulation [13–16] of the engineered symbiotic bacterium, *Pantoea agglomerans*, we demonstrate targeting of the sharpshooter, *H. vitripennis*, under controlled conditions with an alginate hydrogel that is tuned to release its bacterial payload during xylem flow into the foregut of the insect. By deploying a microencapsulation system that permits gated delivery of the bacterial payload to the arthropod, while greatly minimizing release in the environment, we believe that robust field-applicable technologies for paratransgenic control of arthropod-borne diseases will be possible.

Results

A paratransgenic model for the glassy-winged sharpshooter (GWSS)

Pierce's Disease (PD) of grapevines, caused by the bacterium *Xylella fastidiosa*, is a devastating disease of grapes that threatens grape production worth $3.2 billion and wine production worth $18.5 billion in California [17, 18]. Paratransgenic sharpshooters can be an answer to the threat posed by PD. Early attempts to establish paratransgenic

sharpshooters were focused on the GWSS symbiont *Alcaligenes xylosoxidans denitrificans* [10]. However, concerns that this bacterium may be a nosocomial human pathogen led us to a grape endophyte bacterium, *Pantoea agglomerans* E325, currently approved by EPA for control of fire blight in apples and pears [19]. We hypothesized that the sharpshooter can acquire *P. agglomerans* in the same way as it acquires *X. fastidiosa*. To test this we genetically engineered this bacterium to express enhanced green fluorescent protein (EGFP) and ampicillin resistance and used an artificial feeding system (AFS) to deliver recombinant bacteria to GWSS [20]. The GWSS was able to acquire the engineered *P. agglomerans* from the artificial feeding system and EGFP-expressing *P. agglomerans* was found binding to the pre-cibarial and cibarial regions of the insect foregut (Figure 1B). We further demonstrated that over a 15-day period, which is one third of the insect's lifespan, EGFP-expressing *P. agglomerans* persisted in the insect foregut primarily colonizing the pre-cibarial and cibarial regions (Figure 2B). We tested 522 sharpshooters over two seasons to study *P. agglomerans* persistence inside the GWSS foregut and observed 42.2% foregut colonization with EGFP-expressing *P. agglomerans* over a period of 15 days. Carriage of *P. agglomerans* in the foregut of sharpshooters was not affected by time (p=0.1979; chi-squared test). This confirmed that, once acquired by the insect, *P. agglomerans* persisted in the foregut for at least two weeks. Colonization with EGFP-expressing *P. agglomerans* occurs in the same spatial niche as the pathogen *X. fastidiosa* (Figure1B) [21, 22], suggesting that *P. agglomerans* may be used as a delivery vehicle for paratransgenic control of Pierce's Disease.
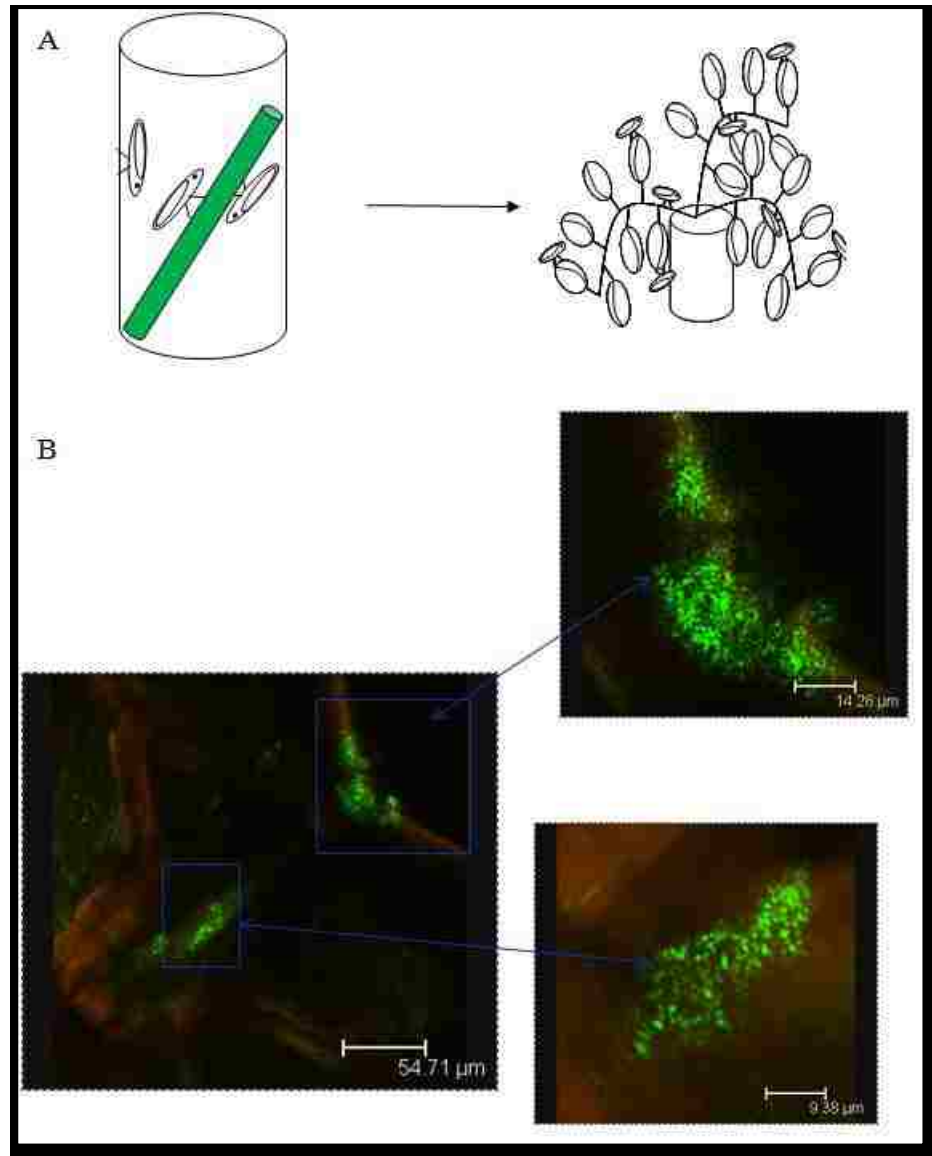
Figure 1 Glassy-winged sharpshooters acquired EGFP-expressing *P. agglomerans* from an artificial feeding system.

(**A**) Glassy-winged sharpshooters were allowed to feed on *P. agglomerans* through artificial feeding system for 48 hours. These insect were then kept on naive plants for 24 hours to flush out the unbound bacteria from the foregut before dissection. (**B**) Image showing EGFP expressing *P. agglomerans* colonizing the foregut of GWSS. Inset: A higher magnification of same image showing *P. agglomerans* colonizing cibarium and precibarium of the GWSS.
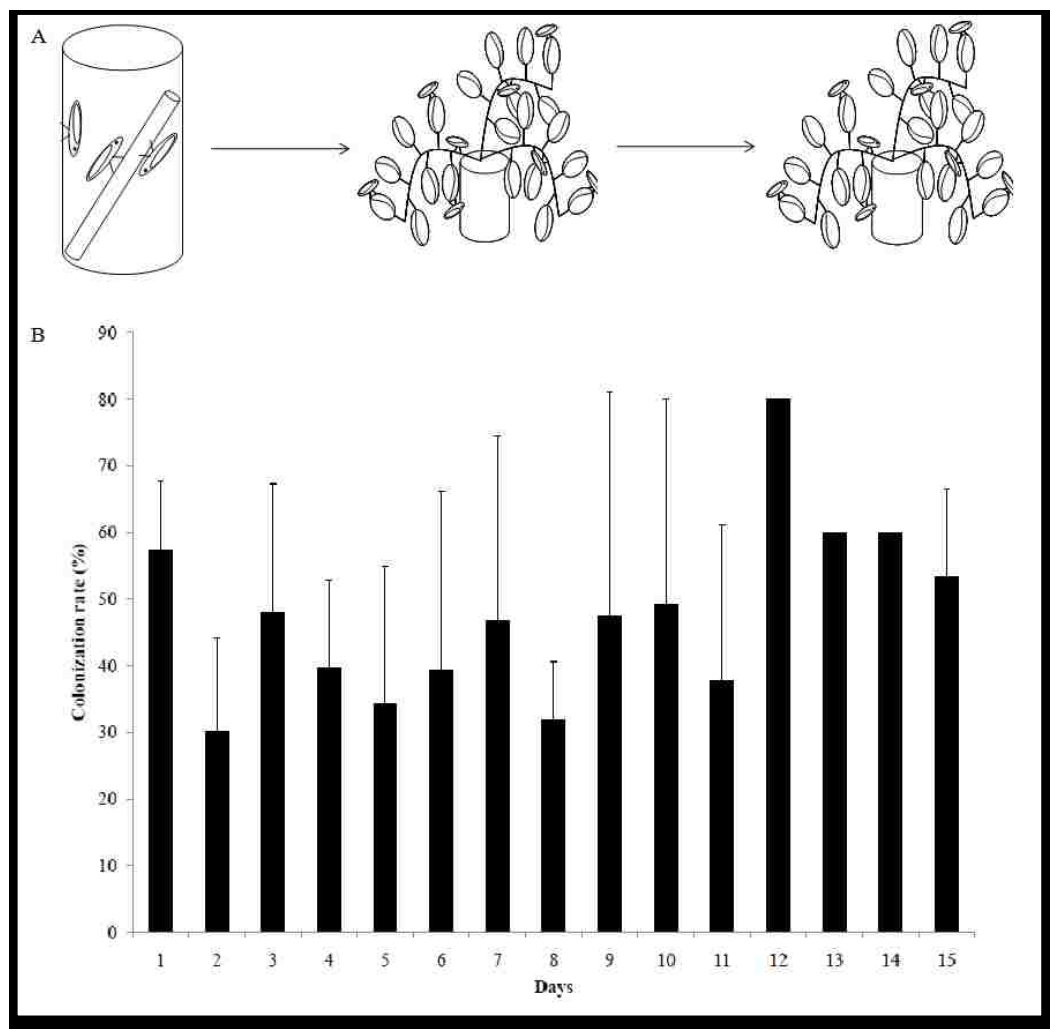
Figure 2 EGFP-expressing *P. agglomerans* persisted inside the foregut of the glassy-winged sharpshooter.

(**A**) GWSS were fed EGFP-expressing *P. agglomerans* via an artificial feeding system for 48 hours. The insects were moved to naive grape plant for 24 hours to wash out unattached bacteria. They were subsequently housed on another naive grape plant for up to 15 days. Insects were collected daily, and processed as described. Homogenates were plated on LB plates to determine the presence of *P. agglomerans*. (**B**) Percent of GWSS carrying EGFP-expressing *P. agglomerans* over a period of 15 days. No difference in proportions of GWSS carrying EGFP-expressing *P. agglomerans* was observed. *p*=0.1979 by chi-squared test for homogeneity.

Microencapsulation strategy for paratransgenic control

We developed a biopolymer microencapsulation system for paratransgenic manipulation of sharpshooters with 4 overall goals: (1) encapsulation of transgenic bacteria to keep them alive, contained and prevent horizontal gene transfer to environmental bacteria (2) release of bacterial payload from capsules tuned to specific physiologic conditions within the arthropod to minimize environmental contamination (3) release of bacteria from the capsules to colonize the target region of the sharpshooter where pathogens reside and (4) use of encapsulation materials that are inert in the insect without adverse physiological impact.

A suitable polymer

We have developed a calcium-alginate microcapsule for containment of EGFP-expressing *P. agglomerans* (Figure 3). To ensure that the particles would fit in the proboscis of the sharpshooter, we used a modified aerosolization-coascervation process to generate micron-sized particles. The particle size varied from 6-90 μm, with 57% of particles in the range of 22-44 μm (Additional File 3: Figure S3). By altering the particle composition, we were able to achieve rapid and sustained release of bacterial payload in response to sudden hydration. Calcium-alginate based microparticles (in absence or presence of glycerol) resulted in sustained release of bacteria over a period of 7 days in response to hydration, which was significantly higher than calcium/barium-alginate microparticles (Additional File 4: Figure S4, p<0.0001). Maximum release of *P. agglomerans* (CFUs = 1.03×10^5) was observed from calcium-alginate microparticles with 10% glycerol over a period of 7 days, while minimum (CFUs=7.33) release was observed from calcium/barium-alginate microparticles. Based on these results we decided
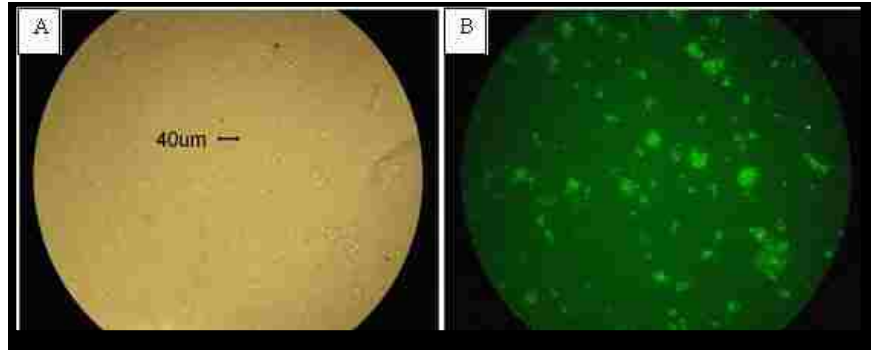
Figure 3 Microparticles containing EGFP-expressing *P. agglomerans*.

(**A**) Calcium-alginate microparticles containing EGFP-expressing *P. agglomerans*(600X). (**B**) Same field under fluorescence. (600X).
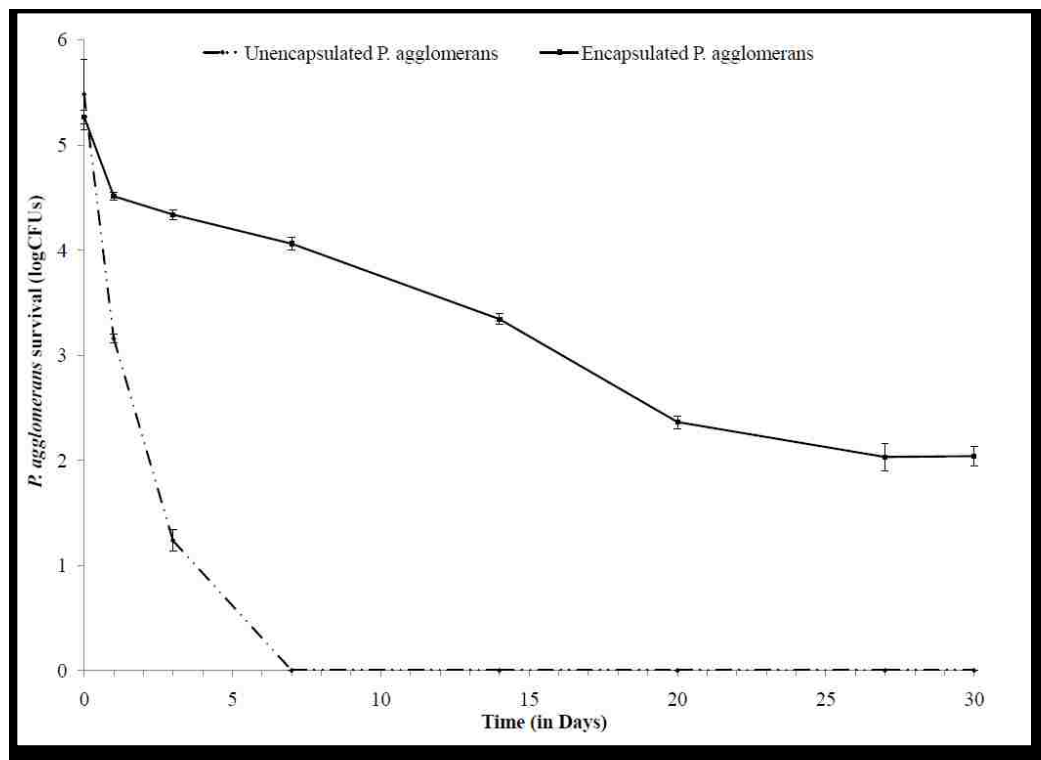
Figure 4 Bacterial survival under extreme dehydration.

Encapsulation of *P. agglomerans* significantly increases bacterial viability compared to unencapsulated controls during desiccating conditions. Unencapsulated *P. agglomerans* died in a week after dehydration, while encapsulated *P. agglomerans* survived for a month.

to engineer calcium-alginate based microparticles for acquisition experiments to achieve

a quick as well as sustained release of bacteria inside the insect foregut after acquisition

of microparticles by the sharpshooters during xylem feeding.

The encapsulation process protected *P. agglomerans* from desiccating conditions,

which are expected during periods of coating on plants prior to ingestion by the

sharpshooter (Figure 4). Under extreme desiccation 100% of unencapsulated *P.*

agglomerans died within 7 days; on the other hand only 2 log decrease in colony forming

units (CFUs) was observed when encapsulated *P. agglomerans* were exposed to

desiccation for the same time period (Figure 4, *p*<0.001). We further observed that

calcium-alginate microparticles were able to keep *P. agglomerans* alive for 30 days,

though the number of CFUs in microparticles decreased over time. This survival of

bacteria during desiccation is important, as similar conditions are expected during

summer in grape growing areas of Southern California.

Addition of 0.5-1.0% of India ink to the particle confers high-level UV-resistance,

gain a desirable quality during periods of application on plants in the summer time

(Figure 5). Exposure of both encapsulated and unencapsulated *P. agglomerans* in the

absence of India ink to UVC resulted in 100% killing in 20 minutes. There was no

significant reduction in CFUs over a period of 60 minutes when *P. agglomerans* mixed

with alginate and India ink were exposed to UVC (Figure 5, *p*=0.051). However, when

Ca-alginate microparticles containing India ink were exposed to UVC there was one log

reduction in *P. agglomerans* CFUs in the first 5 minutes, (Figure 5) and CFU numbers

remained steady thereafter for next 55 minutes. We speculate that bacteria present on the
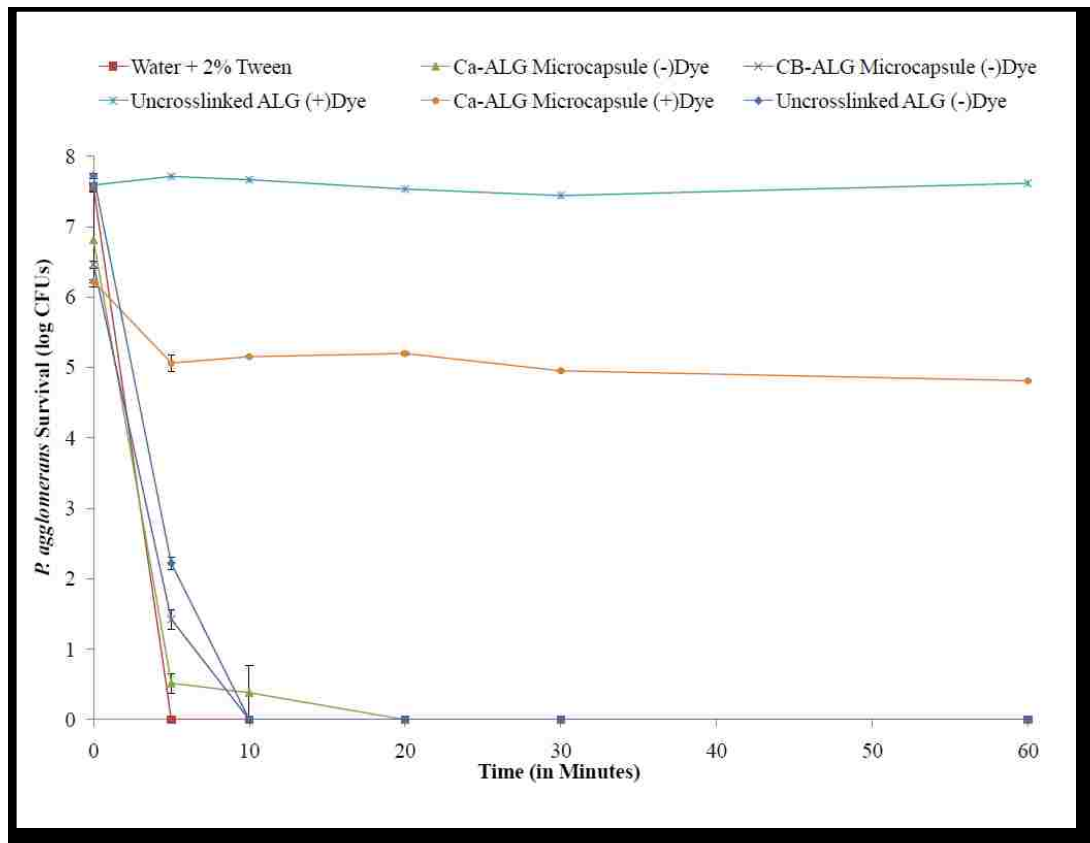
Figure 5 *P. agglomerans* **survival after exposure to UVC light.**

Encapsulated and unencapsulated *P. agglomerans* in the absence of dye were killed after exposure to UVC within 20 minutes, while both encapsulated and unencapsulated *P. agglomerans* in the presence of dye were able to withstand UVC exposure for 60 minutes.

surface of microparticles were exposed to UVC and were killed in the first 5 minutes of exposure, but the bacteria embedded in the microparticles remained protected.

We believe that calcium-alginate-10% glycerol hydrogel particles with India ink, which protected against dehydration and UVC, are ideally suited to deliver transgenic *P. agglomerans* under field conditions to the glassy-winged sharpshooter. We hypothesized that these particles will swell during xylem flow and release the bacterial payload into the foregut of feeding insects.

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A proof-of-concept

Glassy-winged sharpshooters were able to acquire *P. agglomerans* from alginate-based microparticles painted on grape plants. In a control group, GWSS fed on plants painted with unencapsulated *P. agglomerans*, 85.7% of the insects acquired *P. agglomerans*, which was significantly higher than the number of GWSS that acquired *P. agglomerans* from plants painted with encapsulated *P. agglomerans* (Figure 6, *p*=0.016). Amongst the groups of sharpshooters fed on encapsulated *P. agglomerans* maximum colonization was observed in insects acquiring *P. agglomerans* from 1% alginate-based microparticles (51.8%) followed by 2% (38.5%) and 3% (35.7%) alginate-based microparticles. Though an increase in alginate concentration decreased *P. agglomerans* acquisition by the GWSS, it was not statistically different between three tested concentrations of alginate - 1%, 2% and 3% (Figure 6, *p*=0.476). Unencapsulated treatments resulted in the maximum CFUs per insect head (mean CFUs = 245.4±80.4) and minimum CFUs per head were observed in treatments using 3% alginate microcapsules (mean CFUs = 52.1±22.7) (Figure 7). We did not observe significant difference between *Pantoea* CFUs acquired by the insects from microparticles engineered with 1%, 2% or 3% alginate (Figure 7, *p*=0.33).

Though the association between alginate concentration and *Pantoea* acquisition did not attain statistical significance, there was a trend toward decreased *Pantoea* levels in GWSS with increasing alginate in microparticles. This suggests that manipulation of polymer composition may alter levels of bacterial colonization in the sharpshooter foregut.
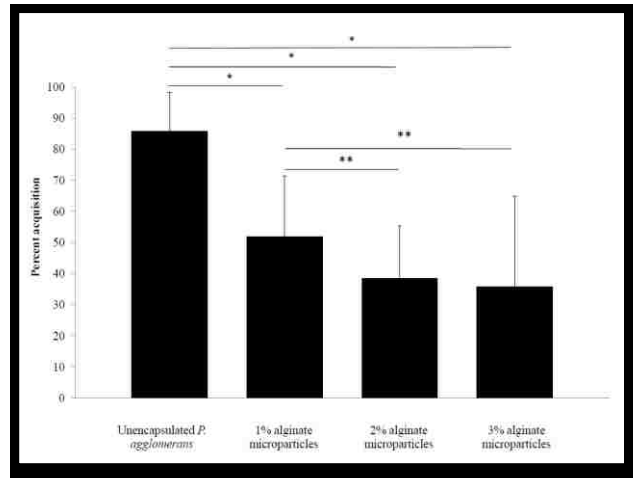
Figure 6 Glassy-winged sharpshooters acquired EGFP-expressing *P. agglomerans* from calcium alginate microparticles.

Percent glassy-winged sharpshooters carrying EGFP-expressing *P. agglomerans* in their foregut. A higher percent of GWSS acquired *P. agglomerans* from plants painted with unencapsulated *P. agglomerans* than from plants painted with encapsulated *P. agglomerans*.* $p<0.05$ by chi-squared test for homogeneity.** $p>0.05$ by chi-squared test for homogeneity.
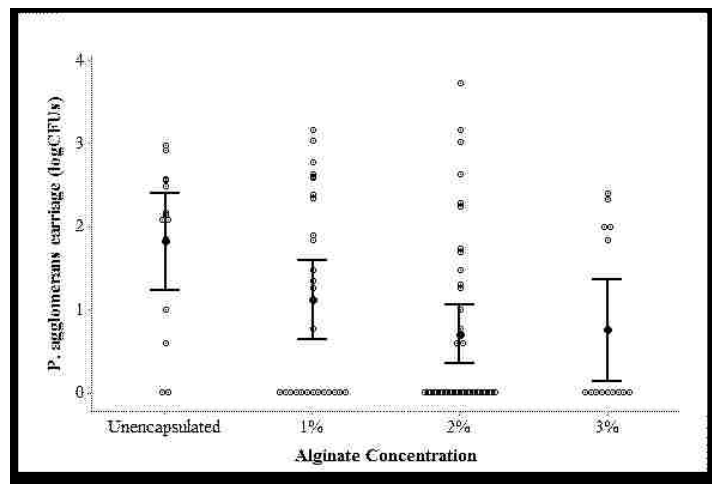
Figure 7 Bacterial CFUs per insect head.

P. agglomerans (EGFP-expressing) colony forming units (log10) per insect head. No difference in *P. agglomerans* CFUs acquired by the GWSS from plants painted with unencapsulated or encapsulated *P. agglomerans* was observed. $p> 0.05$ by one-way ANOVA with Tukey Simultaneous Tests for means with unequal variance.

Discussion

Here, we report a new paratransgenic strategy for control of *Xylella* transmission by the glassy-winged sharpshooter, *H. vitripennis*. *P. agglomerans* E325, an EPA approved agent for biocontrol of certain crop diseases, has been genetically modified using plasmid pT3078, which has the *bla* gene as a marker, to express recombinant EGFP in the foregut of GWSS. We have demonstrated robust EGFP expression in the insect foregut, in the region that harbors the plant pathogen, *X. fastidiosa*. Furthermore, we report the use of a simple and inexpensive microencapsulation strategy to contain engineered *Pantoea* and release the modified bacteria during conditions of xylem flux into the anterior mouthparts of the feeding insect. Our results represent the first iteration of this paratransgenic delivery system. Refinement of polymer chemistry and capsule application may increase the percentage of GWSS that acquire transgenic *Pantoea* in future trials. Chemical functionalization of the polymer backbone to enhance bacterial release along with refined methods of increasing bacterial CFUs per capsule are currently underway.

We propose the use of an EPA approved biocontrol agent, *P. agglomerans* E325*, as a paratransgenic control vehicle for PD. *P. agglomerans* colonized the foregut of the insect from both an artificial feeding system and plants painted with unencapsulated and encapsulated bacteria. *P. agglomerans* persisted in the insect foregut over the entire duration of testing, up to15 days. The 15-day period is significant since it represents close to one third of the insect's lifespan. Additionally, field applications of this approach could involve bi-weekly delivery of encapsulated bacteria to grape plants, thus offering fresh inocula to GWSS on a regular basis. *P. agglomerans* can be engineered to produce anti-

Xylella molecules such as antimicrobial peptides (AMPs) or anti-*Xylella* antibodies to block *X. fastidiosa*. We have used plasmid pT3078, which has the *bla* gene as a marker in addition to an EGFP gene. However, for field application, *Pantoea* lines expressing anti-*Xylella* molecules without antibiotic marker genes will be engineered. *P. agglomerans* lines with fluorophore genes or REDantibody genes as markers are under development for field release. These strategies, coupled with a refined delivery system, will produce a robust toolset in the battle against GWSS-mediated PD transmission.

Field use of paratransgenic control for arthropod-borne diseases remains a future prospect, largely due to concerns about the fate of engineered bacteria. In the current model, we believe that environmental risks associated with release of foreign genes will be reduced. First, plasmid decay for the recombinant *P. agglomerans* (Additional File 2: Figure S2) occurs at the rate of 0.0533% per bacterial generation; thus engineered bacteria are expected to revert to wild-type forms. Furthermore, engineered *P. agglomerans* grows at a rate comparable to wild-type counterparts (Additional File 1:Figure S1), suggesting that it cannot out-compete the native organism. Second, we have encased the recombinant symbiont in an alginate particle that inhibits release of payload into the environment. Physical contact between engineered bacteria and native bacteria of the rhizosphere or phytosphere is greatly impeded by the presence of capsules. Thus horizontal gene transfer between engineered *P. agglomerans* and commonly present bacteria of environmental consortia - a very rare event that occurs in the 10^{-8} to 10^{-9} range (data not shown) - should be further diminished. The overall aim of this strategy is to reduce horizontal gene transfer to levels that are acceptable to regulatory agencies, rather than eliminating gene flow. Abolishing gene flow between environmental bacteria is an

unrealistic goal. However, by providing a physical barrier via microparticles we can minimize exchange between the large number of freshly released bacteria used in plant inundation and environmental microbes, with the aim of reducing unwanted gene flow.

We have contained engineered *P. agglomerans* using calcium-alginate microparticles. To deliver the bacteria to GWSS, we have chosen to paint growing grape shoots, thus facilitating ingestion of the organisms during insect feeding. GWSS and other sharpshooters are xylem feeders [21]. They initially contact a plant surface with the tip of their stylet before penetrating into xylem vessels [22]. We hypothesize that bacteria-laden microparticles that have been painted on plant shoots will contaminate the lumen of the insect's stylet. During the sucking of xylem, hydrostatic pressure gradients as high as 0.24MPa/cm [23], will cause rapid swelling of the alginate hydrogel and release *Pantoea* into the cibarial region of the sharpshooter's foregut. To optimize release of bacteria from the microcapsules and enhance colonization of the insect, hydrogels may be tuned by varying the composition and concentration of divalent cations (Ca^{++}, Ba^{++}) as well as alginate (Figures 6 and 7).

Over 80% of GWSS that were exposed to unencapsulated (naked) *Pantoea* during feeding became colonized with the bacteria, at a mean CFU value of 245.4±80.4. (Figures 6 and 7). We tested microparticles containing 1%, 2% and 3% alginate (w/v) in the hydrogel. In all cases, there was a significant reduction in *Pantoea* acquisition when compared to the control group (GWSS exposed to unencapsulated bacteria) (Figure 6). When an alginate concentration of 1% (w/v) was used, target GWSS acquired *Pantoea* at a rate of 51.8% with a mean CFU count of 186.6±68.9. This rate of acquisition was the highest amongst the tested alginate concentrations and we believe that 1% alginate

particles merit further attention. Rates of acquisition of encapsulated *Pantoea* by target GWSS may have been lowered for several reasons. First, it is likely that the experimental insects represented a heterogeneous population. It is difficult to maintain homogeneous GWSS population under controlled conditions, thus, we used field-collected insects that were previously colonized with environmental bacteria, genetically distinct and of varying ages through the course of the summer. Second, we used a very limited time frame for acquisition of bacteria through feeding by GWSS. It is likely that future field applications that employ multiple treatments of plants with greatly protracted contact time between GWSS and particles will increase colonization rates in the insect. Third, many refinements in particle composition are possible. We are exploring use of particles made of other materials that would dissolve in the presence of enzymes present in the insect saliva to better release the bacterial payload. Attractants and feeding stimulants may be incorporated to enhance bacterial delivery. Indeed, future versions of the microencapsulation-delivery system should result in higher rates of target insect colonization with engineered *Pantoea*.

Microencapsulation is a first step toward addressing concerns associated with paratransgenic manipulation of arthropods. In this proof of principle, we deployed microcapsules to deliver a recombinant bacterium to an insect. We designed the alginate particles to swell under very high hydrostatic pressures encountered during xylem flux and to decrease contact between engineered bacteria and the environment during periods of application and residence on plants. Microencapsulation will decrease direct interactions of released bacteria with other epiphytes when compared to unencapsulated bacteria. This should also decrease the interactions of released bacteria with rhizosphere

organisms. We believe this is a first step toward targeted delivery of recombinant bacteria to arthropod vectors of disease. In ongoing projects, we are designing more advanced particles that respond to specific physiological cues within insects, such as pH and blood products, to establish a platform for field use of paratransgenics.

Based on this technology, microcapsule-based delivery of transgenic bacteria to arthropods of clinical significance (sand flies, kissing bugs, mosquitoes) for human vector-borne disease prevention becomes a real and logical extension, signifying a paradigm shift in paratransgenic techniques from the lab to field settings. A microparticle-based recombinant-bacterial release strategy could make paratransgenic control of Chagas disease and malaria - for which paratrangenic manipulation has been shown to decrease parasite load inside the vector - and other vector-borne diseases a reality.

Conclusion

This is the first example of the use of microencapsulation to deliver recombinant bacteria to an insect gut. We have demonstrated that transgenic symbiotic bacteria can be delivered to the appropriate physiological niche within a disease-transmitting arthropod. Furthermore, by acting as a barrier between transgenic bacteria and the outer environment these microparticles should reduce competition between the recombinant bacteria and native species, environmental contamination and horizontal gene transfer. This platform may be expanded to deliver recombinant bacteria to other disease-transmitting arthropod vectors, thus facilitating field use of paratransgenic control.

Methods

Bacterial culture

P. agglomerans E325 culture was maintained on Luria Bertani (LB) agar at 30ºC or in LB broth at 30ºC at 180 rpm.

Preparation *P. agglomerans* E325 competent cells

Bacterial cells were grown in 100 ml of LB medium at 30ºC with shaking at 175-180 rpm. When OD600 was reached 0.4-0.6 the cells were harvested by centrifugation at 4000 rpm for 10 minutes at 4ºC. These cells were resuspended in 40 ml of ice cold Inoue solution and harvested by centrifugation at 4000 rpm for 10 minutes at 4ºC. Bacterial cells were then resuspended in 10 ml of Inoue transformation buffer. To this solution 0.7 ml of DMSO was added and the cells were stored in ice for 10 minutes. Aliquots of 70 µl were made in microcentrifuge tubes and immediately snap frozen in liquid nitrogen. The competent cells were then stored at -80ºC.

Transformation of *P. agglomerans* E325 with pT3078-5

Competent *P. agglomerans* cells were removed from -80ºC freezer, thawed and kept on ice for 10 minutes. Two µl of plasmid pT3078-5 was added to the cells. pT3078-5 has an EGFP gene and *bla* gene (ampicillin resistant) as markers. The cells were incubated on ice for 30 minutes followed by a heat shock at 42ºC for 90 seconds. The tubes were then transferred to ice for 2 minutes. The cells were recovered by adding 800 µl LB medium and incubated at 30ºC with shaking at 180 rpm for 2 hours. These cells were then transferred on to the LB agar supplemented with carbenicillin (100 mg/liter of media) and incubated overnight at 30ºC. Next day single colonies were plated on LB agar

with carbenicillin. Transformed *P. agglomerans* cells were tested for fluorescence and confirmed by EGFP gene PCR.

Growth comparison

Wild-type *P. agglomerans* and EGFP-expressing *P. agglomerans* were grown overnight in LB and LB+carbenicillin, respectively, to a stationary phase. They were then diluted to 1/100 in 50 ml LB and were incubated at 30°C in a shaker incubator at 200 rpm. OD600 readings were taken hourly until cultures reached stationary phase.

In vitro **plasmid stability**

Transformed *P. agglomerans* were grown in LB supplemented with carbenicillin for 10 hours at 28ºC with shaking at 175-180 rpm. These cells were diluted to ~1000 cells/ml and were grown for 10 hours (approx. 15 generation) in 25 ml LB broth without antibiotic selection. After 10 hours of growth, log phase cells were added to 25 ml fresh LB broth to a final concentration of ~1000 cells/ml and were grown again. This procedure was repeated 5 times (total of 75 generation). Every 10 hours, while transferring the cells to fresh medium, a fraction of cells was aliquoted and grown overnight on LB agar without any selection. The next day 100 random colonies were selected and streaked on LB agar supplemented with carbenicillin.

Acquisition of *P. agglomerans* to the GWSS using artificial feeding system

The EGFP plasmid containing *P. agglomerans* was grown overnight in LB broth supplemented with carbenicillin. The next morning bacteria were centrifuged at 4000 rpm for 10 minutes followed by washing twice with the PBS. The bacterial cells were resuspended in a solution of PBS (0.2X) and sugar (0.2%). This solution was filled into plastic tubes with inner and outer diameter 1/8" and 3/16", respectively. These tubes were

placed in 2.5" diameter vials individually. The vials containing the tubes were called the artificial feeding system (AFS). The control AFS tubes were filled with the PBS-sugar solution without bacteria. *H. vitripennis*, collected from the citrus orchard of Agriculture Operations, UC, Riverside, were put into the control AFS and the bacteria containing AFSs. These insects were given an acquisition access for 48 hours.

Maintenance of sharpshooters

After an acquisition access of 48 hours the insects were transferred to an insect cage containing grape plants for 24 hours to flush out the unattached bacteria. After 24 hours of flushing, the insects were shifted to a new insect cage with clean grape plants. The control (naïve) insects from the acquisition experiment were also transferred to the clean plants. Everyday 5-15 insects were taken out from the cage for bacterial isolation.

Isolation of *P. agglomerans* from insect heads

Everyday 5-15 sharpshooters were taken out from bacteria and control groups and surface sterilized in 70% ethanol and 10% bleach followed by washing twice with autoclaved sterilized water. The heads of surface sterilized insects were cut with a sterile surgical blade and were homogenized in 100 µl PBS using a homogenizer. This solution was plated on carbenicillin supplemented LB agar plates.

General materials and method for alginate experiments

Protonal LF10-60 alginate (FMC Biopolymer, Sweden) was prepared at 1-3% (w/v) concentration in de-ionized water and autoclaved prior to use. Bacterial cultures were grown in LB broth overnight at 30ºC to plateau phase in 15 ml culture tubes, pelleted at 4000 RPM at 4ºC for 15 minutes, washed twice with sterile 1X PBS, re-pelleted at 4000 RPM and supernatant decanted and re-suspended in 3 ml sterile 1X PBS

before incorporating into the alginate. Bacterial-alginate suspensions were utilized immediately after mixing. All experiments were performed in triplicate. Viable colony forming units (CFUs) were evaluated using the pour plate method on selection agar. Particle sizing was accomplished on a Microtrac s3500 laser diffraction analyzer (Microtrac Systems).

Synthesis of calcium-alginate (Ca-ALG) or calcium/barium-alginate (CB-ALG) microparticle

Alginate-bacterial suspension was produced as mentioned above. The resulting mixture was atomized from an alcohol-sterilized airbrush into a vat containing sterile 0.05M $CaCl_2$ with constant agitation from a distance of 20 cm. The resulting microparticles were allowed to harden for 45 minutes and then harvested via vacuum filtration through a sterile Nalgene filter-funnel with two pieces of sterile Nylon mesh (pore sizes of 50 μm and 35 μm respectively) acting as filters. The microparticles were harvested from the mesh and stored for future use.

In the case of CB-ALG microparticles, an equal volume of $BaCl_2$ was added to the $CaCl_2$ solution after the 45 minutes gel time and the microparticles were allowed to further gel for an additional 20 minutes. Microparticles were then collected as described.

Synthesis of carbon-calcium-alginate (CC-ALG) microparticles

For all experiments relating to carbon-calcium alginate particles, the alginate-bacterial suspension was mixed with 1% vol/vol of sterile India Ink prior to particle gellation. The rest of the protocol was left unaltered.

Dehydration of Ca-ALG microparticles

Bacteria-containing Ca-ALG were produced as previously described. Ten gram *P. agglomerans* Ca-ALG microparticles ($1X10^7$ CFUs/g) or 10 gm *P. agglomerans* in 5% glycerol (v/v) slurry ($1X10^7$ CFUs/g) were then under sterile conditions transferred into sterile 10 cm petri dishes and allowed to dry to constant weight under ambient conditions and atmosphere (27ºC, 5-7% RH) overnight. Over the course of several weeks at various time points 1 gm of dry slurry or dehydrated microparticles was transferred into fresh sterile 0.15M sodium citrate and allowed to rehydrate/dissolve for 1 hour and then serially diluted and plated on selection agar and incubated overnight. Viable CFUs were assessed based on colony count.

Survival of encapsulated *P. agglomerans* E325 under highly sterilizing UVC conditions

Bacteria-containing Ca-alginate microparticles both with and without India Ink dye were produced as previously described. Ten grams of encapsulated *P. agglomerans* E325 containing 2.0% (v/v) India Ink were placed in sterile open petri dishes 30 cm directly under a columnated UVC germicidal lamp. Samples were exposed to sterilizing radiation for 0-60 minutes, during which samples were chosen at random at various time intervals and placed in fresh sterile microcentrifuge tubes containing 1ml sterile 0.15M sodium citrate solution and left to dissolve over 1 hour. The liberated microbes were serially diluted and plated on LB agar and left to incubate at 30ºC overnight. CFUs were counted the following day.

Diffusion from CA-ALG and CB-ALG microparticles

Bacteria-laden microparticles were produced as previously described. One gram of microparticle slurry was mixed with 1ml of sterile water in a 1.5ml microcentrifuge tube and allowed to stand at 4ºC for the prescribed time. At various time intervals throughout the experiment, the tubes were vortexed vigorously for 10-15s to re-suspend any diffused bacteria. The microparticles were allowed to settle for 5 minutes and then 50μl aliquots were removed and serially diluted. Fifty μl aliquots of each dilution were then plated on selection agar and the plates were incubated overnight at 30ºC to determine viable CFUs that had diffused from the microparticles.

P. agglomerans **acquisition through microparticles**

EGFP-expressing *P. agglomerans*-containing microparticles were engineered using a 1%, 2% or 3% alginate as described above. Encapsulated bacteria were irradiated three times with UVC for 90 seconds to kill any unencapsulated bacteria during the production process. These microparticles were then mixed with 10% glycerol and 3% guar gum and were applied to the grape plants. Field collected sharpshooters were allowed to feed on these plants for 24 hours. As a control unencapsulated bacteria mixed with 10% glycerol and 3% guar gum were applied to the grape plants. After 24 hours the sharpshooters were removed from the plants and surface sterilized. The foregut and midgut contents were homogenized as described above using a homogenizer. The foregut and midgut contents were plated on LB+carbenicillin agar plates and were incubated at 30ºC. The CFUs were counted after 24 hours.

Statistical analysis

Number of GWSS carrying *P. agglomerans* were compared using Chi-squared test for homogeneity. *P. agglomerans* CFUs in various experiments with different treatments were analyzed by Tukey's test for multiple comparison after taking log values of CFUs. All the values are shown as mean±S.E. Statistical analysis were performed using Minitab version 17 for windows 7. *p* values<0.05 were considered significant.

List of Abbreviations

GWSS - Glassy-winged sharpshooter

EGFP- Enhanced green fluorescent protein

AFS - Artificial feeding system

EPA - Environmental Protection Agency

GE - Genetically engineered

PD - Pierce's Disease

CFU - Colony forming unit

UVC - Ultraviolet C

AMP - Antimicrobial peptides

LB - Luria Bertani

Ca-ALG - Calcium alginate

CB-ALG - Calcium/barium alginate

CC-ALG - Carbon calcium alginate

Competing Interests

The authors declare that they have no competing interests.

Authors' Contribution

AKA, AF, TAM, RD conceived and designed the study. AKA transformed *P. agglomerans* and established recombinant *Pantoea* in sharpshooters gut using artificial feeding system. AF designed microparticles. AKA and AF conducted field simulated experiments with sharpshooters. AKA did statistical analysis. AKA, AF, TAM and RD drafted the manuscript.

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Additional Files

Additional File 1: Figure S1

Comparison of growth of wild type *P. agglomerans* and EGFP-expressing *P.*
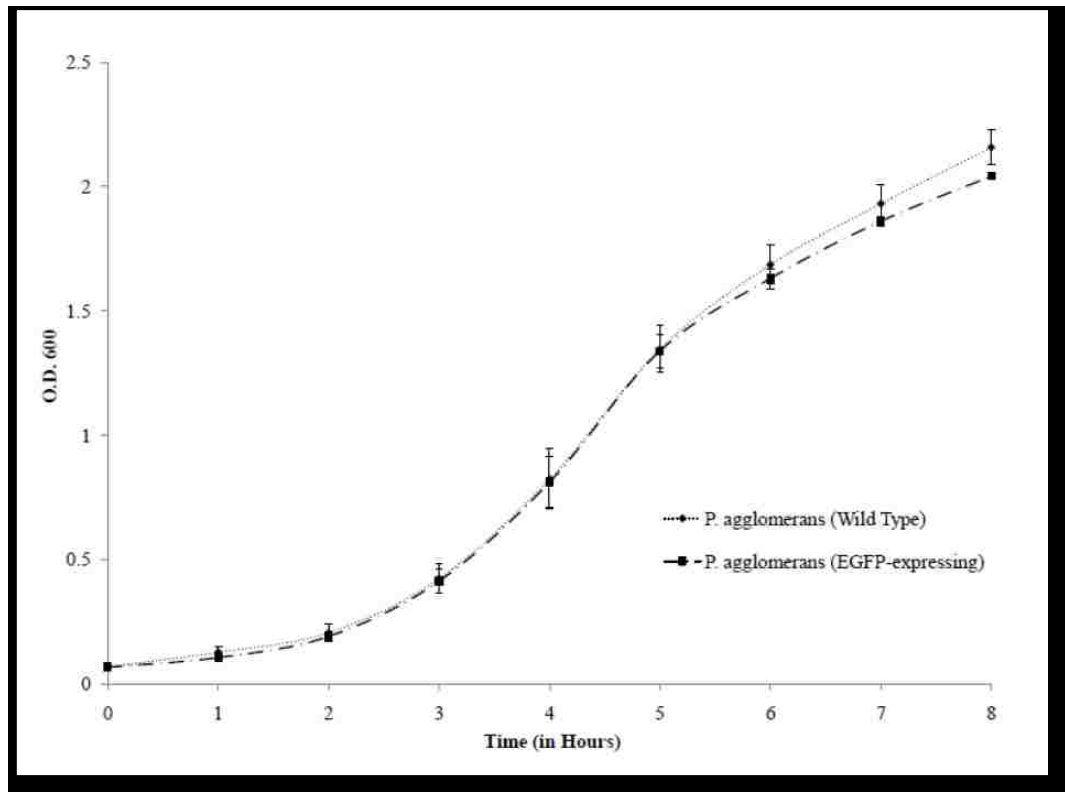
a

Figure S1 Comparison of growth of wild type *P. agglomerans* and EGFP-expressing *P.* agglomerans.

Both the wild type and transformed *P. agglomerans* grew at a similar rate, which shows that the plasmid pT3078 did not negatively affect growth of the transformed bacteria.

Additional File 2: Figure S2

Loss of plasmid pT3078-5 by *P. agglomerans* over time.

Figure S2 Transformed *P. agglomerans* revert to its wild type form over time.

Percent *P. agglomerans* carrying EGFP-containing plasmid pT3078 in a culture kept at mid log phase for 50 hours (~75 generation). *P. agglomerans* lost pT3078 plasmid at a rate of 0.0533 plasmids per generation.
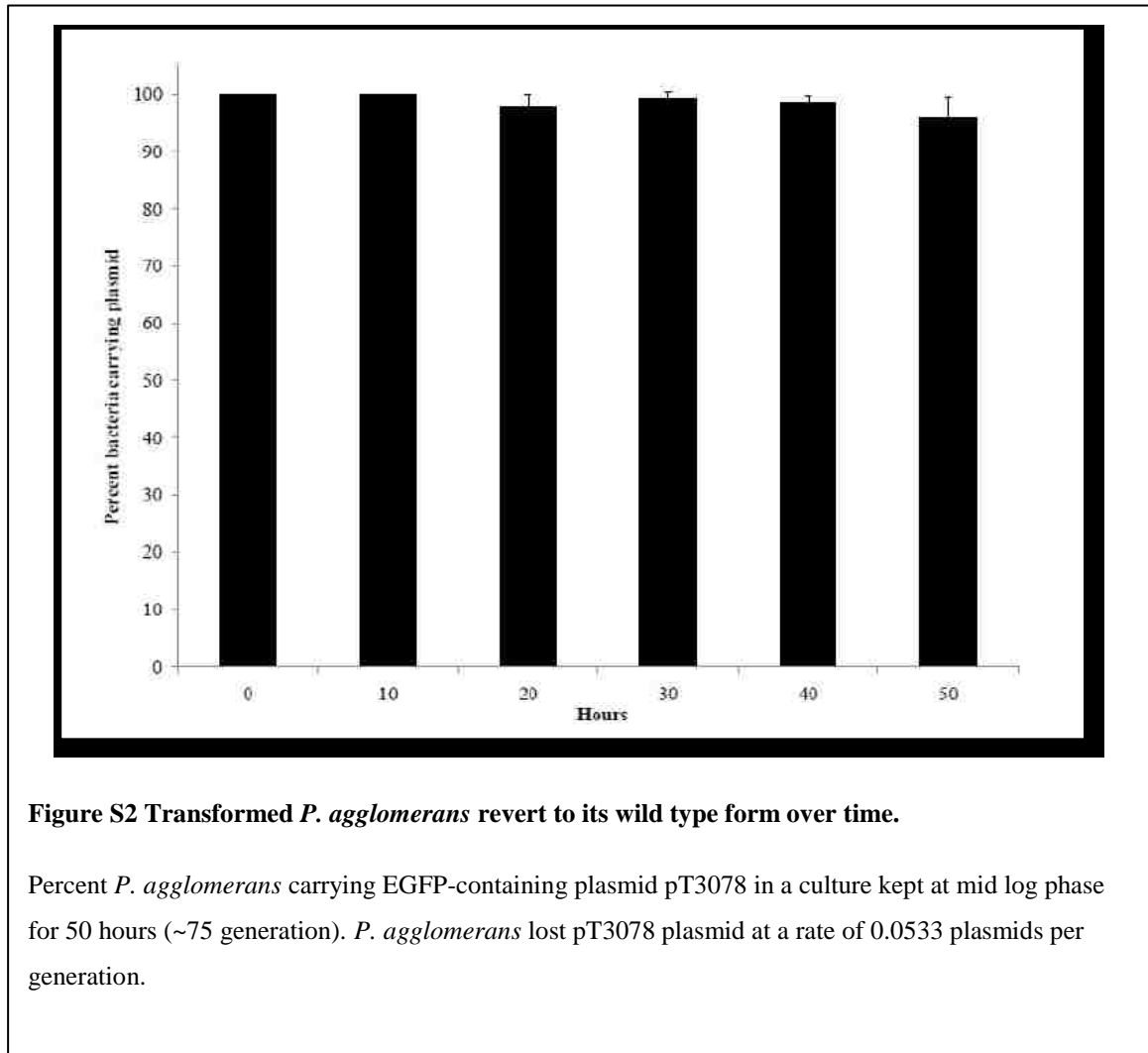
Additional File 3: Figure S3

Size distribution of *P. agglomerans* containing microcapsules.
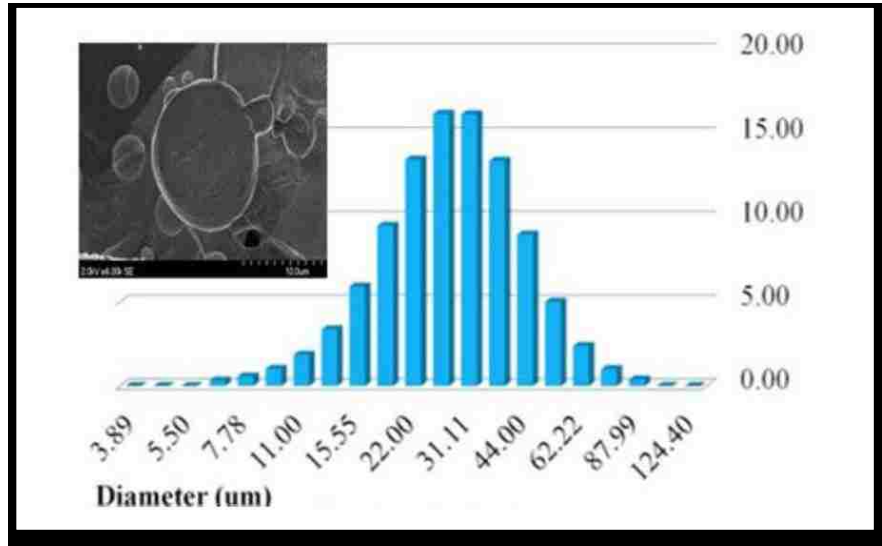
Figure S3 Size distribution of *P. agglomerans*-alginate microcapsules.

Size distribution of *P. agglomerans*-alginate microcapsules generated from a modified aerosolization/coacervation process. (Inset) Electron micrograph of calcium-alginate encapsulated EGFP *P. agglomerans* E325, size = roughly 10um (center) with 3-4μm microcapsules encapsulating single bacteria.

Diffusion of microencapsulated *P. agglomerans* from Ca^{2+} and Ca^{2+}/Ba^{2+} cross-linked microparticles.

Figure S4 Diffusion of microencapsulated *P. agglomerans* from Ca^{2+} and Ca^{2+}/Ba^{2+} cross-linked microparticles.

Use of barium as a cross-linker greatly reduced the diffusion of bacteria from the microparticles.*, **, *** and **** $p < 0.001$ by one-way ANOVA with Tukey Simultaneous Tests for means with unequal variance compared to Ca-Alg and Ca-Alg+10% Glycerol microparticles on days 1, 3, 5 and 7, respectively.

Chapter 3: Paratransgenic Control of Pierce's Disease

Abstract

      Arthropod vectors are the weakest link in the epidemiology and dissemination of vector borne diseases. We can halt disease transmission either by killing the vectors or targeting the pathogen or parasite within the vector's body. Paratransgenesis targets pathogen within the insect gut by utilizing the symbiotic bacteria residing within the same niche. We targeted *Xylella fastidiosa*, a bacterial pathogen that causes Pierce's disease (PD) of grapevine, within the gut of its vector - the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*). We genetically modified *Pantoea agglomerans* E325, utilizing the *Escherichia coli* hemolysin secretion system, to express and secrete two antimicrobial peptides - melittin and scorpine-like molecule (SLM). Melittin and SLM were selected for their expression via *P. agglomerans* after conducting *in vitro* studies wherein both AMPs displayed higher toxicity towards *X. fastidiosa* in comparison to *P. agglomerans*. These AMP-producing *P. agglomerans* lines were tested for their ability to target *X. fastidiosa* within the sharpshooter foregut. Paratransgenic sharpshooters carrying AMP-secreting *P. agglomerans* lines were refractory to *X. fastidiosa* and failed to transmit *X. fastidiosa* to naive grape plants. For the first time, we demonstrated utilization of paratransgenesis to make insect-vectors incompetent to acquire and transmit a pathogen in an agricultural system. This PD paratransgenic strategy will act as model system for other vector borne diseases.

Results and Discussion

Despite advances in public health, arthropod vectors continue to exact a huge toll, either directly through transmission of human pathogens or indirectly by causing diseases in animals and agricultural crops. The global burden of plant diseases that are transmitted by insects such as plant hoppers, aphids, whiteflies and thrips exceeds $10 billion, with profound implications for food security. Toxicity of chemical pesticides, prohibitive costs of sustaining pesticide campaigns and rapid evolution of insect resistance have confounded efforts to control these scourges and underscore the need to develop alternate approaches to pathogen control. Modulation of insects using transgenic symbiotic bacteria- termed paratransgenesis- has been developed for several vectors of human disease such as triatomine bugs, tsetse flies, sandflies and mosquitoes (Durvasula *et al*., 1997; Aksoy *et al*., 2008; Hurwitz *et al*., 2011; Wang *et al*., 2012). This strategy relies on delivery of anti-pathogen molecules within the arthropod vector via engineered symbiotic bacteria to make the insect incompetent to carry and transmit the pathogen (Durvasula *et al*., 1997). Several models of paratransgenic insects have been developed but to date none has been validated as a method to block transmission of a pathogen and prevent disease in a target host. Here, we report the paratransgenic manipulation of an agricultural pest, *Homalodisca vitripennis* (Glassy Winged Sharpshooter), to block transmission of the bacterial pathogen, *Xylella fastidiosa*, to grape plants. *X. fastidiosa* is currently a leading agricultural menace globally, as the causative agent of Pierce's disease of grapevines, citrus variegated chlorosis (CVC) of citrus crops and olive quick decline of olive trees (Purcell, 2013; Saponari *et al*., 2015). Recent reports of decimation of European olive trees (Stokstad, 2015) coupled with impacts on citrus and grape/ wine industries place the

global burden of *X. fastidiosa* at several billion dollars. Leafhoppers commonly known as sharpshooters are vectors of *X. fastidiosa*; amongst them, *Homalodisca vitripennis* (glassy-winged sharpshooter, GWSS), due to its long-range mobility and high fecundity, is the most important (Redak *et al*., 2004). We recently identified *Pantoea agglomerans* as a symbiotic bacterium of *H. vitripennis* and, using an EPA-approved non-pathogenic variant of *Pantoea*, reported both paratransgenic manipulation and a field-applicable strategy to target GWSS with engineered bacteria (Arora *et al*., 2015). Using this platform, we have engineered lines of *P. agglomerans* that secrete antimicrobial peptides that kill *X. fastidiosa* and report here, for the first time, a pathogen-refractory agricultural pest that is unable to infect target plants.

Antimicrobial peptides (AMPs) are defense molecules synthesized by higher eukaryotes that kill pathogens in many ways, ranging from pore formation in the target membrane to disruption of protein or nucleic acid synthesis or signal transduction pathways (Brogden, 2005). Melittin, a 26 amino acid-long peptide having an alpha- helix structure, is found in honeybee venom and kills cells through pore formation or by inducing apoptosis (Oršolić, 2011). We selected melittin for delivery to the sharpshooter gut via *P. agglomerans*. As a second effector molecule, we selected scorpine like molecule (SLM). SLM is an AMP found in the venom of the scorpion *Vaejovis mexicanus*. It is a 77 amino acid-long peptide having 44% homology with scorpine an AMP from the scorpion *Pandinus imperator*. We analyzed its three dimensional structure using I-TASSER (Yang *et al*., 2015; Roy *et al*., 2010), which predicted that it is composed of three coil-helix structures (Fig. 1).

Figure 1. The amino acid sequence and depiction of the three-dimensional structure scorpine-like
molecule (SLM) a) SLM sequence showing predicted domains with helix-coil structure. b) 3-D
structure of SLM as depicted by I-TASSER.
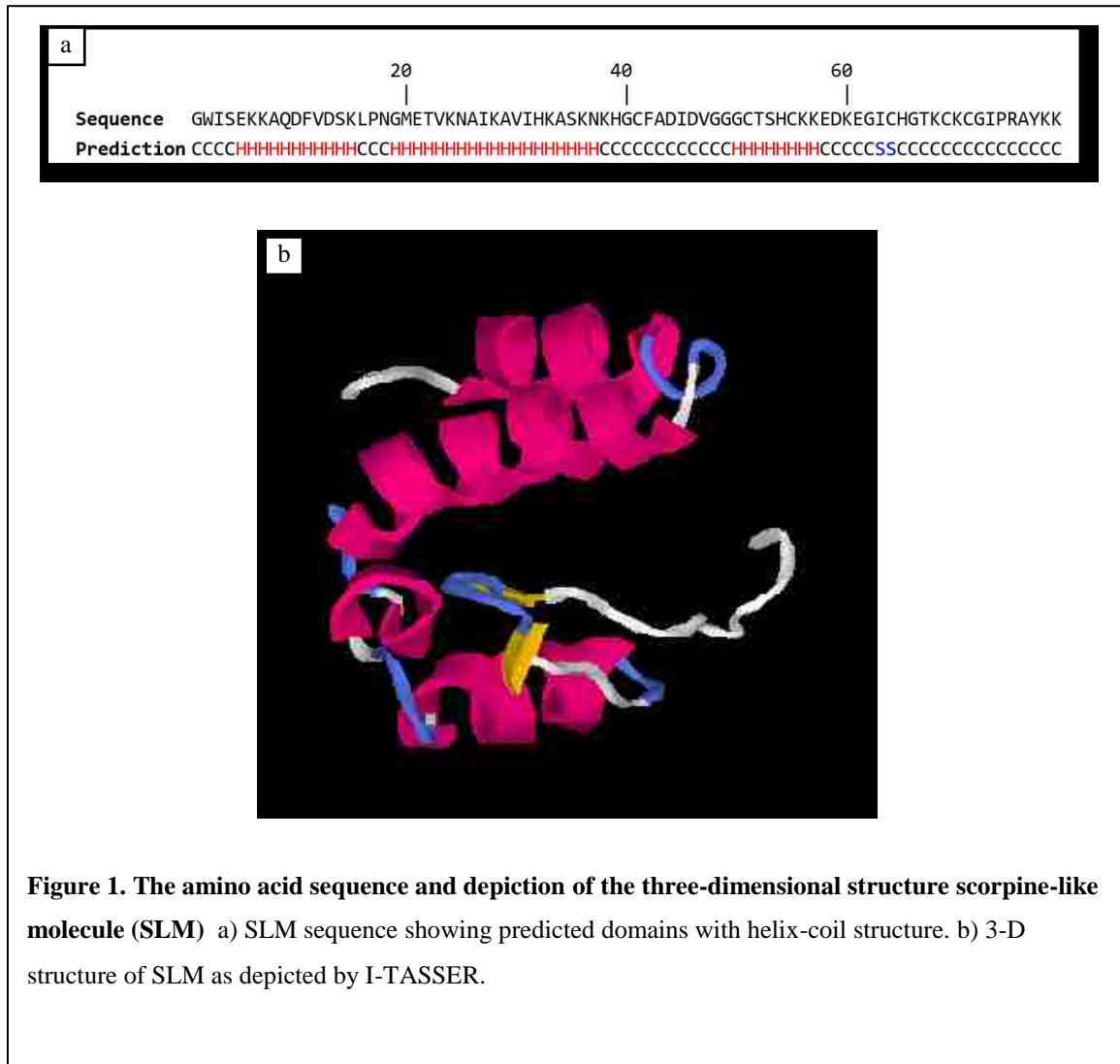
We tested activity of both peptides against *X. fastidiosa* as well as *P.*
agglomerans. Melittin killed *X. fastidiosa* at a concentration of 5µM, which was one-
fifth the concentration needed to kill *P. agglomerans* (25µM) (Fig. 2a and 2b). Similarly,
SLM killed *X. fastidiosa* at a concentration of 10µM, but had no effect on *P.*
agglomerans even at a concentration of 75 µM (Fig. 2c and 2d). This selective toxicity of
these molecules to *X. fastidiosa* rendered them ideal effectors for paratransgenic
manipulation of *H. vitripennis*.

Figure 2. Toxicity of melittin and SLM against *P.agglomerans* and *X. fastidiosa*. 10^5-10^6 CFUs of *P. agglomerans* and *X. fastidiosa* were treated with each AMP. O.D. 600 was measured 24 hours after treatment of *P. agglomerans* with each AMP as it grows at a fast rate; while *X. fastidiosa* grows at a slow rate and it was cultured 24 hours after treatment with each AMP and CFUs were counted. *P. agglomerans* O.D.600 after treatment with a) melittin, c) SLM; *X. fastidiosa* CFUs counts after treating with b) melittin, d) SLM.

To generate *P. agglomerans* strains that secrete melittin and SLM into the foregut of *H. vitripennis*, we chose the *Escherichia coli* hemolysin secretion system, which has earlier been used to secrete active molecules into the outside environment of gram negative bacteria (Fernández *et al*., 2000; Wang *et al*., 2012). This secretion system is composed of the pore forming proteins HlyB and HlyD and a carboxyl terminal *HlyA*
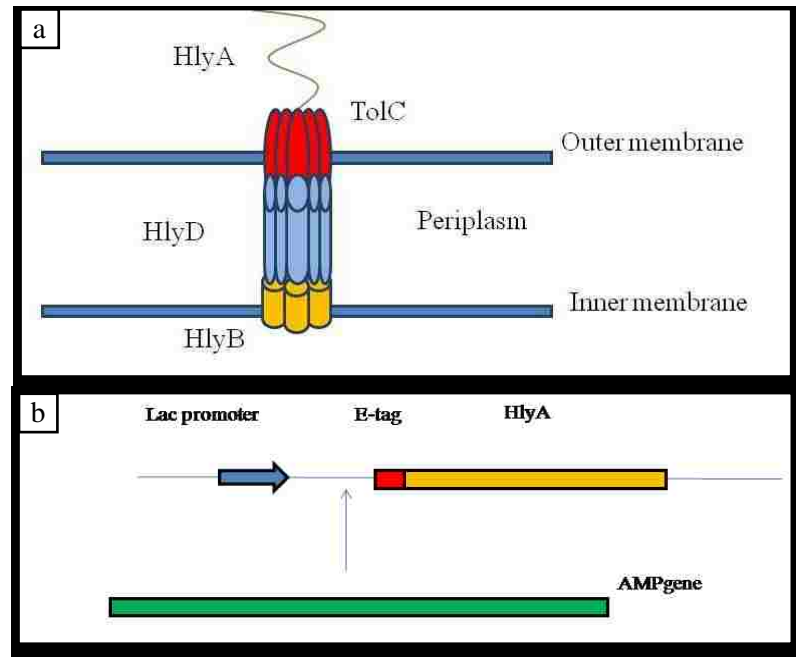
Figure 3. Assembly and working mechanism of *Escherichia coli* hemolysin secretion system. HlyB and HlyD formed pores in the internal membrane of Gram negative bacteria. These pores join pores formed with TolC in the outer membrane and provide a passage to proteins with HlyA secretion signal at their carboxyl end. a) Sketch depicting a protein with HlyA secretion signal passing through pore formed by HlyB and HlyD in combination with TolC in the membrane of Gram negative bacteria. b) Schematic description of introducing effector molecules at 5' end of E-tag in plasmid pEHLYA2-SD, which was in frame with HlyA secretion signal.

secretion signal. Pores formed by HlyB and HlyD in combination with pores formed by TolC provide proteins with an HlyA secretion signal passage to the outside environment (Fig 3a). We introduced genes encoding melittin or SLM in the plasmid pEHLYA2-SD at the 5'end of an E-tag, which was in-frame with the HlyA secretion signal (Fig 3b). We transformed *P. agglomerans* with pVDL9.3, a plasmid with HlyB and HlyD genes, and pEHLYA2-SD. These strains secreted melittin and SLM with the HlyA secretion signal intact (Fig 4a, 5a).

We delivered *P. agglomerans* directly to the sharpshooters via guar gum that was painted on grapevine stems at a concentration of 10^{10} CFUs/ stem as described by Arora *et*

Figure 4. Detection of excretion and accumulation of melittin and SLM conjugated to HlyA secretion signal in the spent medium and within the sharpshooter gut by the transformed *P. agglomerans* lines using an anti E-tag antibody. a) Spent medium of overnight *P. agglomerans* cultures was concentrated using Micron 10kDa filters. This concentrated spent medium was tested using anti-E-tag antibody via Western blot. Lane 1: ladder; lane 2: Wild type *P. agglomerans*; lane 3: HlyA secretion signal only; lane 4: melittin conjugated to HlyA secretion signal; lane 5: SLM conjugated to HlyA secretion signal. **4 (b) and (c).** Single surface sterilized sharpshooter was homogenized in PBS using a homogenizer. Homogenized slurry was centrifuged @ 14000 rpm and supernatant was collected. The collected supernatant was tested for presence of AMPs using an E-tag antibody. (b) Lane 1: ladder; lane 2: sharpshooter fed on *P. agglomerans* expressing melittin conjugated to HlyA secretion signal; lane 3: sharpshooter fed on wild type *P. agglomerans* (c) Lane 1: ladder; lane 2: sharpshooter fed on *P. agglomerans* expressing SLM; lane 3: sharpshooter fed on wild type *P. agglomerans*.

Figure 5. Confirmation of excretion and accumulation of melittin conjugated to HlyA secretion signal by transformed *P. agglomerans* lines in the spent media as well as within the sharpshooter gut. (a) Spent medium was concentrated as described earlier and was analyzed using anti-melittin serum via Western blot. lane 1: melittin conjugated to HlyA secretion signal; lane 2: synthetic melittin without HlyA secretion signal; lane 3: ladder. (b) Solution from sharpshooter was prepared as described earlier. This solution was analyzed using anti-melittin bleed. Lane 1: ladder; lane 2: sharpshooter fed on *P. agglomerans* expressing melittin conjugated to HlyA secretion signal; lane 3: sharpshooter fed on wild type *P. agglomerans.*

al. (2015). GWSS were allowed to feed on these plants for 48 hours. After *P. agglomerans* acquisition, sharpshooters were infected with *X. fastidiosa* by feeding on infected grape plants for 48 hours. These sharpshooters were collected and placed, two at a time, on naïve grape plants for 24 hours to determine transmission of *X. fastidiosa*. Sharpshooters that harbored AMP-producing *P. agglomerans* were refractory to *X. fastidiosa* acquisition; insects that carried melittin- or SLM-secreting *P. agglomerans*, on an average, had *X. fastidiosa* burden that was 4.28% and 0.22%, respectively, of the

pathogen burden in control insects (*p*<0.00001) (Fig 6a). Furthermore, the number of

paratransgenic sharpshooters that carried *X. fastidiosa* in their foregut also decreased,

significantly: 80.55% of control sharpshooters acquired *X. fastidiosa*, while only 15.38%

of GWSS harboring melittin- and SLM-secreting *P. agglomerans* were found to carry *X.*

fastidiosa in their foregut (*p*<0.00001) (Fig 6b). Secretion of the HlyA signal peptide

alone by *P. agglomerans* in GWSS also decreased acquisition of *X. fastidiosa*; 43.75%

sharpshooters in this group acquired *X. fastidiosa* in their foregut. This is not surprisingly

as Wang et al. (2012) have also seen a decrease in *Plasmodium* acquisition by the

mosquitoes that carry HlyA secretion signal expressing bacteria.

Our next step was to determine whether decreased acquisition of *X. fastidiosa* by

H. vitripennis results in decreased pathogen transmission to naïve grape plants.

Sharpshooters that carry *X. fastidiosa* transmit the pathogen to grape plants

approximately 20 percent of the time (Almeida and Purcell, 2003). Paratransgenic

sharpshooters, that acquired melittin- and SLM- producing *P. agglomerans* strains prior

to acquisition of *X. fastidiosa*, failed to transmit *X. fastidiosa* to the naïve grape plants

(Fig. 7). Control sharpshooters and sharpshooters carrying wild type *P. agglomerans*

transmitted *X. fastidiosa* 16.67% and 20% of the time, respectively (Fig. 7).

Sharpshooters that carried *P. agglomerans,* which secreted only the HlyA signal protein

(and not the AMP molecules) also failed to transmit *X. fastidiosa* to the naïve plants. In

our laboratory model of *Xylella* transmission, we allowed 24 hours of inoculation access

and used 2 sharpshooters/plant. Under field condition more than 2 sharpshooters can feed

on the same plant for extended periods of time or even multiple times, which could result

in higher pathogen transmission than we observed in our experimental model.

We tested sharpshooters for the presence of recombinant AMP molecules to confirm that decrease in *Xylella* transmission to grape plants was a result of AMP activity in the insect gut. Grape plants were coated with 10^{10} CFUs of *P. agglomerans* per plant via guar gum. GWSS were allowed to feed on these plants for 48 hours, then removed, surface sterilized and tested for accumulation of peptides. Western blot analysis established presence of both melittin and SLM within the insects (Fig Fig 4b, 4c). Further, we confirmed presence of melittin using anti-melittin serum, which did not cross-react with any of the sharpshooter proteins (5b).

Prior studies with paratransgenic arthropod vectors of disease demonstrated reduction or elimination of pathogens in disease-transmitting insects (Durvasula et al. 1997, Wang et al. 2012). Here, we report a paratransgenic strategy that completely eliminates transmission of a pathogen from an arthropod to a target plant. Three molecules- the HlyA protein, melittin and SLM- when expressed in the sharpshooter via engineered *P. agglomerans*, aborted transmission of *X. fastidiosa* to grape plants. Melittin and SLM decreased *Xylella* CFU in paratransgenic sharpshooters to levels that should eliminate pathogen transmission even during periods of feeding that exceed the 24 hour window. Additionally, under field conditions, several sharpshooters may feed on a single plant, unlike our experimental model in which only 2 insects were placed on target plants. Again, the level of elimination of *X. fastidiosa* in the insect achieved with melittin and SLM would stop transmission under such real world conditions. HlyA alone did reduce *X. fastidiosa* acquisition by the sharpshooter and eliminated transmission in our study.
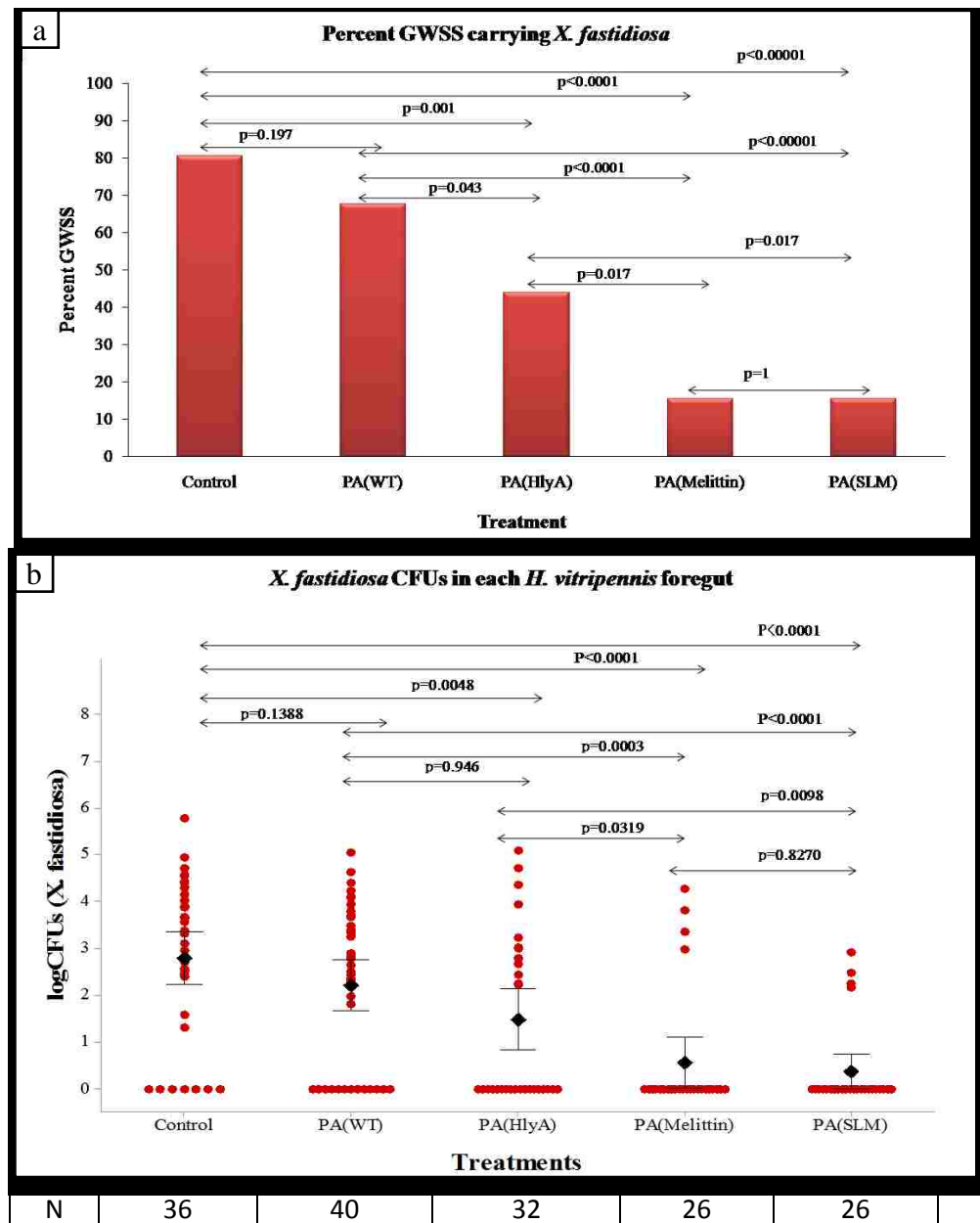
Figure 6: Graphs showing a decrease in *X. fastidiosa* acquisition by paratransgenic sharpshooters. *P. agglomerans* was painted on the grape stems after mixing with guar gum. The sharpshooters were allowed to feed on *Pantoea* painted plants for 48 hours before putting them in cage containing *X. fastidiosa* infected plant for 48 hours. Subsequently the sharpshooters were collected and two sharpshooters were caged on single naive grape plant for 24 hours. These sharpshooters were surface sterilized and *X. fastidiosa* presence was tested using rt-PCR. Graph depicting a) Prevalence of *X. fastidiosa* in the sharpshooter heads; a) *X. fastidiosa* CFUs per insect head.

However, the degree of *X. fastidiosa* reduction in the insect may not prevent transmission under enhanced feeding settings.

Spread of Pierce's disease and other vector-borne diseases is kept under check by controlling vector populations with insecticides. There are reports of the development of resistance in many insect vectors including mosquitoes and triatomine bugs against various insecticides (Vassena, 2000; Picollo, 2005; Riaz, *et al.*, 2013; Liu, 2015). Paratransgenic control of these diseases is an alternative to insecticidal control, which can be employed in the field to decrease transmission. It can also be included in integrated vector management. We believe that paratransgenic control will reduce disease spread and also will help to decrease over-reliance on chemical pesticides. We did not observe adverse physiological effects in the sharpshooters carrying *P. agglomerans* strains, such as decreased feeding or early mortality. Paratransgenic sharpshooters should be able to complete their life cycle and there will not be any selection pressure on these insects, which is the main cause of resistance development.

The sharpshooter foregut as compared to the grape plants carries far fewer *X. fastidiosa* CFUs, which makes the insect gut an ideal place to attack *X. fastidiosa*. AMPs expressed by *P. agglomerans* will encounter very few bacteria exerting less selection pressure on the bacteria. Further, a low quantity of active molecules can combat the pathogen within the gut. These are all factors that contribute to delay in resistance development. Our lab has also developed single chain fragment variables (scFvs) specific to *X. fastidiosa* surface protein, mopB (Azizi *et al.*, 2012). These antibodies can be expressed in tandem with active AMPs or as antibody:AMP chimeras to increase in the
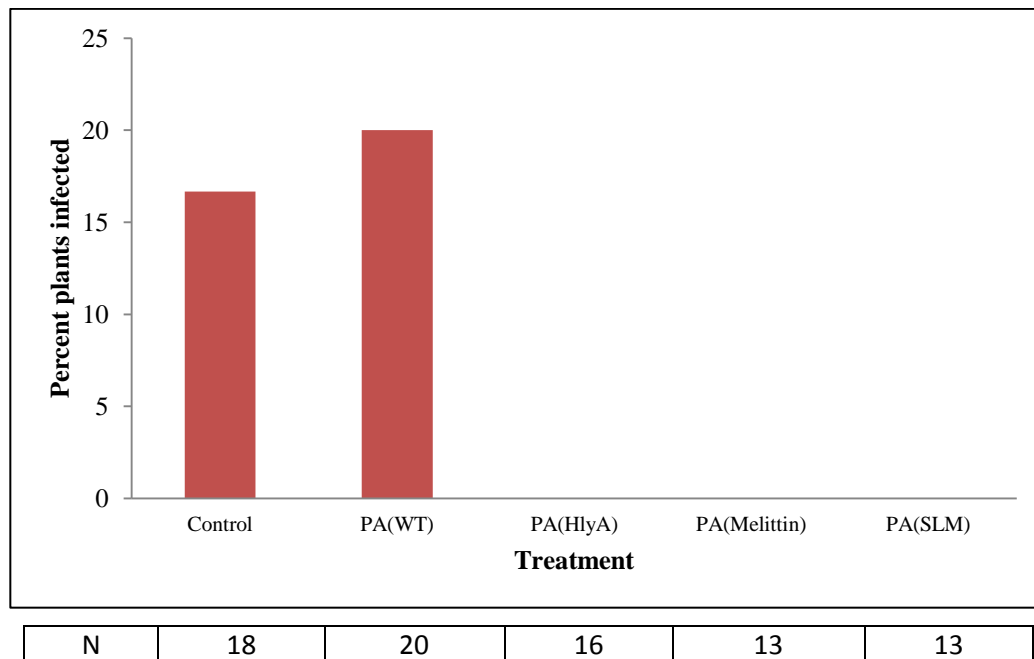
Figure 7 : Graphs showing a decrease in *X. fastidiosa* **transmission by paratransgenic sharpshooters..** *P. agglomerans* were painted on grape stems after mixing with guar gum. The sharpshooters were allowed to acquire *P. agglomerans* from *P. agglomerans* painted plants for 48 hours before acquisition access of 48 hours on *X. fastidiosa* infected grape plants. Subsequently the sharpshooters were collected and two sharpshooters were then confined on each naive grape plant. After 24 hours of inoculation access the insects were removed and the plants were kept in greenhouse for 30 weeks before testing them for presence of *X. fastidiosa* using rt-PCR. a) Percent plants infected with *X. fastidiosa* in each treatment.

efficacy. The tandem expression of antibodies will further help to delay the development

of resistance.

The true potential of paratrangenesis in the field has not been realized yet largely

due to lack of application methods. We have already developed a strategy based on use of

calcium-alginate microparticles to disseminate the genetically modified bacteria in the

field. These microparticles not only provide a physical barrier between the bacteria and

the environment to decrease the environmental contamination, but also resulted in

tolerance against desiccation and UV radiations (Arora *et al*., 2015). We can apply

microparticle containing recombinant *P. agglomerans* to fields in late spring for the

newly emerging sharpshooters to acquire AMP-expressing bacteria. This will make the

sharpshooter incompetent of transmitting the pathogen and thereby facilitating

transmission blockage.

The success of this PD paratransgenic model writes a new chapter in the control

of vector borne diseases in the field of agriculture. Whitefly, aphids, leafhoppers and

thrips transmit deadly diseases to crop plants ranging from cotton to papaya to rice. These

insects carry different symbionts that enhance their fitness and also provide them

resistance against biotic and abiotic stresses. We can use these symbionts as "Trojan

Horse" to block transmission of pathogens that are transmitted by these insects.

We are encouraged by the demonstration of complete transmission blockage by

the AMP-expressing *P. agglomerans* and we are confident that the transgenic bacteria

can be used individually or can be included as a component of integrated vector

management to save the grapevine from the menace of PD.

Materials and Methods

Sharpshooter maintenance

The glassy-winged sharpshooters (*H. vitripennis*) were collected from crepe

myrtle (*Lagerstroemia* sp.) trees planted in parking lot 9 of UC, Riverside. These

sharpshooters were kept on basil plants in cages until they were used.

Bacterial strains, culture conditions and painting on to the plant

Escherichia coli strain XL1-Blue was used to maintain plasmids and for gene cloning. *Pantoea agglomerans* E325, an EPA approved biological control agent, was used to express and deliver different AMP molecules inside the sharpshooter gut. Both *E. coli* and *P. agglomerans* were grown in Luria Barteni agar or broth. *P. agglomerans* and *E. coli* were cultured on agar plates at 30°C and at 37°C, respectively. Broth cultures were grown at the same temperatures in a shaker incubator (200 rpm). Carbenicillin or chloramphenicol were added at a concentration of 100 µg/mL and 35 µg/mL, respectively, when needed.

X. fastidiosa Temecula strain was used in toxicity assays and was cultured in PD3 agar at 28°C or in PD3 broth at 28°C. The shaker was agitated at 175-200 rpm to grow *X. fastidiosa* in broth culture.

Toxicity of AMPs against *P. agglomerans* and *X. fastidiosa*

P. agglomerans was grown in LB broth overnight at 200 rpm in an shaker incubator at 30°C. Next morning *P. agglomerans* was diluted 1/100 in 3 ml LB broth and grown at 30°C to mid log phase. At mid log phase the bacteria were diluted (CFUs/mL) in LB medium to 10^5-10^6 colony forming units/ml. Ninety µl of diluted *P. agglomerans* was pipetted into sterilized 0.2 mL PCR tubes and to this 10 µL of 10X test concentration of either melittin or SLM was added. These tubes were incubated overnight at 30°C and next morning OD 600 was determined.

X. fastidiosa strain Temecula was grown in PD3 broth to its log phase. The culture was then taken out and diluted to a concentration of 10^5-10^6 CFUs/mL in PD3 medium. 90 µL of diluted *X. fastidiosa* was mixed with 10 µL of 10X final concentration

either AMP in a sterilized 0.2 mL PCR tube and was incubated overnight at 28°C in a

shaker incubator. *X. fastidiosa* is a slow growing bacterium, which makes measuring

change in OD 600 of overnight cultures unfeasible. Hence, after overnight treatment with

AMPs *X. fastidiosa* was plated on to PD3 agar. These plates were incubated at 28°C for

10 days and CFUs were counted.

Plasmid Construction

Melittin gene's sense and antisense sequences with NheI and XmaI overhang were

ordered from IDT(Coralville, Iowa, USA) and were annealed to themselves by lowering

the temperature by 1°C/min from 95°C to 50°C.

Fig 8. Cloning of melittin or SLM gene into pEHLYS2-SD
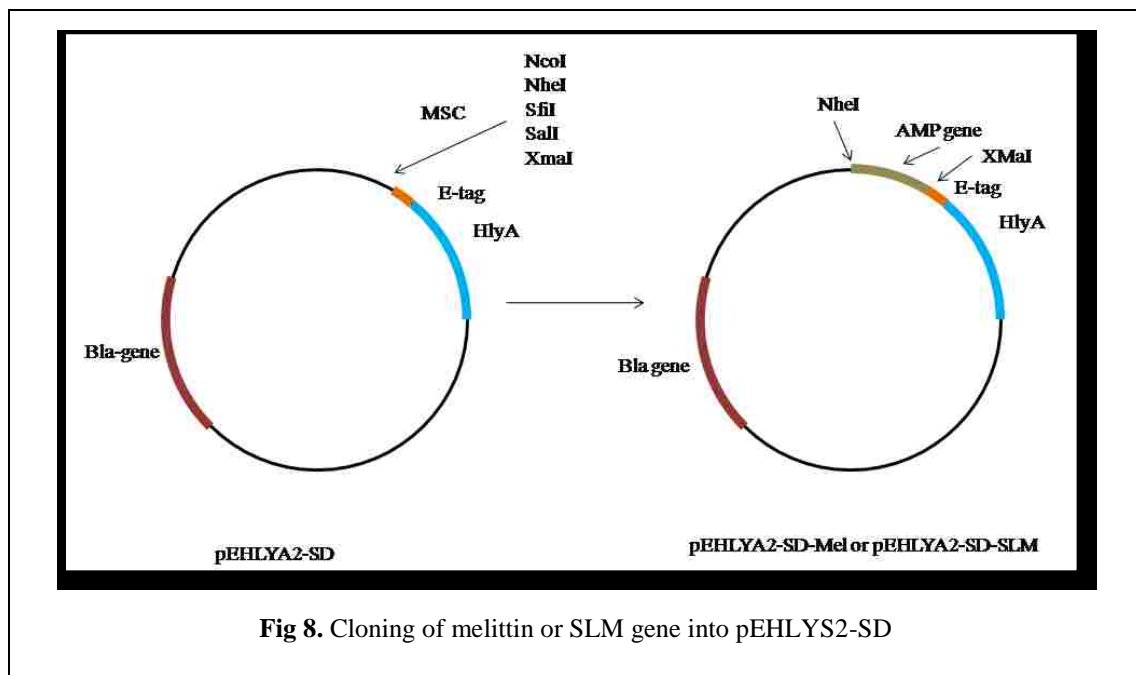
Scorpine like molecule (SLM), an AMP from *Vaejovis mexicanus* venom, gene

was amplified from a plasmid (kindly provided by Dr. Lorival D. Possani) using forward

primer (ScoHlyAF1.1) CAGCTAGCGGTTGGATAAGCGAG; and reverse Primer

(ScoHlyAR1.1) TTTTTTATAGGCACGGGGTATACC. The product was cut using

restriction enzymes NheI and SmaI.

The plasmid pEHLYA2-SD (kindly provided by Dr. Luis A. Fernandez) - having the hlyA secretion signal of the *E. coli* hemolysin secretion system- was also cut using restriction enzymes NheI and SmaI. Melittin or SLM genes were ligated into linearized pEHLYA2-SD plasmid. The in frame presence of both melittin and SLM genes was confirmed by sequencing. The successful, inframe insertion of melittin or SLM gene resulted in plasmid pEHLYA2-SD-Mel or pEHLYA2-SD-SLM.

P. agglomerans transformation

Overnight *P. agglomerans* was diluted in LB broth and grown to an OD600 of 0.6-0.7 (mid log phase). These cells were centrifuged at 4°C and 8000 rpm for 10 minutes and supernatant was removed. The cells were washed with ice cold autoclaved water. The final cell pellet of competent cells was re-suspended in 1ml 10% glycerol. Eighty µl of competent cell suspension was aliquoted into microcentrifuge tubes. One µl of pVDL9.3 plasmid (Fig. 9) was added to 80

Fig. 9: Plasmid pVDL9.3
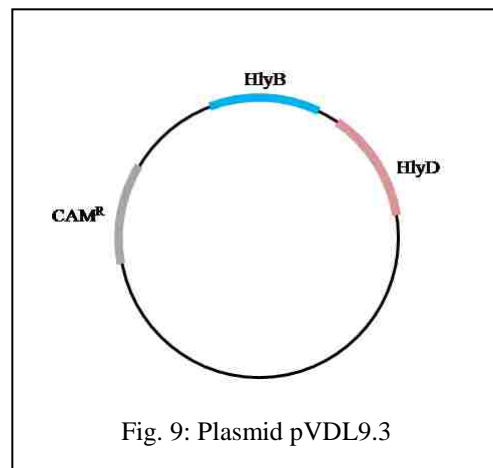
µl of competent cells and transferred to an ice cold 1 mm cuvette. These cells were electroporated at 2.0 kv, 25 microF. The cells were then plated onto chloramphenicol-containing LB agar. Next morning the colonies were selected and presence of plasmid was confirmed.

pVDL9.3 plasmid-containing *P. agglomerans* cells were made competent using the above mentioned protocol and were transformed with plasmid pEHLYA2-SD or

pEHLYA2-SD-Mel or pEHLYA2-SD-SLM. *P. agglomerans* containing both the plasmids were selected on LB agar containing carbenicillin and chloramphenicol.

P. agglomerans Growth Curve

Wild type *P. agglomerans* and *P. agglomerans* secreting melittin, SLM or only HlyA secretion signal were cultured overnight at 30°C in LB broth. Carbenicillin and chloramphenicol were added to LB broth where needed. Next morning each culture was diluted 1/50 in LB broth without antibiotic. The cultures were grown in a shaker incubator at 30°C and 200 rpm and OD600 was measured every hour.

Detection of melittin and SLM in spent medium

Overnight cultures of *P. agglomerans* were centrifuged at 10000 rpm and the supernatants were collected. The supernatant from each culture was concentrated using 10kDa NMWL filter (catalog #MRCPRT010, EMD Millpore, Temecula, CA, USA). Twenty µL of concentrated spent medium was mixed with 5 µL of loading dye and run on a 8-16% precast polyacrylamide gel (Catalog # 456-1103, Biorad, Hercules, Califirnia, USA) at a constant electric potential of 150V. The proteins were then transferred to a nitrocellulose membrane. The nitrocellulose membrane was first incubated with primary rabbit anti-E-tag antibody, which was diluted to a dilution of 1:1000 in 10% milk-TBST, at room temperature. This membrane was washed 5 times with TBST and incubated with mouse anti-rabbit antibody with AP conjugate, which was diluted in milk-TBST to a dilution to 1:5000. This membrane was washed 5 times with TBST and was developed using NBT and BCIP.

Presence of melittin in the supernatant was reconfirmed using rabbit anti-melittin serum using the protocol as mentioned above. This serum was generated by immunizing

rabbit with synthetic melittin and later collecting the serum. The serum was tested and confirmed for its binding to melittin via ELISA.

X. fastidiosa transmission blocking assays

P. agglomerans lines were cultured in LB broth and overnight cultures were washed twice with PBS. After washing, 10^{10} CFUs of *P. agglomerans* lines were suspended in 3 ml PBS. Each suspension was mixed with 20 ml 3% guar gum (w/v). One ml glycerol and 500 μl India Ink were added to it before this slurry was painted on to grape stems. The plants were kept overnight to let the guar gum dry. These stems were then covered with sleeve cages and field collected sharpshooters were released on these plants. The sharpshooters were kept on these plants for 48 hours before putting them on *X. fastidiosa* infected plants for another 48 hours. After acquisition access of 48 hours on *X. fastidiosa* infected plants the sharpshooters were collected and two of each sharpshooters were confined on naive grape plants for 24 hours. We used 2 sharpshooters to inoculate *X. fastidiosa* on each naive grape plant to increase the percent transmission, which is usually around 20 percent (Almeida and Purcell, 2003). The insects were removed after 24 hours, surface sterilized and DNA was extracted before running real-time PCR. The inoculated grape plants were kept in the greenhouse for 30 weeks and were tested for *X. fastidiosa* infection via real-time PCR.

DNA extraction from the insect head

The sharpshooters were surface sterilized by washing them in 70% ethanol for 2 minutes followed by washing in 10% bleach for 2 minutes. Subsequently these sharpshooters were washed twice in sterilized water for 2 minutes. The heads were removed from the sterilized sharpshooters' bodies using surgical blade. The

sharpshooters' heads were then homogenized in 200µL PBS using a Kontes homogenizer and DNA was extracted using DNeasy Blood and Tissue Kit (Catalog # 69504, Qiagen, Valencia, CA, USA) following manufacturer's instructions.

DNA extraction from plant tissues

After 30 weeks of inoculation stems of ~ 8 cm were cut from plants. These stems were sterilized by washing in 70% ethanol and 10% bleach for 2 minutes each, followed by 2X washing in sterilized water for 2 minutes. These stems were put in Adagia bags (Catalog # ACC 00930/0100, Elkhart, IN, USA) and homogenized in 800 µL of lytic buffer (20 mM Tris-Cl pH 8.0, 70 mM Sodium EDTA, 2 mM Sodium Chloride, 20 mM Sodium Metabisulfite) using mortar and pestle. 200 µl of plant tissue suspension in lysis buffer was placed in a 1.5 ml microcentrifuge tube. This suspension was incubated at 55°C for 1 hour after adding 40µl of 5% sodium sarkosyl and 1.5 µl of proteinase K. After 1 hour of incubation this suspension was centrifuged at 13,000 rpm for 15 minutes and supernatant was collected. DNA was purified from the supernatant using a GeneClean kit (Catalog #111001200, MP Biomedicals, Santa Ana, CA, USA) following manufacturer's instructions.

Real-time PCR

We used ITS-specific primers and probes described in Schaad *et al*. (2002) to run real time-PCR. The 20µl reaction was performed in 0.1ml strip tubes containing 10 µl 2X IQ Supermix (Biorad), 100nM forward primer, 200 nM reverse primer, 200 nM Taqman probe with dye, 5.8 µl of PCR-grade water and 2 µl of template DNA. The real-time PCR was performed on the Eppendorf Realplex at 95ºC for 3 minutes for enzyme activation

followed by denaturation at 95ºC for 15 seconds, and extension and annealing at 62ºC for 1 minute. The PCR was run for 40 cycles.

Detecting accumulation of AMPs inside the insect body

The glassy-winged sharpshooters were surface sterilized as mentioned above. The whole sharpshooters were then homogenized in PBS using a Kontes homogenizer. The homogenized solution was then centrifuged at 13,000 rpm for 10 minutes and supernatant was used for AMP detection. Twently µl of supernatant was mixed with 5µl of reducing marker and was run on precast Mini PROTEAN TGX gels. Proteins were transferred on to nitrocellulose membranes as mentioned above and accumulation of protein was detected using primary rabbit anti-E-tag antibody as mentioned above.

Accumulation of melittin inside the insect body was confirmed using rabbit anti-melittin serum. The protocol for Western blot was used as mentioned above.

Statistical analysis

Chi-square test for homogeneity was employed to compare number of GWSS carrying *X. fastidiosa* in their foregut. *X. fastidiosa* CFUs present in GWSS foregut in various treatments were analyzed by Tukey's test for multiple comparison after taking log values of CFUs. All values are shown as mean±S.E. Statistical analysis were performed using Minitab version 17 for windows8. *p* values<0.05 were considered significant.

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Chapter 4: Ribosome Display of Combinatorial Antibody Libraries Derived from Mice Immunized with Heat-Killed *Xylella fastidiosa* and the Selection of MopB-Specific Single-Chain Antibodies.

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Azizi, A., Arora, A., Markiv, A., Lampe, D. J., Miller, T. A. and Kang, A. 2012. Ribosome display of combinatorial antibody libraries derived from mice immunized with heat-killed *Xylella fastidiosa* and the selection of mopb-specific single-chain antibodies. *Applied Environmental Microbiology*. **78**(8): 2638-2647.

Chapter 4: Ribosome Display of Combinatorial Antibody Libraries Derived from Mice Immunized with Heat-Killed *Xylella fastidiosa* and the Selection of MopB-Specific Single-Chain Antibodies

<u>Abstract</u>

Pierce's disease is a devastating lethal disease of *Vitus vinifera* grapevines caused by the bacterium *Xylella fastidiosa*. There is no cure for Pierce's disease, and control is achieved predominantly by suppressing transmission by the glassy-winged sharpshooter insect vector. We present a simple robust approach for the generation of panels of recombinant single-chain antibodies against the surface-exposed elements of *X. fastidiosa* that may have potential use in diagnosis and/or disease transmission blocking studies. *In vitro* combinatorial antibody ribosome display libraries were assembled from immunoglobulin transcripts rescued from the spleens of mice immunized with heat-killed *X. fastidiosa*. The libraries were used in a single round of selection against an outer membrane protein, MopB, resulting in the isolation of a panel of recombinant antibodies. The potential use of selected anti-MopB antibodies was demonstrated by the successful application of the 4XfMopB3 antibody in an enzyme-linked immunosorbent assay (ELISA), a Western blot assay, and an immunofluorescence assay (IFA). These immortalized *in vitro* recombinant single-chain antibody libraries generated against heat-killed *X. fastidiosa* are a resource for the Pierce's disease research community that may be readily accessed for the isolation of antibodies against a plethora of *X. fastidiosa* surface-exposed antigenic molecules.

<u>Introduction</u>

  Xylella fastidiosa includes a group of closely related pathogens, each affecting a specific plant target. One subspecies affects grapevines, but little else, another subspecies affects almonds and grapevines, and still another affects only oleanders (1). The subspecies *Xylella fastidiosa* subsp. *fastidiosa* (*Xanthomonadales*: *Xanthomonadaceae*) is a pathogen of the grapevine (14), an economically important crop in California. This bacterium is the causative agent of Pierce's disease (PD) of grapevines and is vectored by the leaf hopper *Homalodisca vitripennis* (*Germar*) (*Hemiptera*: *Cicadellidae*) (formerly *H. coagulata*), also known as the glassy-winged sharpshooter (GWSS) (39). The precise pathogenic mechanisms resulting in disease are not clearly understood; however, the ability of the pathogen to colonize in both the xylem of the grapevine and in the foregut of the GWSS vector is well established (11). The *X. fastidiosa* genome has been sequenced, and comparative analysis has provided valuable information of genes, metabolic pathways, and potential virulence factors that may be involved in pathogenicity (43).

  Investigating the interfaces between the plant-pathogen-insect interactions may reveal sites for molecular interventions that could confer resistance or reduce transmission of the pathogen. It is possible to predict and explore the surface-exposed components that may play a role in bacterial virulence and/or be involved in attachment or biofilm formation in either the plant or arthropod. The function of surface-displayed targets may also be probed using lectins or polyclonal antibodies (29). However, if monoclonal antibody (MAb) tools were available, they would allow a more detailed

study of the assembly, distribution, function, and the role of such targets in plant-vector interactions.

The desire for the efficient rapid generation of MAbs to biologically important protein antigens over the past 2 decades has driven the development of a range of *in vitro* technologies based on combinatorial immunoglobulin repertoire cloning (26), high-throughput screening, phage display (4, 27, 37), and ribosome display (22, 23, 36). For ribosome display, immunoglobulin mRNA transcripts can be isolated from antibody-producing cells, converted to cDNA, and assembled *in vitro* to create linear DNA templates encoding libraries of single-chain-fragment variable antibodies (scFv's), which can be transcribed *in vitro* into mRNAs that lack a termination codon. Translation of mRNA templates in the absence of a stop codon results in each ribosome stalling at the last codon and retaining the correctly folded antibody polypeptide and the mRNA, creating tripartite antibody-ribosome-mRNA complexes (ARMs) (see Fig. 2). The library of ARMs can then be affinity enriched to select the desired ARM for recovery. The essence of the approach is the linking of the recognition function to the replication function, i.e., linking the phenotype to the genotype (27). Recombinant protein display technologies allow ready access to genetically encoded ligands or receptors for defined targets, an approach pioneered more than 25 years ago (44).

The aim of this study was to create an antibody resource from mice immunized with *X. fastidiosa* that would permit the isolation of recombinant antibodies against surface-accessible molecules on *X. fastidiosa* and to validate the approach by selecting scFv's against the outer membrane protein MopB (8).

Materials and Methods

Bacterial strains and plasmids

The oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. The pSANG10-3F vector (42) was provided by John McCafferty (University of Cambridge) and used with the XL1-Blue *Escherichia coli* strain (Stratagene) for plasmid construction and the BL21(DE3) or Rosetta gamiB (DE3) *E. coli* strain (Novagen, United Kingdom) for recombinant protein expression. David Lampe provided the pMALc2x_mopB plasmid. Plasmid pAHAHis for scFv bacterial cytoplasmic expression was based on modified pET32a (unpublished data). The plasmid pFab1-PhoA-H (47) was provided by Masataka Takekoshi (Tokai University, Japan). The anti-NANP repeat monoclonal antibody 2A10 (38, 49) V_H and V_L sequences were provided by Anthony A. James (University of California Irvine). The *Xylella fastidiosa* Temecula1 strain was prepared at the University of California Riverside in the laboratory of Thomas A. Miller.

MopB

The complete amino acid sequence of the *X. fastidiosa* Temecula1 strain (NCBI NP_779898.1) MopB was submitted to web-based protein prediction programs, namely, SIG-Pred (signal peptideprediction;http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.html) (6) and the PRED TMBB website, which hosts a Hidden Markov Model method, capable of predicting transmembrane beta-strands of outer membrane proteins of Gram-negative bacteria (http://biophysics.biol.uoa.gr/PRED-TMBB/) (2, 3, 45), to identify the signal peptide and the putative surface-exposed domains, respectively. The predicted encoded mature domain (MopB = 354 amino acids [aa]) and surface-exposed domain (MopB =

182 aa) were PCR amplified using primers designed to include the NdeI (5′) site

encoding an in-frame methionine start codon and the NotI (3′) site encoding an in-frame

triple alanine (primers are listed in Table S1 in the supplemental material). The MopB-

encoding inserts were PCR amplified using a pMALc2x_mopB template. The PCR

products were restricted with NdeI and NotI and inserted into pSANG 10-3F vector to

produce proteins with in-frame C-terminal sequence

[AAASA(H)$_6$KLDYKDHDGAYKDHDIAYK(D)$_4$K]. The molecular masses and

isoelectric points were predicted using the ExPASy bioinformatics resource portal

(http://web.expasy.org/compute_pi/) (20). The plasmids were used to transform XL1-

Blue *E. coli* cells and confirmed by DNA sequencing.

Bacterial expression of recombinant MopB

Plasmids encoding recombinant MopB were transformed into BL21(DE3) *E.

coli* cells (Novagen) and grown in 200 ml of LB medium containing kanamycin (30

μg/ml) at 37°C with shaking at 250 rpm until an optical density (OD) at 600 nm of 0.4 to

0.6 was reached. Protein expression was induced by the addition of isopropyl β-D-1-

thiogalactopyranoside (IPTG; final concentration, 0.3 mM), and incubation was resumed

for 3 h before harvesting was performed by centrifugation at 5,000 rpm and 4°C for 20

min (using a Sorvall Super T 21 benchtop centrifuge, with an SL-250T rotor). Cell pellets

were frozen and stored at −80°C.

Recombinant MopB extraction

The cell pellet obtained from 200 ml of culture was resuspended in 5 ml of

denaturing lysis buffer (6 M guanidine hydrochloride, 10 mM Tris-HCl, 500 mM NaCl,

10 mM imidazole, pH 8.0). The cells were lysed by sonication on ice (6 30-s pulses) in an

ultrasonic cell disruptor and centrifuged at 16,000 rpm for 45 min to pellet the cellular debris. The supernatant containing soluble material was retained for the subsequent purification using Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) agarose affinity resin. A 1-ml volume of nickel-chelating agarose slurry (G-Biosciences) was applied to a 10-ml column and equilibrated with 5 ml of wash buffer (8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0). The supernatant containing the hexahistidine-tagged protein was loaded onto the resin. The flowthrough was collected and the resin washed with 5 ml of wash buffer (8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 6.3). The bound recombinant MopB fusion proteins were eluted in 1 ml of elution buffer five times (8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 4.5). Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27) using 12% Tris-glycine gels and visualized using a fast microwave-assisted Coomassie stain technique described elsewhere (34). Protein concentrations were estimated using the Bradford dye binding assay (6).

Mouse immunization

The *X. fastidiosa* Temecula 1 strain was grown in PD3 media (13) (100 ml) in a 0.5-liter flask at 28°C at 180 rpm for 10 days until an OD at 600 nm of 0.5 was reached. The bacteria were pelleted and resuspended in 100 ml of PBS twice, the final pellet wet weight was determined, the pellet was resuspended in PBS at 10 mg/ml (wt/vol) and 1-ml aliquots were prepared. An aliquot of the *X. fastidiosa* bacteria was incubated at 28°C (viability; positive control) and the remainder heat treated at 55°C for 1 h and checked for viability by plating on PD3 agar and incubation for 15 days at 28°C; the remainder of the bacterial vials were frozen at −80°C. Once it had been confirmed that no viable bacteria

were present in the heat-treated ampules, aliquots were sent to ProSci Inc. (Poway, CA) for immunization of BALB/c mice ($n = 5$). The initial immunization (0.5 ml/mouse) was followed by boosting at weeks 4 and 8 and 12. Test bleeds were taken a week after the third boosting for enzyme-linked immunosorbent assay (ELISA) and dot blot analysis. Following testing, mice were boosted and 3 days later underwent a splenectomy; each spleen was placed in 10 ml of TRIzol (Invitrogen, Carlsbad, CA) for use in RNA isolation.

MopB ELISA

Recombinant MopB (10 μg/ml) or bovine serum albumin (BSA) (10 μg/ml) in phosphate-buffered saline (PBS) (pH 7.4; 0.1 ml/well) were used to coat a 96-well plate (Nunc) at 4°C overnight and blocked with 0.2 ml of 2% BSA–PBS at room temperature for 1 h. Polyclonal sera from mice ($n = 5$) previously immunized with whole heat-killed *X. fastidiosa* were diluted to 1/20,000 in PBS containing 0.05% Tween 20 (PBS-T), added in duplicate to the wells (0.1 ml/well), and incubated at 37°C for 2 h. The wells were washed with PBS-T, and 0.1 ml/well of rabbit anti-mouse antibody (Sigma-Aldrich) diluted 1:20,000 in 1% BSA–PBS-T was added and incubated at 37°C for 2 h. The plates were washed as before, and 0.1 ml/well of goat-anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) (diluted 1:40,000 in 1% BSA–PBS-T) was added and incubated at 37°C for 2 h. The plates were washed as before with an additional final wash with PBS alone and developed with *p*-nitrophenyl phosphate (1 mg/ml)–0.2 M Tris buffer (pH 8.0) (0.1 ml/well) at 37°C for 30 min. The absorbance was measured at 405 nm on an ELISA plate reader (VersaMax; Molecular Devices, United Kingdom).

Bacterial dot blot assay

Strips of nitrocellulose ∼5 mm wide were spotted with 5 μl of 5% (wt/vol) skimmed milk powder–PBS, 5 μl of a bacterial suspension of *E. coli*, and 5 μl of the *X. fastidiosa* Temecula1 strain, allowed to air dry, blocked with 1% BSA–PBS, and incubated with immune sera (bleed 3) diluted 1/20,000 in PBS-T with 1% BSA for 1 h. Following washing with PBS-T, the mixture was incubated with rabbit anti-mouse alkaline phosphatase antibody (Sigma-Aldrich), diluted 1:20,000 in 1% BSA–PBS-T for 1 h, washed as before, and developed with a mixture containing 0.02% 5-bromo-4-chloro-3-indolylphosphate (BCIP), 0.03% nitroblue tetrazolium (NBT), 10 ml of 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, and 0.05% Tween 20 (pH 9.5).

Mouse immunoglobulin library assembly

The mouse spleens, each in 10 ml of TRIzol (Invitrogen), were shipped on dry ice and stored at −20°C prior to processing. Upon thawing at room temperature, each sample was macerated using an IKA T8 Ultra-Turrax homogenizer and processed following the instructions for total RNA isolation (TRIzol; Invitrogen). The resulting RNA pellets were dissolved in 50 μl of RNase-free water and analyzed using a NanoDrop apparatus (Thermo-Fisher, United Kingdom) to determine relative purity and RNA concentrations and stored at −20°C until required. First-strand cDNA was synthesized from ∼1 μg of total RNA by the use of a ProtoScript First Strand cDNA synthesis kit (M-MuLV) and reverse transcriptase (RT; New England BioLabs) with Oligo-dT29VN for light chains and MVHLink2 for the heavy chains, following the instructions provided. For antibody library construction, the PCR primers were based on published sequences (26, 28) with minor modifications (see Table S1 in the supplemental material). The
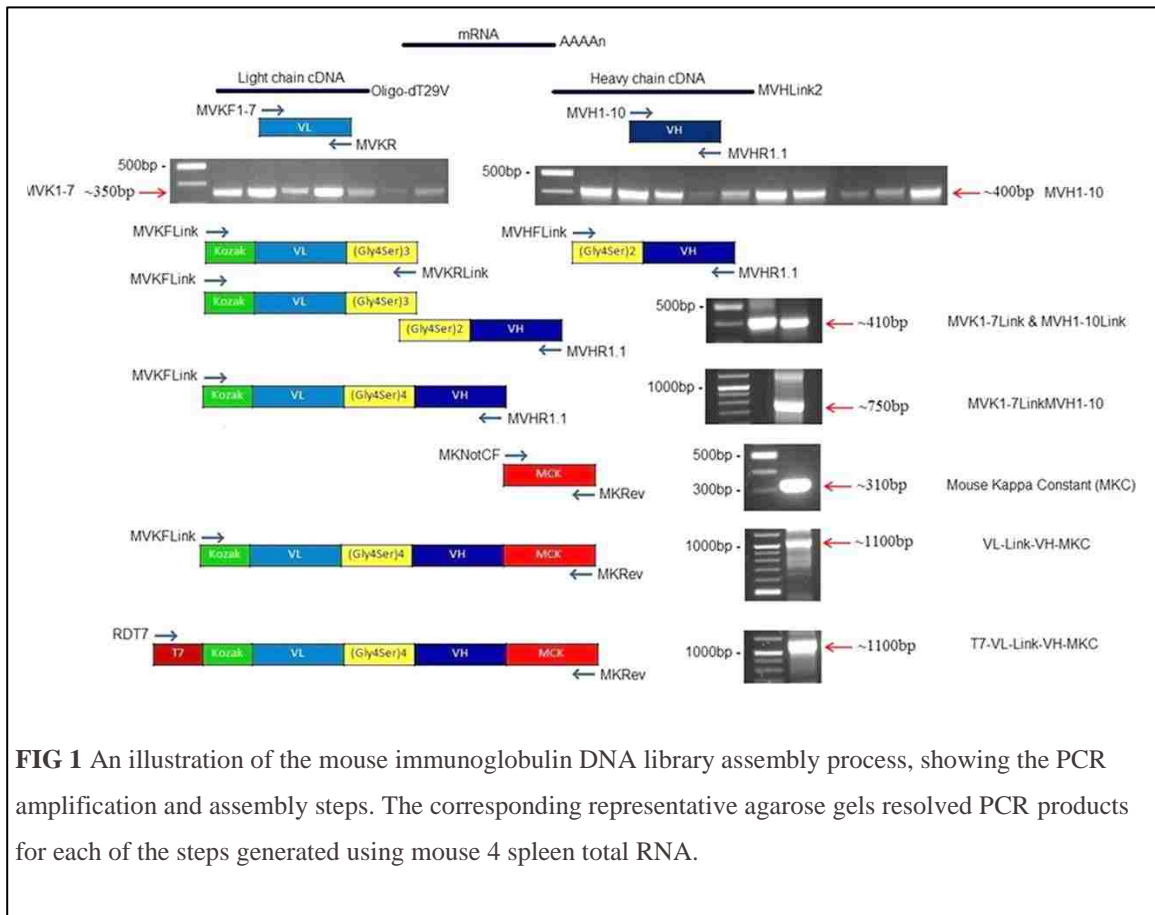
FIG 1 An illustration of the mouse immunoglobulin DNA library assembly process, showing the PCR amplification and assembly steps. The corresponding representative agarose gels resolved PCR products for each of the steps generated using mouse 4 spleen total RNA.

primers were designed to introduce in-frame NcoI and NotI restriction sites to the 5′ end of the V_L sequence and to the 3′ end of the V_H sequence, respectively, as shown in Fig. 1. Members of the light-chain families were individually amplified by PCR using the Oligo-dT29VN cDNA template and combinations of MVKF1–7 with MVKR. Members of the heavy-chain families were individually amplified by PCR using MVHLink2 cDNA templates and combinations of MVHF1–10 and MVHR1.1. The amplified light-chain products were purified and pooled, and an aliquot was subjected to another round of PCR amplification using MVKFLink and MVKRLink to introduce part of the T7 site and Kozak sequence (32) on the 5′ end and an overlap extension on the 3′ end to facilitate joining to the variable heavy-chain libraries. The amplified heavy-chain products were processed and modified in a similar manner using primers MVHFLink and MVHR1.1.

The modified variable light- and heavy-chain products were combined and amplified using MVKFLink and MVHR1.1. A synthetic mouse kappa constant (MKC) domain (optimized for *E. coli* codon usage and synthesized by Epoch Biolabs, Sugar Land, TX) was amplified using MKNotCF and MKRev. The MKC was joined to the V_L-link-V_H combinations by a PCR overlap extension reaction using MVKFLink and MKRev (Fig. 1). Finally, the PCR product encoding all the variable light-chain and heavy-chain combinations was amplified with primers RDT7 and MKRev to produce the DNA encoding the anti-*X. fastidiosa* immunoglobulin scFv libraries. The initial PCR amplification reactions were performed at a 50°C annealing temperature with 30 cycles, and the subsequent library assembly step used 16 cycles with *Taq* DNA polymerase and 20 pmol of each primer pair per reaction. DNA fragments were resolved by gel electrophoresis on 2% (wt/vol) agarose gels. DNA isolation from agarose gels was carried out following Qiagen DNA gel purification kit instructions. In total, five combinatorial scFv antibody DNA libraries were constructed.

Antibody ribosome display

To select for single-chain antibodies, a modified eukaryotic ribosome display (23) was used as outlined in Fig. 2. The PCR-generated DNA library of antibody-coding genes derived from mouse 4 were expressed in a coupled rabbit reticulocyte lysate system (Promega; TNT quick-coupled transcription-translation system). An *in vitro*-coupled transcription-translation reaction was set up in a 0.5-ml tube as follows: to 40 μl of TNT T7 Quick Master Mix, 2 μl of DNA library (0.1 to 1.0 μg), 1 μl (1 mM) of methionine, 1 μl of DNA enhancer, and 6 μl of water were added, and the reaction mixture was incubated at 30°C for 90 min. Then, 6 μl of RNase-free DNase I (Roche)

FIG 2 A schematic of the steps involved in *in vitro* antibody ribosome display. Step 1: the scFv antibody library DNA template was prepared flanked with a T7 sequence and mouse kappa constant sequence. Step 2: the antibody library DNA was transcribed to mRNA and translated to form an antibody-ribosome-mRNA complex. Step 3: the mixture was incubated with immobilized truncated MopB. Step 4: the unbound components were removed by washing. Step 5: the retained complexes were released. Step 6: the mRNA was reverse transcribed and amplified by PCR. Step 7: the PCR products were cloned into TOPO vectors and recovered plasmids sequenced. Full-length in-frame scFv sequences were inserted into pAHAHis vector for recombinant antibody production.

(10,000 U/ml) was added, and the mixture was incubated for 20 min at 30°C (to degrade input DNA). To select specific antibody fragments, a 0.5-ml PCR tube was coated with 0.1 ml of recombinant truncated MopB (10 μg/ml)–PBS at room temperature for 1 h, washed with PBS, and blocked with 100 μl of molecular-biology-grade BSA–PBS (10 mg/ml) (New England BioLabs) for 1 h. The translation-transcription mixture containing

the antibody-ribosome-mRNA complexes was added to a MopB-coated tube and incubated on ice for 1 h. The tube was washed by filling once with PBS (0.5 ml) and decanting and carefully removing the residual liquid by the use of a sterile pipette tip. The retained RNA was recovered using an RNeasy (350 μl) Mini protocol following the manufacturer's instructions and used in OneStep (Qiagen) RT-PCR with MVKFLink and MKRD2 primers to amplify the antibody encoding DNA in a 50-μl reaction mixture by the use of 6 μl of RNA template and 5 μl of each primer (20 pM). The mixture was cycled at 50°C for 30 min and 95°C for 15 min and then 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1 min, and 72°C for 5 min and finally kept at 8°C. The RT-PCR product from a single round of ribosome display was purified by agarose gel electrophoresis and cloned into pCRII-TOPO vector (TOPO TA cloning kits; Invitrogen) according to the manufacturer's instructions and used to transform XL1-Blue competent cells. Plates containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) were used to select for disruption of the β-galactosidase. Four white colonies were randomly selected and inoculated into 10 ml of LB media with carbenicillin (100 μg/ml); plasmid DNA was isolated from *E. coli* by the use of standard procedures and a QIAprep Spin Miniprep kit (Qiagen) following the manufacturer's instructions, and DNA was sequenced at the core facility at The Wolfson Institute for Biomedical Research, University College London.

ScFv expression

An scFv antibody cytoplasmic expression vector with a carboxyl-terminal-tag peptide sequence derived from influenza virus hemagglutinin (HA) (Asp Val Pro Asp Tyr Ala Ser [DVPDYAS]) followed by a hexahistidine tag designated pAHAHis was

constructed based on pET32a. The recovered single-chain antibody-encoding sequences were inserted into pAHAHis plasmid as NcoI-NotI fragments for cytoplasmic expression in Rosetta gami B (DE3) *E. coli*. The pAHAHis encoding the scFv was transformed into Rosetta gami B (DE3) *E. coli* cells (Novagen) that were then inoculated into 200 ml of LB medium containing carbenicillin (100 μg/ml) and chloramphenicol (34 μg/ml) and grown at 37°C with shaking at 250 rpm until the optical density at 600 nm reached 0.4 to 0.6. Protein expression was induced by the addition of IPTG (final concentration, 1 mM), and cells were then incubated for 20 h at 20°C with shaking at 250 rpm. Cells were harvested in four 50-ml tubes by centrifugation at 5,000 rpm and 4°C for 20 min (using a Hettich Universal 320 centrifuge and a model 1617 rotor). Cell pellets were frozen and stored at −80°C before undergoing further processing. The cell pellet was resuspended in 3 ml of native lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 0.15% Triton X-100 [pH 8.0], and 250 μl of lysozyme at 1 mg/ml). The cells were lysed by sonication on ice as described earlier and centrifuged at 18,000 rpm for 45 min. The supernatant containing the soluble fraction was retained for affinity purification. A 1-ml volume of nickel-chelating resin suspension (G-Biosciences) was packed in a MicroSpin column and equilibrated with 600 μl of native lysis buffer. The supernatants containing the hexahistidine-tagged recombinant antibodies were loaded onto the equilibrated 1-ml Ni^{2+}-NTA spin column and centrifuged (using a Hettich 320R Universal centrifuge and a 1689-A rotor) at 1,600 rpm for 5 min. The flowthrough was collected and the Ni^{2+}-NTA column washed two times with 600 μl of wash buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0) at 2,900 rpm for 2 min. The bound recombinant antibodies were eluted into 100-μl fractions using 500 μl of elution buffer (20 mM Tris-HCl, 500

mM NaCl, 500 mM imidazole, pH 8.0) at 2,900 rpm for 2 min, and the purified scFv was stored at 4°C.

Functional assays

Recombinant scFv binding to MopB was examined by Western blot, ELISA, and immunofluorescence analysis. The scFv antibody binding to recombinant MopB was detected using the DVPDYAS peptide tag and a secondary recombinant scFv antibody with a $(Gly_4Ser)_3$ linker based on the V_H and V_L sequences of the mouse 26/9 anti-DVPDYAS monoclonal antibody (12) fused to alkaline phosphatase (*phoA*) (46) (anti-HA scFv-AP) in both the ELISA and Western blot analysis. ELISA with either full-length mature or truncated MopB was performed as described earlier with a modification using 5% (wt/vol) milk–Tris-buffered saline (TBS) containing 0.1% NaN_3 as a negative control. Purified antibodies, selected scFv antibody for MopB, and negative-control anti-NANP repeat scFv based on a 2A10 MAb diluted 1 in 10 using TBS-NaN_3 were incubated for 6 h at 8°C. After washing with TBS containing 0.05% Tween-20 (TBST-NaN_3), anti-HA scFv-AP antibody (diluted 1:10 in 1% [wt/vol] milk–TBS–NaN_3) was added and the mixture was incubated overnight at 8°C, washed as before, and developed with *p*-nitrophenyl phosphate as described earlier.

For the Western blot analysis, recombinant MopB proteins were resolved by SDS-PAGE as described earlier and blotted onto a polyvinylidene fluoride (PVDF) membrane, blocked in 5% (wt/vol) milk–TBS at room temperature for 1 h, and then incubated with the scFv 4XfMopB3 as the primary antibody (diluted 1:10 in 1% [wt/vol] milk–TBS) for 6 h at 8°C. After washing with TBST incubated with the secondary antibody, anti-HA scFv-AP antibody (diluted 1:10 in 1% [wt/vol] milk–TBS–0.1% NaN_3) was added and

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the mixture was incubated at room temperature for 1 h on a rocking platform followed by

washing as before and a final rinse with TBS (5 min) prior to the addition of BCIP-NBT

substrate as described earlier.

Heat-killed *X. fastidiosa* was air dried on glass slides and blocked with 2%

(wt/vol) milk–PBS for 30 min. Purified scFv antibodies (selected scFv antibody for

MopB and negative-control scFv 2A10 antibody) were diluted 1 in 10 with 1% (wt/vol)

milk–PBS. The slides were incubated with XfMopB3 (anti-MopB), 2A10 (nonspecific),

or no scFv for 1 h. After washing with PBS, anti-polyhistidine-fluorescein isothiocyanate

(FITC) conjugate (AbCam, United Kingdom) (diluted 1:10,000 in 1% [wt/vol] milk–

PBS) was added. The slides were incubated for 1 h at the ambient temperature in the

dark. Slides were washed with PBS and viewed under oil immersion on an Axioscop 50

fluorescence microscope (Zeiss). Images were captured using a charge-coupled-device

(CCD) camera (PowerShot digital camera; Canon) and AxioVision software (Zeiss).

Nucleotide sequence accession numbers

The GenBank accession numbers for the 4XfMopB1 and 4XfMopB3 scFv's

are JQ606804 and JQ606805, respectively.

Results

MopB bioinformatic analysis

The *X. fastidiosa* Temecula1 MopB protein sequence downloaded from the NCBI

database was analyzed using SIG-PRED to predict the putative signal sequences, and

Met1-Ala36 and Met15-Ala36 were identified (Fig. 3A). The β-barrel transmembrane

domain identified using PRED TMBB was determined to be between Trp43 and Tyr177.
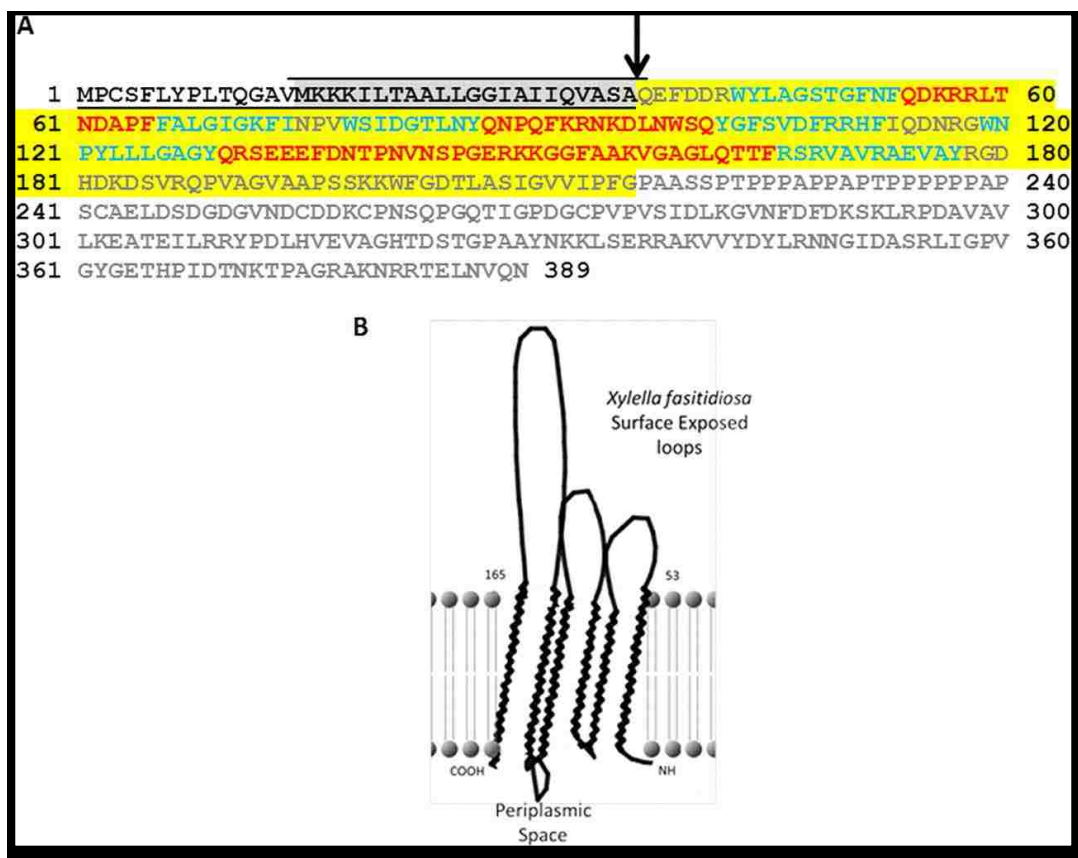
FIG 3 The amino acid sequence of *Xylella fastidiosa* Temecula1 MopB and the predicted model for the B-barrel outer membrane domain. (A) The putative leader sequence starting at Met1 is indicated with black underlining; a shorter potential leader sequence starting at Met15 (previously identified by Bruening et al.) (8) is indicated with a black line over the sequence and gray highlighting. The arrow between Ala36 and Gln37 denotes a signal peptide cleavage site, gray denotes periplasmic regions, blue denotes membrane-spanning regions, and red denotes surface-exposed loops. The yellow highlighted region corresponds to the truncated MopB. The sequence scored a value of 2.929, which is lower than the threshold value of 2.965. The difference between the measured value and the threshold indicates the possibility of the protein being an outer membrane protein. (B) A two-dimensional figure of the predicted MopB transmembrane β-barrel with the protruding surface-exposed loops generated using PRED TMBB. The amino acid numbers of the beginning and end of the β-strand traversing the outer membrane are in gray.

A two-dimensional representation of the result shows the exposed loops, with the last

loop being the longest (Fig. 3B). The recombinant mature and truncated MopB proteins

expressed in pSANG 10-3F were predicted to have a pI of 6.36 and molecular mass of

42.98 kDa and a pI of 6.79 and molecular mass of 24.64 kDa, respectively.

Expression and purification of the full-length mature and truncated MopB proteins

The genes encoding full-length mature Met-Ala36-Asn389 and truncated Met-

Ala36-Gly252 MopB from *X. fastidiosa* were PCR amplified and cloned into the

pSANG10-3F vector and verified by DNA sequencing. Protein analysis by SDS-PAGE

revealed abundant full-length and truncated MopB proteins at 3 h postinduction with

FIG 4 (A) SDS-PAGE analysis of mature full-length MopB expression and purification under

denaturing conditions. Lane 1, protein molecular mass markers; lane 2, noninduced; lane 3, induced;

lane 4, final wash; lanes 5 and 6, elutions 2 and 3. (B) SDS-PAGE analysis of truncated MopB

expression and purification under denaturing conditions. Lane 1, protein markers; lane 2, uninduced;

lane 3, induced; lane 4, soluble fraction; lane 5, flowthrough; lane 6, final wash; lanes 7 to 10, elutions

1 to 4.

IPTG at 37°C. The SDS-PAGE results indicated proteins with an apparent molecular

mass of about 43 kDa for full-length MopB and 25 kDa for truncated MopB (Fig. 4A and

B, respectively). The recoveries of recombinant mature and truncated MopB proteins were estimated to be approximately 50 mg/liter of culture.

Serum ELISA and dot blot analysis

The ELISA performed with immobilized recombinant truncated MopB protein and a 1/20,000 dilution of sera from bleed 3 of the mice immunized with whole heat-killed *X. fastidiosa* indicated that 4 of 5 mice produced antibodies that recognized the recombinant surface-exposed portion of MopB with little or no cross-reactivity with the BSA control protein (Fig. 5A). The immune sera from bleed 3 were also evaluated in a dot blot analysis using whole intact bacteria of *E. coli* and the *X. fastidiosa* Temecula1 strain (Fig. 5B). Mouse sera 1, 2, 4, and 5 had clear antibody binding to *X. fastidiosa* at a dilution of 1/20,000, whereas mouse 3 serum did not. The antibody activity against milk proteins was absent or very weak in mouse sera 2 and 4.

Mouse immunoglobulin library assembly and ribosome display

The amounts of total RNA isolated from the mice ranged from 200 to 50 μg with various degrees of purity as shown in Table S2 in the supplemental material, providing ample material for the subsequent cDNA reactions. The immunoglobulin libraries were assembled as outlined in the schematic; the PCR amplification results and steps of the library construction performed using mouse 4 spleen-derived cDNA are shown in Fig. 1. Mouse V_k6 and V_H4 and -8 were present at lower levels; all other immunoglobulin families were readily amplified. The final DNA template encoding the library flanked by a T7 site and a synthetic mouse kappa constant chain was used in an *in vitro* ribosome display with a single selection step wittruncated MopB as outlined in Fig. 2. The affinity-enriched mRNAs were amplified by RT-PCR and cloned into pCRII-TOPO vector. From
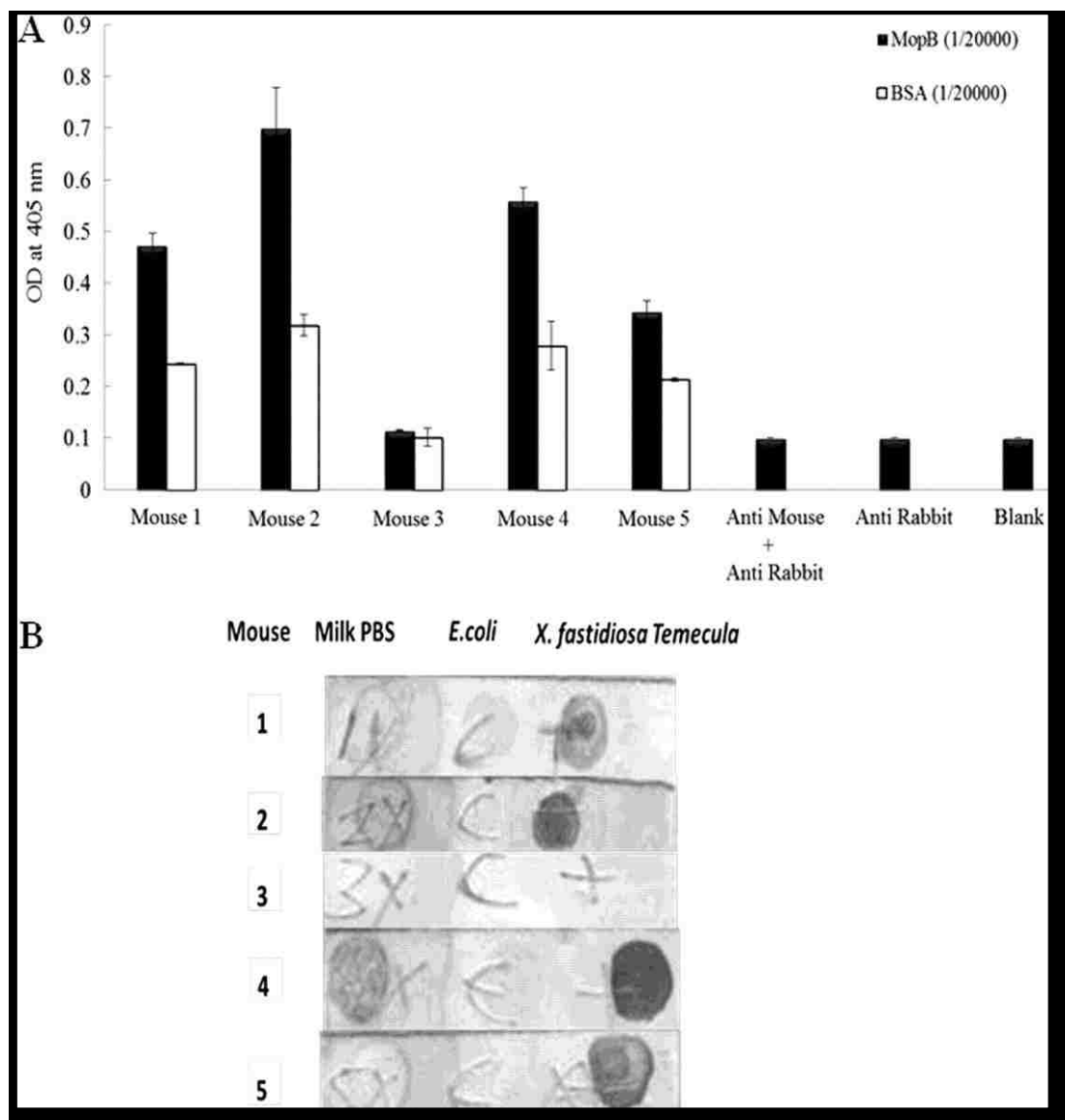
FIG 5 Mouse immune serum evaluation by ELISA and immuno-dot blot analysis. (A) Mouse 1 to 5 sera from bleed 3 diluted 1/20,000 in the ELISA performed with truncated MopB (10 μg/ml) or BSA (10 μg/ml) and h blank, rabbit anti-mouse (1/20,000) plus anti-rabbit alkaline phosphatase, and anti-rabbit alkaline phosphatase (1/40,000) without anti-mouse antibody. (B) Immuno-dot blot screen with a 1/20,000 dilution of bleed 3 sera from mice 1 to 5 on 5% (wt/vol) milk protein control or 5 μl of *E. coli* or *X. fastidiosa* spotted directly onto nitrocellulose strips and probed with rabbit anti-mouse alkaline phosphatase and developed with NBT-BCIP.

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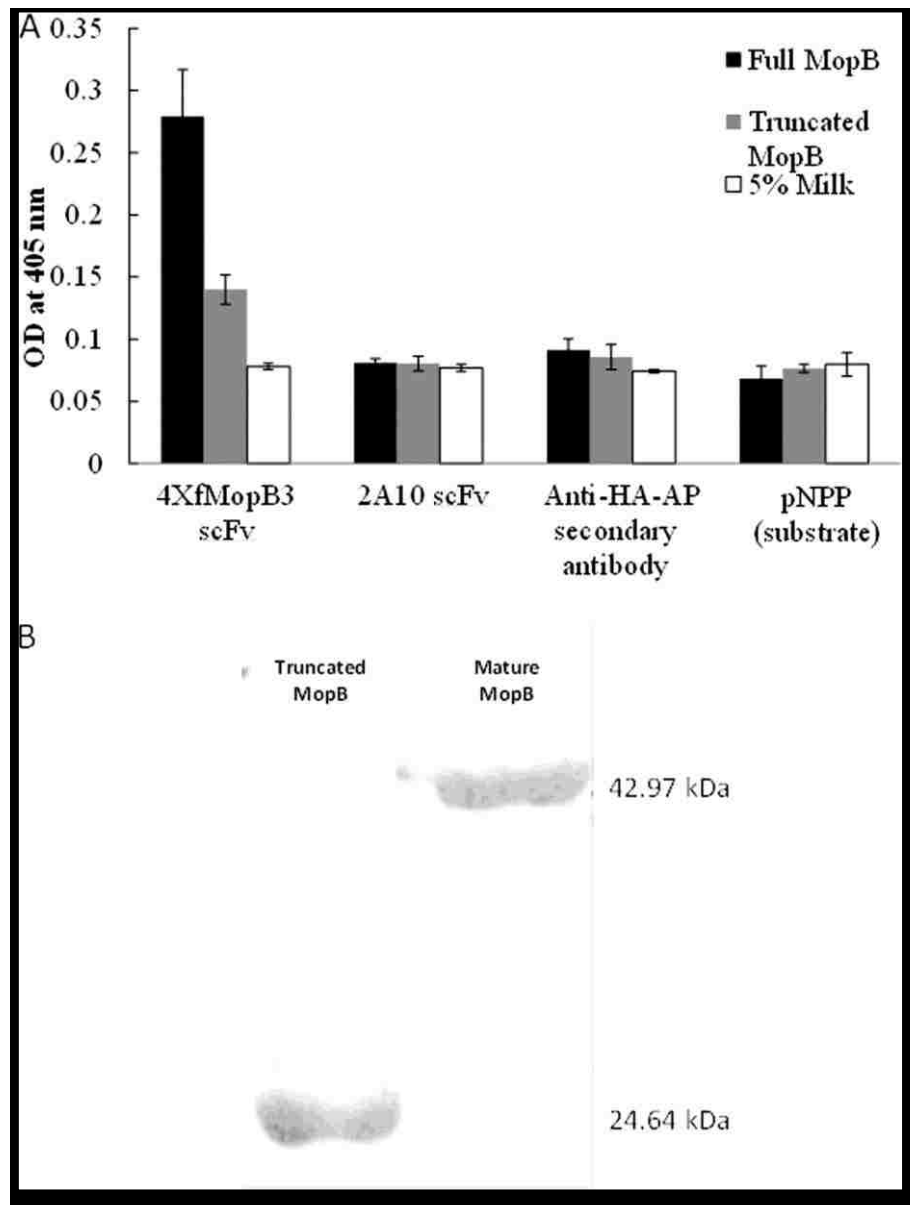
FIG 6 Functional analysis of 4XfMopB3 HA scFv by ELISA and Western blot analysis. (A) The ELISA with full-length and truncated MopB and 5% milk protein as a control. 2A10 scFv expressed in pAHAHis control antibody (against NANP repeat) was used as an irrelevant negative-control scFv. The detection of anti-HA alkaline phosphatase without a first antibody and detection of the substrate alone were used as additional controls. (B) MopB Western blot analysis using 4XfMopB3 HA scFv with mature full-length and truncated MopB on nitrocellulose membrane and detected with anti-HA alkaline phosphatase.

approximately 100 white colonies, 4 were randomly selected to prepare plasmids for

sequencing. Three with confirmed intact in-frame VL and VH combinations were

subcloned into pAINFHis for scFv expression and characterization (4XfMopB1,

4XfMopB2, 4XfMopB3). Analysis of the variable region sequences (see Table S3 in the

supplemental material) shows that different gene segments of the antibody sequences are

present in the selected recombinant antibody clones. 4XfMopB3 scFv antibody detected

recombinant mature and truncated MopB in the Western blot analysis and ELISA (Fig.

6), whereas clone 4XfMopB1 detected MopB in the Western blot analysis only (data not

shown). Furthermore, the Xf4MopB3 scFv was used to image *X. fastidiosa* by

immunofluorescence microscopy as shown in Fig. 7.

Discussion

Investigations of plant-pathogen and vector-pathogen interfaces in PD would be

greatly facilitated with the availability of highly specific monoclonal antibodies.

Although it is possible to use conventional hybridoma technology (30) to obtain

monoclonal antibodies and then clone and reassemble the corresponding antigen binding

site genes, recombinant antibody display technology is a more cost-effective option for

the nonspecialized laboratory, requiring general molecular biology skills, reagents, and

techniques to build and access antibody libraries. Moreover, genetically encoded scFv's

are amenable to further engineering to optimize the desired characteristics, such as

affinity, specificity (21), or formation of multimers (25) or as fluorescent molecules

(34, 35). The use of a complex immunogen (i.e., whole bacteria) with a mixture of
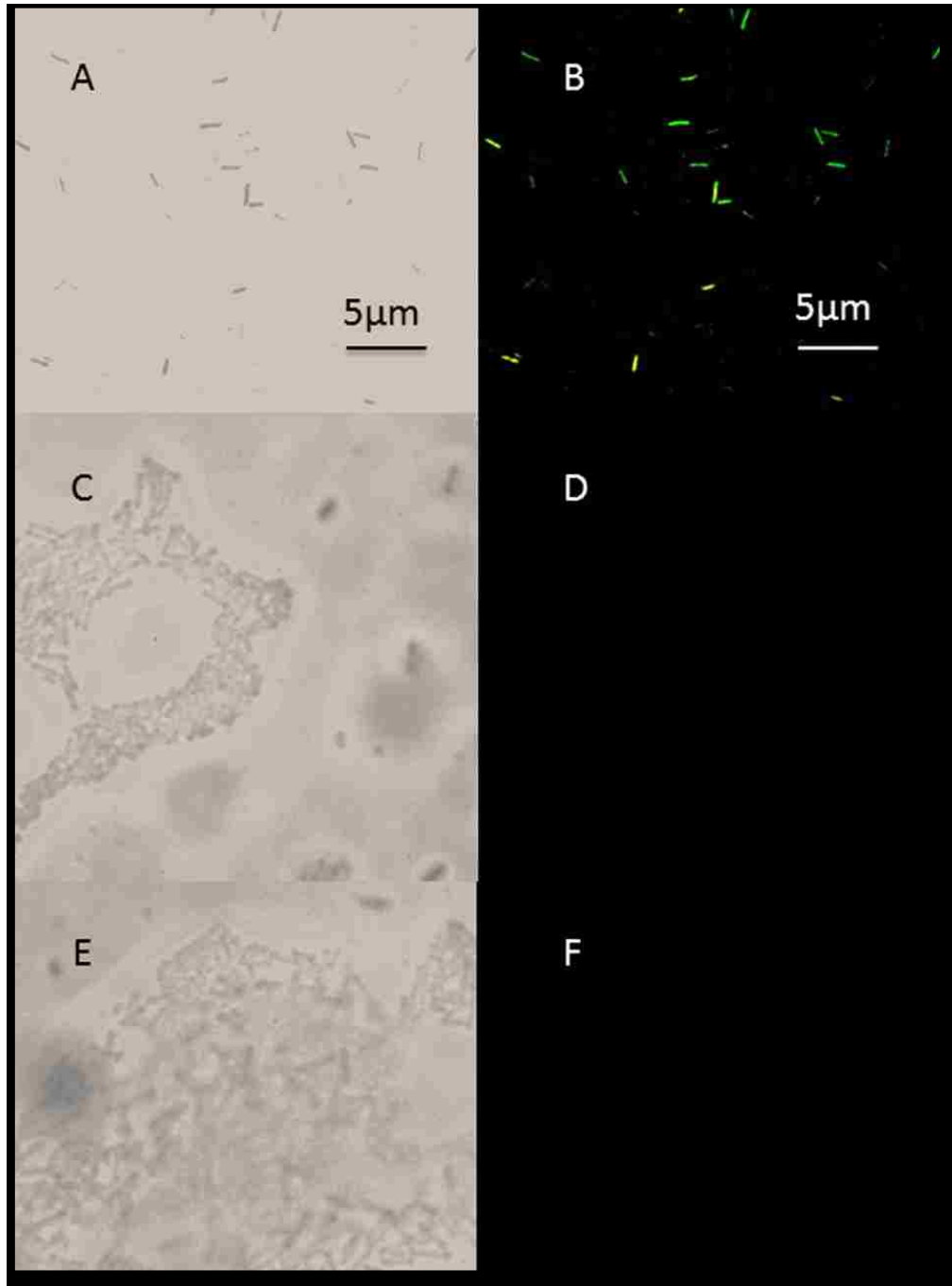
FIG 7 Immunofluorescence detection of *X. fastidiosa* with Xf4MopB3 scFv. Panels A, C, and E were viewed with bright-field microscopy and B, D, and F with fluorescence microscopy. (A and B) *X. fastidiosa* with primary 4XfMopB3 scFv probed with secondary anti-polyhistidine FITC. (C and D) *X. fastidiosa* with primary anti-NPNA scFv probed with secondary anti-polyhistidine FITC. (E and F) *X. fastidiosa* probed with primary anti-polyhistidine FITC only. The size bars shown in panels A and B are 5 μm.

antibodies against a mixture of targets (on the cell surfaces) (5) results in combinatorial complexity and invariably requires deconvolution at some point (50). When initially validating a technique, a simpler approach is to anchor one component and search through a complex mixture of potential partners. In this study, we assembled combinatorial libraries of recombinant antibodies from mice immunized with whole heat-killed *X. fastidiosa*. To deconvolute the mixture, we chose a surface-exposed molecule, MopB, as bait.

Although the role of MopB in *X. fastidiosa* is not known, it is an abundant surface protein and may potentially be a target for bacterial agglutination via engineered recombinant antibodies. Earlier studies that attempted to isolate natural MopB and to make recombinant MopB protein had met with limited success (7, 8). An *in silico* analysis of the target identified key features that assisted in the design of an appropriate recombinant bait molecule for use in antibody selection. MopB has a characteristic signal leader sequence (Met15-Ala36) with a classical cleavage motif, Ala Ser Ala, followed by a transmembrane β-barrel (with surface-exposed loops), Trp43-Tyr177, and a periplasmic domain, Arg178-Asn389 (Fig. 3). The protein is extremely toxic to the heterologous host *E. coli* when inserted into the outer membrane by the use of either its natural leader sequence or an alternative such as *ompA*. A leaderless construct results in proteins not traversing or inserting in the inner membrane but being retained in the bacterial cytoplasm and aggregating due to the hydrophobic nature of the transmembrane β-sheets. Full-length mature (43-kDa) MopB proteins and truncated (25-kDa) recombinant MopB proteins were both efficiently expressed without a leader sequence. Extraction using denaturing conditions released the recombinant MopB into the soluble fraction. SDS-

PAGE analysis tracking the expression and purification revealed that the recombinant proteins were enriched, and the major products eluting from the affinity resin corresponded to the predicted sizes for the mature and truncated MopB (Fig. 4), with a yield of ~50 mg/liter of bacterial culture.

Sera (diluted 1/20,000) from *X. fastidiosa*-immune mice 1, 2, 4 and 5 had activity against truncated MopB protein and *X. fastidiosa* in the ELISA and immuno-dot blot analysis, respectively. Mouse 3 serum produced the lowest signal in both assays (Fig. 5). The very high dilution used in both the evaluations of the sera suggests a very robust response to the whole-bacterial-antigen preparation.

Five antibody libraries were assembled following the steps outlined in Fig. 1, and the mouse 4 DNA antibody library was used in the subsequent selection procedure. After a single enrichment step using ribosome display, scFv's specific for MopB were readily isolated. This rapid enrichment may be a common feature of antibody libraries constructed from intentionally immunized or naturally immune sources and is in agreement with our findings determined using antibody ribosome display to access human scFv's against HIV-1 gp120 constructed from an HIV-1-positive donor (48), where only a single round of selection was required.

One scFv, 4XfMopB3, recognized the mature and truncated recombinant MopB proteins in both the ELISA and Western blot analysis (Fig. 6) and heat-killed *X. fastidiosa* by IFA (Fig. 7), suggesting that the epitope is nonconformational and possibly one of the three predicted loops exposed on the surface of the bacteria. These results provide evidence that immunization with whole heat-killed *X. fastidiosa* clearly induces antibodies against surface-exposed antigenic molecules such as MopB, indicating that the

mild heat treatment used to kill the bacteria preserved the surface-accessible protein immunogenicity. We speculate that the immune sera may also have contained antibodies against other putative surface-exposed molecules possibly associated with attachment in either the GWSS or the*V. vinifera* grapevine (31).

The *in vitro* anti-*X. fastidiosa* scFv libraries generated in this study and the strategy for preparing recombinant putative membrane proteins provide approaches for rapidly discovering additional scFv's against surface components involved in aggregation (19) and/or motility (15, 24, 33). The anti-MopB or other anti-*X. fastidiosa* scFv molecules may also have use in diagnostic applications for pathogen surveillance and could be assembled with in-built fluorophores as described recently (34, 35). With the availability of genetically encoded molecules that target the surface of *X. fastidiosa*, alternative ways to investigate the biology of *X. fastidiosa* host-vector interactions may be explored to develop interventions. One such approach is paratransgenesis, which in concept involves the genetic manipulation of a vector's naturally occurring microorganisms such as bacteria, fungi, or viruses to compromise competence to transmit a particular agent.

Engineering symbiotic bacteria to express and secrete recombinant molecules inside an arthropod was initially demonstrated using the bacterium *Rhodococcus rhodnii* to express cecropin (16) in the kissing bug *Rhodnius prolixus*, the vector of Chagas' disease, and subsequently to express an scFv (17). Recently, scFv's that target *Trypanosoma cruzi* epimastigote glycans have been developed for use in this paratransgenic application (34). This strategy may also be implemented with symbionts

or commensal organisms of the GWSS (40). Alternatively, other "Trojan horse" vehicles may be developed.

Recently, an scFv against a *Plasmodium falciparum* sporozoite surface protein isolated from a malaria-immune individual (10) and shown to inhibit sporozoite invasion of human hepatocytes (9), has been used to reduce *P. falciparum* sporozoite levels in *Anopheles gambiae* via an engineered entomopathogenic fungus (18), basically using an infection to fight an infection (41). In the malaria study, the recombinant antibody PfNPNA-1 targeted a conserved surface-accessible repetitive target on the highly abundant circumsporozoite protein and was engineered to agglutinate the parasite, preventing migration to and invasion of the salivary gland. Such an approach is independent of disrupting host cell-specific interactions and may be a general strategy against targets that are present at high density on the pathogen surface. Recombinant antibodies against MopB and other abundant surface-exposed molecules on *X. fastidiosa* could also be engineered in a similar way to agglutinate the bacteria and evaluated in the GWSS via paratransgenic organisms such as engineered commensals or symbionts or by infection with *Metarrhizium* spp. or *Beauvaria bassiana*, providing novel alternative platforms to investigate in the control of PD.

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Primer	5'-Sequence-3'
MopBNdeF	ATA<u>CATATG</u>GCCCAGGAATTCGATGACCGGTGGTATCTCGCTGG
MopB-MatNot	ATAAT<u>GCGGCCGC</u>ATTCGTAACGTTCAACTCAGTAC
MopB-TrunNot	GCAT<u>GCGGCCGC</u>ACCAAATGGGATCACGACACC
Oligo dT29VN	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
MVHLink2	GGCAGCAGATCCAGGGGCCAGTGGATAGAC
MVKF1	CGAATTCCA<u>CCATGG</u>CAGACRTCMAGATRAYCCAGWCTMCA
MVKF2	CGAATTCCA<u>CCATGG</u>CARAMATTKTGCTGACYCARTYTCC
MVKF3	CGAATTCCA<u>CCATGG</u>CAGATRYTKTGATGACCCAAACTCCA
MVKF4	CGAATTCCA<u>CCATGG</u>CASRAAWTSTTCTCWYMCAGTCTCC
MVKF5	CGAATTCCA<u>CCATGG</u>CARRCRTTSWGATGWCACAGTCKCCA
MVKF6	CGAATTCCA<u>CCATGG</u>CAGATATTGTGATRACKCAGGMTRMA
MVKF7	CGAATTCCA<u>CCATGG</u>CARRYATTGTGATGACCCARWCWC
MVKR1	GAACCACCA<u>CCACC</u>ACSTWKBABHKYCARYTTKG
MVHF1	GGTGGTGGTGGTTCTMAGCTTCAGGAGTCRGGACC
MVHF2	GGTGGTGGTGGTTCTCAGCTGAAGSASTCAGGACC
MVHF3	GGTGGTGGTGGTTCTMWGSKGGTGGAGTCTGGGGGA
MVHF4	GGTGGTGGTGGTTCTARSSTGGWGGAATCTGGAGGA
MVHF5	GGTGGTGGTGGTTCTARGSTGRTSGAGTCTGGAGG
MVHF6	GGTGGTGGTGGTTCTCARSYGCAGCARYCTGGG
MVHF7	GGTGGTGGTGGTTCTCAGYTGSWGCARTCTGGA
MVHF8	GGTGGTGGTGGTTCTCAGCTGCAGCAGTCWGTG
MVHF9	GGTGGTGGTGGTTCTMASYTGSWGGWGWCTGGAGG
MVHF10	GGTGGTGGTGGTTCTCAGMTSCAGCAGYCTGG
MVHR1.1	GGA AAC TGT CGG T<u>GC GGC CGC</u> CGM RGA RAC DGT GAS HRD RG
MVKFLink	GTAATACGACTCACTATAGGGCGAATTCCACCATGG
MVKRLink	GGAGCCGCCGCCGCCAGAACCACCACCACCAGAACCACCACCACCAC
MVHFLink	GGCGGCGGCGGCTCCGGTGGTGGTGGTTC
MKNotCF	CG<u>G CGG CCG C</u>AC CGA CAG TTT CC
MKRev	AGA AGC TTG CTC GAG GAT TCG
RDT7	CTATAGAAGGGTAATACGACTCACTATAG
MKRD2	GCACTCCATTCTGGCGT CGC
IUPAC-IUB symbols for nucleotide nomenclature	**M**=A/C, **R**=A/G, **W**=A/T, **S**=G/C, **Y**=C/T, **K**=G/T, **V**=A/G/C, **H**=A/C/T, **D**=A/G/T, **B**=G/C/T and **N**=A/G/C/T

S Table 1. The oligonucleotide primers used in the construction of recombinant MopB and the mouse scFv immunoglobulin libraries, the restriction site NdeI, NcoI and NotI are underlined.

Mouse number	RNA concentration (mg/mL)	A 260/280	A 260/230
#1	2.2	1.66	2.30
#2	4.03	1.75	1.83
#3	1.2	1.81	2.65
#4	2.6	2	2.08
#5	0.88	1.64	0.47

S Table 2. Mouse spleen total RNA concentrations and A260/280nm and A260/230nm ratios.

ScFv	Mouse IGVK	Mouse IGVKJ	VL CDR3	Mouse IGVH	Mouse IGVHJ	Mouse IGVHD	VH CDR3
4XfMopB1	17-127*01F	2*01F	L Q S D N M P Y T	2-9-1*01F	4*01F	1-1*01F	A R D Q V Y G S S Y YA M D Y
4XfMopB2	12-46*01F	5*01F	L Q H G E S P L T	5-6-5*01F	1*01F	-	A R Y Y Y G T S F Y W Y F D V
4XfMopB3	12-44*01F	1*01F	Q H H Y G T P W T	14-3*02F	2*02F	-	A F P H Y
4XfMopB4	2-59*01F	5*01F	Q Q R S S Y P L T	1-82*01F	4*01F	2-1*01F	A R S R D G N Y G Y Y YA M D Y

S Table 3. The junction analysis and alignment of the VL and VH CDR3 sequences of the selected anti-MopB scFv's. The nomenclature for numbering the scFv is as follows, mouse number (#1-5), bacterial strain antigen (Xf), bait (MopB) and clone in order of isolation i.e., 4XfMopB1-4. The sequences of the scFv's analysed using the International ImMunoGeneTics (IMGT®), information system® (33, 50) to assign the mouse variable genes and alleles. For each isolated scFv the light chain variable kappa region(VK),variable J region (VKJ) and complementary determining region 3 (VL CDR3) and for the heavy chain variable region (VH), variable J region (VHJ), variable D segment (VHD) and complementary determining region 3 (VH CDR3).

Chapter 5: Conclusions

Paratransgenic control is a Trojan Horse strategy, which has been designed to control various vector borne diseases (Durvasula *et al.*, 1997; Wang *et al.*, 2012). We have utilized the same strategy to control Pierce's disease (PD) of grapevines, a disease caused by a bacterium, *Xylella fastidiosa* and transmitted by the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*). Based on its acquisition and persistence within the sharpshooter foregut, we selected *Pantoea agglomerans* E325 as the paratransgenic control agent to block *X. fastidiosa* transmission. The Environmental Protection Agency (EPA) has already approved use of *P. agglomerans* E325 to control fire blight of apples and pears. We genetically modified *P. agglomerans* to express enhanced green fluorescent protein (EGFP) to study the interaction of *P. agglomerans* with *H. vitripennis*. *H. vitripennis* was able to acquire EGFP-expressing *P. agglomerans* from the artificial feeding system (AFS) and *P. agglomerans* persisted within the GWSS foregut for 15 days (Chapter 2).

Different paratransgenic models have been conceived and a decrease in vector competence to carry parasites such as *Trypanosoma cruzi* and *Plasmodium* sp. was achieved under lab conditions (Durvasula *et al.*, 1997; Wang *et al.*, 2012). However,the paratransgenic strategy is still a lab concept and field application of any of the paratransgenic agents yet to be realized, mainly due to the absence of a safe delivery system. We developed calcium-alginate based microparticles for field application of genetically modified *P. agglomerans*. These microparticles were designed to open up under high hydraulic pressure. When the GWSS fed on plants painted with *P. agglomeran*-containing microparticles, these microparticles swell due to pressure exerted

by the xylem flow resulting in bacterial release within the sharpshooter foregut (Chapter 2). We engineered microparticles with different alginate concentrations (1%, 2%, and 3%). Microparticles engineered using 1% alginate showed promise as 51.8% GWSSs were able to acquire EGFP-expressing *P. agglomerans* from plants painted with EGFP-expressing *P. agglomerans*-containing microparticles. These microparticles provide a physical barrier between the bacteria and the outer environment, which will help to decrease environmental contamination and horizontal gene transfer. Additionally, bacteria present within microparticles were able to withstand UVC radiation and desiccation. This resistance is significant and a necessity to accomplish paratransgenic control of PD as direct sunlight in the grape fields results in very dry conditions and also imposes a high dose of UV radiations (Chapter 2).

Antimicrobial peptides (AMPs) are small proteins, which are amphiphatic in nature. These molecules in general, make holes in the cell membrane of bacteria and other microorganisms and kill them. We tested toxicity of two AMPs, melittin and scorpine like molecule (SLM), against *X. fastidiosa* and *P. agglomerans*. Both these AMPs exhibited higher toxicity against *X. fastidiosa* in comparison to *P. agglomerans* and we chose them to be delivered via *P. agglomerans* (Chapter 3). Using the *Escherichia coli* hemolysin secretion system we genetically modified *P. agglomerans* to express melittin and SLM. We confirmed presence of AMPs in spent medium through Western blot (Chapter 3). Both melittin and SLM-expressing *P. agglomerans* lines were tested for their efficacy to block *X. fastidiosa* acquisition and transmission by *H. vitripennis*. We observed a decrease in acquisition and abolishment of transmission of *X. fastidiosa* by paratransgenic *H. vitripennis* carrying melittin or SLM-expressing *P.*

agglomerans lines (Chapter 3). Presence of AMPs within insect gut resulted in decreasing vector competence was confirmed by detecting melittin and SLM in insect bodies via Western blot (Chapter 3). This is first time, when paratransgenic control has been shown to prevent experimental transmission of a plant disease. Further, this is the first time, when not only decrease in acquisition, but abolishment of pathogen transmission has been achieved using paratransgenic insects.

To control PD transmission we used AMP-expressing *P. agglomerans* lines. Though the AMPs are very potent molecules, they lack specificity. We can increase the safety of paratransgenic control by utilizing molecules with higher specificity, such as single chain atibodies (scFv). To achieve this goal we engineered scFvs specific to *X. fastidiosa* surface protein mopB (Chapter 4). We used ribosomal display to engineer scFvs against mopB. To produce scFv we harvested the spleens from the mice that were previously injected with heat killed *X. fastidiosa* (Chapter 3). In parallel, we expressed and purified mopB with histidine tag in *E. coli* cells. We utilized purified mopB protein to select for antibodies that bound to mopB protein. Using ribosomal display technology we were able to engineer scFvs, which can bind to purified mopB as well as to whole *X. fastidiosa* cells (Chapter 4).

This dissertation is focused on achieving paratransgenic control of PD using *P. agglomerans* E325 as a delivery vehicle. We developed microparticles for field release of bacteria to limit the environmental contamination, horizontal gene transfer and site specific release of payload. We engineered *P. agglomerans* lines that rendered *H. vitripennis* incompetent of transmitting *X. fastidiosa*. We also engineered scFv specific to mopB surface protein of *X. fastidiosa*.

We believe that in the future paratransgenic control will be used in the field to control PD and this model will act as a framework to test other paratransgenic models.