Molecular intervention in Pierce's Disease

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MOLECULAR INTERVENTION IN PIERCE'S DISEASE

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Abstract

Pierce's Disease (PD) is one of the most devastating diseases threatening grapevines in the USA caused by the Gram negative bacterium *Xylella fastidiosa*. There is no effective control method for Pierce's Disease and control is mainly achieved by suppressing transmission of the glassy winged sharpshooter (GWSS) insect vector. The study of the surface proteins of *X. fastidiosa* may provide targets that may be attractive sites for intervention. MopB is an outer membrane protein, which has been identified on the *X. fastidiosa* surface. Blocking MopB with antibodies may prevent or reduce transmission of the pathogen.

The main goal of this research was to generate recombinant antibodies to be used to investigate the interaction of MopB with the plant and GWSS. Towards this goal, recombinant MopB has been produced and used with sera from five mice immunized with whole heat killed *X. fastidiosa* in an Enzyme-Linked Immunosorbent assay (ELISA). The ELISA results revealed the presence of antibodies against the recombinant MopB protein. *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 the 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 ELISA, Western blot and immunofluorescence assays.

Pantoea agglomerans, a candidate bacterium for PD paratransgenic approach was engineered to express and secrete selected anti-MopB (4XfMopB3) single chain antibody. The expression cassette that include the *pelB* secretion signal was created and tested for its efficiency in secreting the 4XfMopB3 antibody in *E. coli* and *P. agglomerans*. The 4XfMopB3 antibody was successfully expressed and secreted in *E. coli* while it was only expressed in *P. agglomerans.*

Declaration

I hereby declare that the thesis submitted for the degree of Doctor of Philosophy (PhD) at the University of Westminster, is the result of my own original work and investigation carried out in the School of Life Sciences, except where published work has been cited and where collaborators have been acknowledged.

This work has not been submitted or accepted in substance, in part or in any form, for any other degree.

Armaghan Azizi

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Abbreviation

Abbreviation	Full name
ALS	Almond Leaf Scorch
AP	Alkaline Phosphatase
Arg	Arginine
ARM	Antibody-Ribosome-mRNA
BLS	Bacterial Leaf Scorch
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
bp	Base pair
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CDR	Complementarity-Determining Region
CVC	Citrus Variegated Chlorosis
C-terminal	Carboxyl-terminal
dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Exopolysaccharide
FITC	Fuorescein Isothiocyanate
Gly	Glycine
GWSS	Glassy Winged Sharpshooter
HAs	Haemagglutinin Adhesins
IFA	Immunofluorescence Assay
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Ile	Isoleucine
kDa	Kilodaltons
LB	Luria Bertani
Leu	Leucine
MKC	Mouse Kappa Constant
mRNA	Messenger RNA
NBT	Nitro Blue Tetrazolium
OD	Optical Density
OLS	Oleander Leaf Scorch

Abbreviation	Full name
ORF	Open Reading Frame
P. agglomerans	*Pantoea agglomerans*
PTG	Paratransgenic
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Pierce's Disease
PG	Polygalacturonase
PMSF	Phenylmethanesulfonylfluoride
PNPP	p-Nitrophenyl Phosphate
PRM	Protein-Ribosome-mRNA
Pro	Proline
RBS	Ribosomal Binding Site
RFP	Red Fluorescent Protein
RT	Reverse transcriptase
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
ScFv	Single-Chain Variable Fragment
SD	Shine-Dalgarno
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TBST	Tris Buffered Saline Tween-20
TEMED	Tetramethylethylenediamine
tRNA	Transfer RNA
UV	Ultraviolet
VH	Variable Region of Heavy Chain
VL	Variable Region of Light Chain
Vκ	Variable Region of Kappa Chain
WIBR	Wolfson Institute for Biomedical Research
X-gal	5-Bromo-4-Chloro-3-Indolyl-ß-D-Galactopyranoside
X. fastidiosa	*Xylella fastidiosa*

Chapter 1.0
Introduction

Chapter 1: Introduction

1.1 Pierce's Disease Background

One of the most devastating agricultural and economically important diseases threatening grapevines (*Vitis vinifera*) in the USA is Pierce's Disease (PD). This disease occurs mainly the parts of America, which have mild winters (Purcell *et al*., 1979). The prevalence of this particular disease decreases in regions with colder temperatures (i.e. places in higher altitudes and/or further away from coastal regions). However, the most affected regions in America occupy the stretch from Florida to California.

In 1882 the plant pathologist Newton B. Pierce studied the first outbreak of the disease known as "Anaheim grapevine" or "grape mystery" disease in the Santa Ana River Valley near Anaheim in Southern California. Pierce reported the disease in 1891 without identifying the cause of the disease. This disease is now known as Pierce's disease of grape.

During 1939 -1945 PD was identified as a vector-borne disease. Xylem sap-sucking insects such as sharpshooters (subfamily Cicadellinae in the leafhopper family Cicadellidae) and spittlebugs (family Cercopidae) were discovered as vectors of the PD pathogen (Morano *et al*., 2008, Ramirez *et al*., 2008, Myers *et al*., 2007). These insects play an important role in spreading the pathogen from plant to plant.

Initially, PD was considered to be a viral disease (Hewitt *et al*., 1946) until the disease was suppressed by chemotherapy (tetracycline antibiotic) and thermotherapy in 1971, which indicated that PD might not be a viral disease. Furthermore a "rickettsia-like" bacterium was identified in the infected grapevine xylem vessels by the use of electron microscopy (Hopkins and Mortense, 1971, Hopkins and Mollenha, 1973, Goheen *et al*., 1973). In 1987, the causative agent of PD was grown in culture and named *Xylella fastidiosa* (Wells *et al*., 1987).

1.2 *Xylella fastidiosa* Bacterium

Xylella fastidiosa is a rod-shaped Gram negative xylem limited fastidious phytopathogen bacterium with dimensions of 0.25 to 0.35 µm in radius and 0.9 to 3.5 µm in length (De la Fuente *et al*., 2007). It is non-flagellate, non-sporulating, has distinctive rippled cell walls, two types of pili (Type I and Type IV) and a twitching motility (Li *et al*., 2007, Nyland *et al*., 1973).

X. fastidiosa is a nutritionally fastidious aerobic bacterium that can survive in high turbulence, low nutrients, and can withstand host defense responses of plants and insects (O'Toole *et al*., 1999). This bacterium resides in plant xylem environment which contains dilute solutions of essential nutrient sources such as specific amino acids, mainly glutamine and asparagine, organic acids and inorganic ions. *X. fastidiosa* has specialised mechanisms to concentrate and absorb the essential nutrients in such environments (Purcell and Hopkins, 1996). These nutrient sources are necessary for the bacterium to produce energy and grow. Thus, understanding the carbohydrate metabolism of *X. fastidiosa* provides important information about the plant-pathogen interactions.

X. fastidiosa has more than 100 host plant species (Hopkins and Purcell, 2002). Strains of *X. fastidiosa* are involved in several important economically important crop diseases such as PD in grape (Davis *et al*., 1978), citrus variegated chlorosis (CVC) (Chang *et al*., 1993), almond leaf scorch (ALS) (Mircetich *et al*., 1976), alfalfa dwarf (Goheen *et al*., 1973), phony peach, plum scald (Wells *et al*., 1981), several species of ornamental plants, e.g. oleander leaf scorch (OLS) (Purcell *et al*., 1999) and bacterial leaf scorch (BLS) of *Ulmus* spp., *Quercus* spp. (Hearon *et al*., 1980), *Acer* spp. (Sherald *et al*., 1987), and *Platanus* spp. (Sherald *et al*., 1982). This bacterium has also been detected asymptomatically in many plant species such as wild grasses, sedges, lilies, and various bushes and trees (Raju *et al*., 1983, Hopkins and Adlerz, 1988, Costa *et al*., 2004, Henneberger *et al*., 2004, Hill and Purcell, 1995).

1.2.1 *Xylella fastidiosa* Genome Sequence

X. fastidiosa is the first non-viral plant pathogen to have its genome (2Mb) completely sequenced (Van Sluys *et al*., 2003, Simpson *et al*., 2000). Comparative genome analysis has provided valuable information of genes, metabolic pathways and potential virulence factors that may be involved in pathogenicity (Simpson *et al*., 2000).

Based on *X. fastidiosa* genomic sequence analysis potential virulence factors are known as pili, exoploysaccharides, biofilm formation and cell aggregation, degradative enzymes and systematic movement from vessel to vessel that may be involved in pathogenicity (Simpson *et al*., 2000). In addition the open reading frames (ORF) encoding a polygalacturonase (PG), three endo-1-4β-glucanases, a cellobiohydrolase, three endo-β-xylanases and twoβ-xylosidases have been identified. These enzymes, especially PG, may have an important role in pathogenicity via digesting the pit membrane, the degraded primary cell walls and middle lamella of vessels that acts as porous filters allowing free passage of the water and nutrients while limiting passage of large objects such pathogens (Van Sluys *et al*., 2003, Perez-Donoso *et al*., 2010, Roper *et al*., 2007). The carbohyrate metabolism of *X. fastidiosa* has also been studied and suggests that this pathogen can only use a few sugars or sugar polymers such as glucose, fructose, mannose, ribose, N-acetylglucosamine, cellulose and glycerol (Almeida *et al*., 2004, Chang and Donaldson, 2000, Chatterjee *et al*., 2008).

1.2.2 Pathology

The precise mechanism by which *X. fastidiosa* causes disease is not clearly understood however the ability of the pathogen to colonize both the xylem of the grape vine and in the foregut of the GWSS vector is well established (Almeida *et al*., 2004).

Bacterial biofilm formation plays an important role for *X. fastidiosa* to survive and replicate in both plant and insect with low nutrient environments (Voegel *et al*., 2010,

Guilhabert and Kirkpatrick, 2005, de Souza *et al*., 2003, Rodrigues *et al*., 2008). The attachment of the bacterium to the host surface is the first step of biofilm formation. On the *X. fastidiosa* surface there are fimbrial and afimbrial adhesins which are involved in bacterium attachment (Li *et al*., 2007).

Two classes of fimbriae have been identified for *X. fastidiosa*, type IV encoded by *pil* genes and type I encoded by the *fim* operon (Meng *et al*., 2005). The type I pili are responsible for the bacterium-bacterium adhesion, aggregation and biofilm formation by an exopolysaccharide (EPS) secretion, while type IV polar pili are responsible for twitching motility (Simpson *et al*., 2000, de La Fuente *et al*., 2007, Li *et al*., 2007). The afimbrial adhesins such as haemagglutinin adhesins (HAs) are involved in bacterial attachment to the plant host cells (Voegel *et al*., 2010).

1.3 Pierce's Disease Life Cycle

After an epidemic of PD in Northern California (1939 to 1945), xylem sap-sucking insects such as sharpshooter (subfamily Cicadellinae in the leafhopper family Cicadellidae) and spittlebugs (family Cercopidae) were discovered as vectors of PD pathogen (Morano *et al*., 2008, Ramirez *et al*., 2008, Myers *et al*., 2007). In 1989 a new shapshooter, *Homalodisca vitripennis,* also known Glassy-winged sharpshooter (GWSS) was introduced to California from Florida (Blua *et al*., 1999).

X. fastidiosa is transmitted between plants by xylem sap-sucking insects. While these insects are feeding from the infected plants, they rapidly (less than 2 hours) pick up the bacterium (Backus *et al*., 2009, Morano *et al*., 2008, Ramirez *et al*., 2008, Myers *et al*., 2007, Backus and Morgan, 2011). *X. fastidiosa* attaches itself to the buccal cavity in the foregut of the insect (Hill and Purcell, 1995) and while infected insects are feeding again using their sucking mouthparts to access the xylem sap via the water conducting xylem vessels the bacteria can immediately transfer to the plant (Purcell *et al*., 1979, Nadarasah and Stavrinides, 2011, Myers *et al*., 2007, Ramirez *et al*., 2008, Jackson *et al*., 2008).

The bacterium is directly injected into the xylem vessels through the insect food canal

(Wayadande *et al*., 2005, Myers *et al*., 2007, Ramirez *et al*., 2008, Jackson *et al*., 2008, Nadarasah and Stavrinides, 2011). The xylem sap can pass through the bordered pits (channels that connect vessels to each other), but due to the presence of pit membrane, the degraded primary cell walls and middle lamella of vessels that acts as porous filters allowing free passage of the water and nutrients while limiting passage of large objects such pathogens, *X. fastidiosa* is blocked (Baccari and Lindow, 2011, Choat *et al*., 2003, Chatterjee *et al*., 2008).

From the site of inoculation *X. fastidiosa* attaches to the xylem walls, multiplies and spreads to colonize the xylem, forming biofilm-like colonies, which can clog the xylem vessels and block water transport network, causing scorch like symptoms. The colonization process requires *X. fastidiosa* to move within the xylem. The bacterium must pass through the pit membrane, which acts as porous filters, in order to move from one vessel to another (Stevenson *et al*., 2004). However, *X. fastidiosa* can not use the water flow to pass from one vessel to another via the pit membrane due to the size of the bacterium. Thus, movement of the bacterium is an active process dependent on the pathogens ability to disrupt the pit membrane (Figure 1.1). For intervessel migration and spread through the plant, the pathogen needs to digest pectin in the pit membrane, which also provides a nutrient source supplementing the low amount of nutrients in the xylem sap and also leads to the exposure of additional cell wall polysaccharides that could be digested by other extercellular enzymes such as glucanases and xylanases (Chatterjee *et al*., 2008).

The blockage of the xylem is related to the large cell aggregation of the pathogen in the xylem and not the number of vessels that are colonized. The bacterium spread in the xylem is limited due to the pit membrane and possibly because of the tyloses and polysaccaride-rich gels plants produce to block the xylem in response to infection (Chatterjee *et al*., 2008, Cheng *et al*., 2009, Perez-Donoso *et al*., 2007, Hopkins, 2005, Fry and Milholland, 1990).

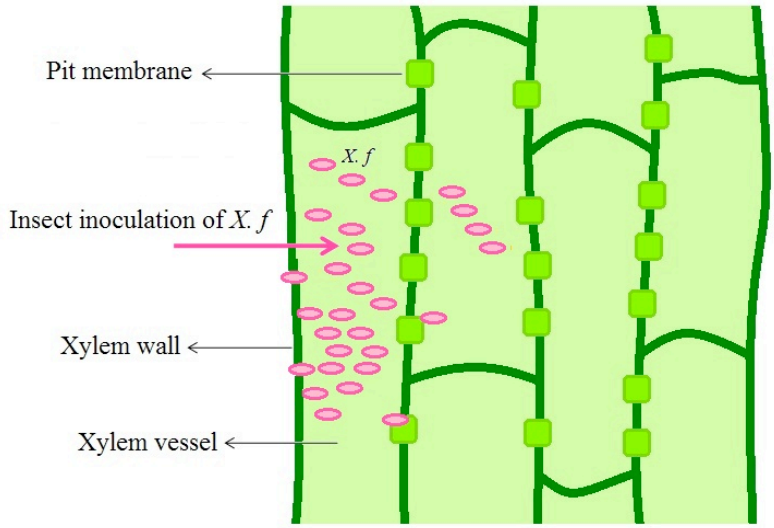

Figure 1.1 *X. fastidiosa* is delivered directly into the xylem vessels by xylem sap-feeding insect vector. *X. fastidiosa* is xylem-limited and can only spread to the neighboring xylem vessel by disrupting the pit membrane (PM). Picture is adapted from (Chatterjee *et al*., 2008).

1.4 Pierce's Disease Symptoms

Usually PD symptoms are not noticed until the time of fruit maturation, or in late fall when the host plant is senescing (Hopkins, 1989).

The initial symptom of the infection is delayed growth in the spring, leaf chlorosis (yellowing) and sudden drying or scorching of the margin of the leaf. These symptoms are followed by loss of the affected leaves while the petioles still remain on the canes, brown and green patches appear on the vine. In the late stage of the infection, wilting of the fruit and drying up will occur and the infected plant produces stunted, chlorotic shoots. The grape plants stop growing and the plant dies within a year or over a few years (Stevenson *et al*., 2005, Morano *et al*., 2008, Hopkins, 2005).

1.5 Detection Methods for *Xylella fastidiosa*

Currently detection methods for *X. fastidiosa* are based on light microscopic and electron microscopic (French *et al*., 1977) visualization of the bacterium in vessels in cross-sections of petioles or isolating the bacteria using suitable selective media (Raju

et al., 1982, Wells *et al*., 1983, Davis *et al*., 1983) followed by biochemical and serological analysis. Microscopic detection is slow and inefficient. Another method to detect *X. fastidiosa* is culturing methods, which in comparison with other methods such as serology, is very slow. To detect and identify *Xylella* bacterium, serological methods (ELISA or fluorescent antibodies) are easier than culturing and microscopic methods, but less sensitive (French *et al*., 1978, Hopkins and Adlerz, 1988, Sherald and Lei, 1991). ELISA reliably detects only 10^5 or greater cells isolated per gram plant tissue due to its low sensitivity, thus low number of cells are not detectable. In addition, different strains show different quantitative reaction to anti-sera and false positive results may occur due to unidentified plant components, which can interfere with the assay (Carbajal *et al*., 2004). Recently, for *X. fastidiosa* detection, specific DNA hybridization probes and polymerase chain reaction (PCR) primers have been developed (Minsavage *et al*., 1994, Oliveira *et al*., 2002). Moreover, by the use of such specific and sensitive methods, low numbers of *X. fastidiosa* in its insect vectors are detectable (Yonce and Chang, 1987). A high throughput DNA isolation method also has recently been developed to detect *X. fastidiosa* in both plant and insect tissues (Brady *et al*., 2011).

Other methods such as grafting to susceptible indicator plants or vector tests (Hutchins *et al*., 1953), SDS-PAGE (Bazzi *et al*., 1994), dot immunobinding assay (DIBA) and Western blot (Chang *et al*., 1993, Hill and Purcell, 1995) are still routinely used to detect *X. fastidiosa* (Oliveira *et al*., 2002).

1.6 Management of Pierce's Disease

There is no efficient control for PD at the present. It is difficult to control this disease due to several factors: 1) no efficient methods for controlling infected plants, 2) sharpshooter vectors are extremely mobile and have a large host-plant range that includes many common ornamental and crop plants and 3) *X. fastidiosa* is capable of infecting a wide range of plant hosts without causing symptoms. Furthermore, new strains of *X. fastidiosa* may be spread via the international movement of plants and animal materials (Blua *et al*., 1999).

To prevent PD, a single quick efficient solution is required but, until now, several different strategies have been attempted to control the disease. In 1989, Van Emden suggested that protection against plant diseases could be classified as cultural, chemical, biological or strain improvement.

Culture Method

Vines infected with *X. fastidiosa* that show PD symptoms should be removed completely or isolated because they act as sources of inoculum for infection of other plants (Gubler *et al*., 2006). This strategy does not decrease the subsequent rate of increase of PD (Hopkins and Purcell, 2002).

Chemical control

- Chemical control can be used to reduce the insect vector (sharpshooters) populations using xylem-translocated insecticide such as the neonicotinoid, imidacloprid (Purcell *et al*., 1999, Krewer *et al*., 2002). Vector control efforts have not succeeded in California or in Florida (Hopkins and Purcell, 2002).

- Plant disease control (antibiotics and antimicrobials): antibiotics such as tetracycline and penicillin, which are effective in inhibiting the bacterium; and micronutrients such as zinc, manganese and copper that are toxic to the bacterium can be utilised to avoid a potential proliferation of the pathogen and hence transmission of the disease from the infected plants to a healthy recipient. In fact, the use of chemicals is classified as the second stage response. Unfortunately, chemicals do not offer a long term solution to PD (Hopkins and Purcell, 2002).

Biocontrol

Traditional biological control of PD relies on a natural enemy or a pathogen that attacks a particular pest. However, one should discover the natural habitat of the competing organism in question (Torchin *et al*., 2003).

- Parasitoid wasps: several mymarid and trichogrammatid species such as *Gonatocerus ashmeadi* Girault, *G. triguttatus* Girault, *G. morrilli* Howard, *G. fasciatus* Girault, and most recently a strain of *Anagrus epos* from Minnesota have

been identified as the most common natural enemies of the GWSS eggs (Triapitsyn and Phillips 2000, Pilkington et al., 2005, Krugner *et al*. 2008).

- Entomopathogenic fungi: *Pseudogibellula formicarum*, *Metarhizium anisopliae* and *Hirsutella homalodiscae* nom. prov., are the two possible entomopathogenic fungi species that can be used for PD biocontrol (Kanga *et al.* 2004, Boucias *et al.* 2007). *Hirsutella homalodiscae* nom. prov., was determined to be the most dominant and widespread pathogen of GWSS in its native range (Boucias *et al.* 2007).

Host resistance

Using plants bred for resistance to *X. fastidiosa* (Hopkins and Purcell, 2002), is the only efficient control mechanism for PD in Southeastern United States and parts of California. To maintain the productive lifespan of grapes in these regions it is necessary to replace the existing grapevines with varieties resistant to the pathogen (Mortensen *et al*., 1977, Hopkins and Purcell, 2002).

Investigations revealed that PD kills almost all European types of grapes (*V. vinifera*), American type (*V. labrusca*) and French-American hybrid grapes. However the native species to the southeastern United States such as muscadine grapes (*V. rotundifolia)* and *V. vinifera* were found to be resistant or tolerant to PD (Raju and Goheen, 1981, Hopkins and Purcell, 2002).

A rethink of approaches and novel solutions are urgently required. One such approach is to attack *X. fastidiosa* in the insect vector. This can be achieved by using resident symbiotic organisms that reside in the same vicinity as *X. fastidiosa* in the insect. Manipulation of resident microorganisms to express and secrete an anti-*Xylella* factor, and then be re-introduced into the GWSS is an example of paratransgenic (PTG) control (Ramirez *et al*., 2008). In this way the transmission of the pathogen from the insect vector into the plant xylem region may be prevented (Miller, 2007).

According to the College of Natural Resources, University of California, Berkeley there are more than 145 strains of *X. fastidiosa* that cause PD. Future disease

management may be based on the complete genome sequence of *X. fastidiosa*. A few *X. fastidiosa* strains are completely sequenced and annotated. These genomes revealed pathogenicity factors for this bacterium such as toxins, antibiotics, and iron sequestration systems (Hopkins and Purcell, 2002).

1.7 Pierce's Disease Paratransgenesis Approach (PTG)

1.7.1 Paratransgenesis Approach

Dr. Frank Richards and his colleagues introduced a novel mechanism, symbiotic control, for eradicating pest-borne diseases. He established the advanced stage of genetic transformation of a gut symbiont bacterial *Rhodococcus rhodnii*, with a plasmid to express a foreign gene in *Rhodnius prolixus* the Chagas disease vector (Beard *et al.,* 1992, Durvasula *et al.,* 1997, Miller, 2011). Indeed, symbiotic control does not primarily target the insect vector, which is normally-directly linked to the associated disease. Rather, symbiotic control targets the pathogen, the causative agent of the disease by genetic manipulation of a vector's naturally occurring microorganisms such as bacteria, viruses or fungi to compromise competence to transmit a particular agent (Miller, 2011, Ramirez *et al*., 2008). The pathogen's ability to survive is therefore, undermined. An indirect way to put the pathogen's survival in jeopardy is to actually prevent its transmission from the insect vector into the host (Miller, 2007, Coutinho-Abreu *et al*., 2010). Figure 1.2 shows an illustration of paratransgenesis using a bacterial symbiont.

Currently, paratransgenic approaches are being investigated for many agricultural pests and diseases. Paratransgenesis is used to interfere with triatomid bug transmission of the Chagas disease pathogen (Beard *et al*., 2001, Ben Beard *et al*., 2002, Fieck *et al*., 2010, Matthews *et al*., 2011), to interfere the HIV attachment to its target cells (Chang *et al*., 2003, Rao *et al*., 2005), and to eliminate *Candida* infections from biofilms in chronically infected patients (Beninati *et al*., 2000). Paratransgenesis also has been used for malaria (Ren *et al*., 2008, Bisi and Lampe, 2011), to reveal colitis by cytokines delivery to mammalian guts (Steidler *et al*., 2000, Steidler, 2001), Dengue fever (Coutinho-Abreu *et al*., 2010) and Pierce's Disease (Bextine *et al*.,

2004).

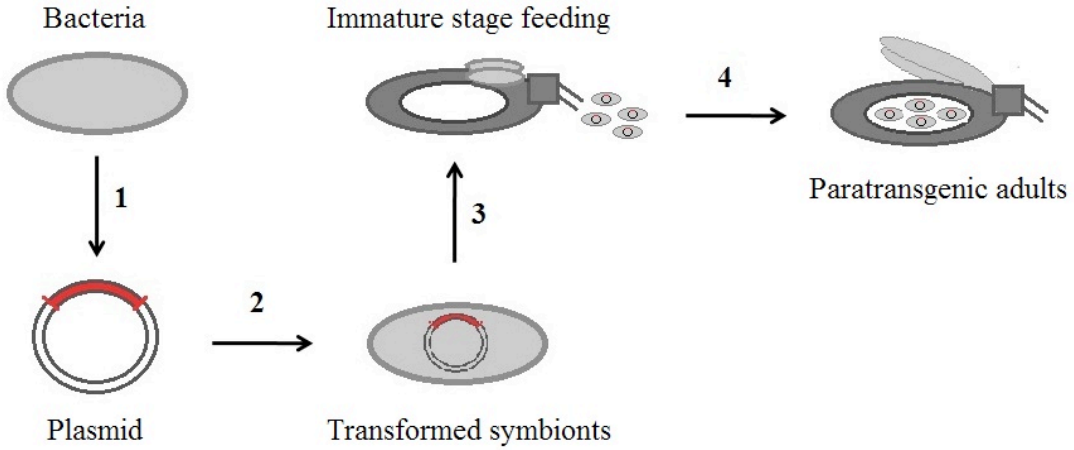

Figure 1.2 Paratransgenesis. An illustration of insect transformation using the transgenic symbiont procedure is presented. 1) Bacterial symbionts can be genetically modified to express a gene blocking pathogen development in vectors; 2) Symbiotic bacteria are transformed with plasmids expressing a gene (red) to inhibit the pathogen development in the insect gut; 3) The transformed symbionts are acquired by insect hosts though larvae or nymph feeding; 4) Once an insect acquires the transformed symbionts, these microorganisms can express proteins to inhibit the pathogen development. Picture is adapted from (Coutinho-Abreu *et al*., 2010).

1.7.2 Pierce's Disease Paratransgenesis

In order to apply a PTG approach for PD, several components are required: 1) symbionts of the GWSS need to be identified and cultured *in vitro*, 2) methods for transforming symbionts need to be developed, 3) methods for reintroducing the symbionts into the GWSS devised, 4) strategies for retaining and spreading the paratransgenic bacteria investigated and finally 5) specific anti-*X. fastidiosa* effector molecules developed.

Bextine *et al.,* (2004) and Lacaval *et al.,* (2009) identified probable bacterial candidates for PTG approaches for PD, which are *Alcaligenes* sp., *Chryseornonas* sp., *Ralstonia* sp. from the GWSS and additionally *Pantoea agglomerans, C. flaccumfaciens* and *Methvlohacterium* spp. (Lacaval *et al*., 2009). All of the suggested candidates can be cultured and the transmission mode of these endophytes is similar to the transmission of *X. fastidiosa.* In 2005, Bextine *et al.,* reported that

Alcaligenes xylosoxidans (Axd) shows suitable properties as a delivery vehicle for anti-*Xylella* strategies (Bextine *et al*., 2005).

In 2004, a genetically modified Axd containing a DsRed fluorescent marker gene (DsAxd) was introduced to GWSS. These modified Axd were able to occupy the same region in the foregut of the insect that the pathogen occupies and colonize the cibarial region of the insect (Bextine *et al*., 2004). The presence of DsRed was traced in different plants such as lemon (Citrus limon), orange (Citrus sinensis ‘‘sweet orange’’), grapevine (*Vitis vinifera* cv. Chardonnay), periwinkle (*Vinca rosea*), crepe myrtle (*Lagerstroemia indica*) and chrysanthemum (*Chrysanthemum grandiflora* cv. ‘‘White Diamond’’) as a direct result of Axd transmission by GWSS. Many reports suggested that the DsAxd can also be introduced into the grapevines but as the GWSS samples were originally obtained from citrus groves in the Agricultural Operations plot at the University California Riverside it seems that they are more adapted to citrus than to grapevine (Miller, 2007, Bextine *et al*., 2005).

1.8 Anti-*Xylella* Proteins

According to *X. fastidiosa* genome sequencing analysis it is possible to predict and explore the surface exposed components that may potentially play a role in bacterial virulence and or be involved in attachment or biofilm formation in either the plant or insect. If identified, such molecules may be used as targets to further understanding and ultimately develop strategies to inhibit and/or prevent PD by disrupting colonization in either the GWSS or the plant. Moreover plentiful surface displayed proteins also provide targets for agglutination of the pathogen independent of the role in virulence, providing a generic alternative mode of transmission disruption.

To develop anti-*Xylella* proteins, it should be considered that these proteins should be: (1) specific for the target organism and (2) if possible prevent some pathogenicity characteristic, for example to inhibit the ability of attaching to the insect bucal cavity or to the plant xylem. There are several anti-*Xylella* antimicrobial peptide candidates. Kuzina *et al.,* (2006) reported that antibiotics and antimicrobial peptides show activity against *X. fastidiosa,* which may protect the plants from developing PD. However antimicrobial peptides show less specificity. The production of single chain

antibodies (scFv's) is a potential avenue for anti-*Xylella* factor generation. Lampe *et al.,* (2007) attempted to screen single chain antibodies (scFvs) against the outer protein coat of *X. fastidiosa* from a phage antibody library (Miller, 2007, Lampe *et al*., 2006).

Recently the expression of *X. fastidiosa* fimbrial and afimbrial proteins has been investigated during biofilm formation. These proteins showed different distribution pattern during biofilm formation in the xylem vessels (Caserta *et al*., 2010). Furthermore, haemagglutinin adhesin, an outer membrane protein, has been localized and characterized in *X. fastidiosa* (Voegel *et al*., 2010). Developing scFv antibodies against the candidate surface protein targets on *X. fastidiosa* may be a key to inhibit bacterial attachment and to prevent PD.

1.9 *Xylella fastidiosa* Outer Membrane Protein MopB

The study of the surface proteins of *X. fastidiosa* may provide molecules that attach either in the plant or insect, and which may be attractive targets for intervention. According to previous studies, and sequence analysis of outer membrane proteins, MopB has been identified on the surface of *X. fastidiosa*. This protein belongs to the OmpA family and is partially exposed on the surface of the bacterium (Bruening, 2002, Bruening, 2003, Bruening, 2004, Fjellbirkeland *et al*., 2000). MopB is identified as an important target for disruption of the *X. fastidiosa* cell or interference with the *X. fastidiosa* infection cycle because 1) MopB is the most abundant protein in membrane preparations, 2) the MopB sequence was identical in all *X. fastidiosa* subspecies and 3) MopB is accessible on the *X. fastidiosa* surface (Dandekar *et al*., 2012, Bruening, 2004). Blocking MopB with antibodies may prevent the initial attachment of bacteria to the xylem or the buccal cavity of the GWSS.

Although the *X. fastidiosa MopB* gene has been identified, the production of high levels of the encoded protein has not previously been reported. Membrane proteins are notoriously difficult to express at high levels in *Escherichia coli* (*E. coli*) (Bruening, 2003, Bruening, 2005).

To develop an anti-MopB antibody first, a leaderless *X. fastidiosa MopB* gene should be engineered, to retain the expressed MopB protein in the *E. coli* cytoplasm. Then the recombinant MopB protein should be characterized using mice sera previously immunized with heat-killed *X. fastidiosa* and used as a selection bait to pull out recombinant single chain antibodies from an *in vitro* ribosome antibody display library (Mattheakis *et al*., 1994, Hanes and Pluckthun, 1997, He and Taussig, 1997) assembled from immunoglobulin genes rescued from the same *X. fastidiosa* immune mouse spleens.

1.10 *In vitro* Ribosome Display

Display technology is used to isolate the DNA or RNA encoding protein sequence. In this technology not only the genetic information is recovered but also functional protein is developed. Based on this technology there are numerous methods developed and validated. These methods could be divided into two categories: (1) cell based methods such as phage display (Winter *et al*., 1994) and surface display (Georgiou *et al*., 1997, Shusta *et al*., 1999) as well as (2) cell-free methods such as ribosome display (Mattheakis *et al*., 1994, Hanes and Pluckthun, 1997, He and Taussig, 1997) and mRNA display (Roberts and Szostak, 1997).

Ribosome display technology is a fully *in vitro*, cell-free method, which overcomes many limitations of cell-based methods by producing *in vitro* protein-mRNA complexes. The advantages of ribosome display comparing to cell-based methods are efficient screening of large libraries without compromise of the library size by transformation efficiency, selecting high affinity combining sites, and eukaryotic cell-free systems are capable of post-translational modifications. Furthermore, as no cell culture is involved it is quick and efficient (He and Taussig, 2007). The only limitation in ribosome display is the accessible functional ribosome in the reaction for the library, which depends on the library size.

This technology is based on *in vitro* protein evolution to produce proteins, which are able to bind to the desired ligand by using the principal of coupling individual proteins (phenotype) to their corresponding mRNA (genotype) to produce stable protein-ribosome-mRNA (PRM) complexes (Figure 1.3). The stable protein

(antibody)-ribosome-mRNA (ARM) complexes are the association of individual antibody fragments with their related mRNA (He and Taussig, 2002).

The PRM complexes formation is achieved via deletion of the stop codon from the mRNA, which causes stalling of the translating ribosome at the end of mRNA. The linkage of protein-mRNA allows simultaneous isolation of the desirable proteins (antibodies) through affinity for an immobilized ligand. The protein-mRNA complex, which binds tightly to the ligand will first be reverse transcribed to cDNA and then PCR amplified to recover the DNA encoding protein sequence (He *et al*., 2004).

In vitro ribosome display can be used to select functional single chain antibodies (scFv's) against recombinant *X. fastidiosa* surface protein MopB using constructed libraries from mouse spleens previously immunized with whole heat-killed *X. fastidiosa.*

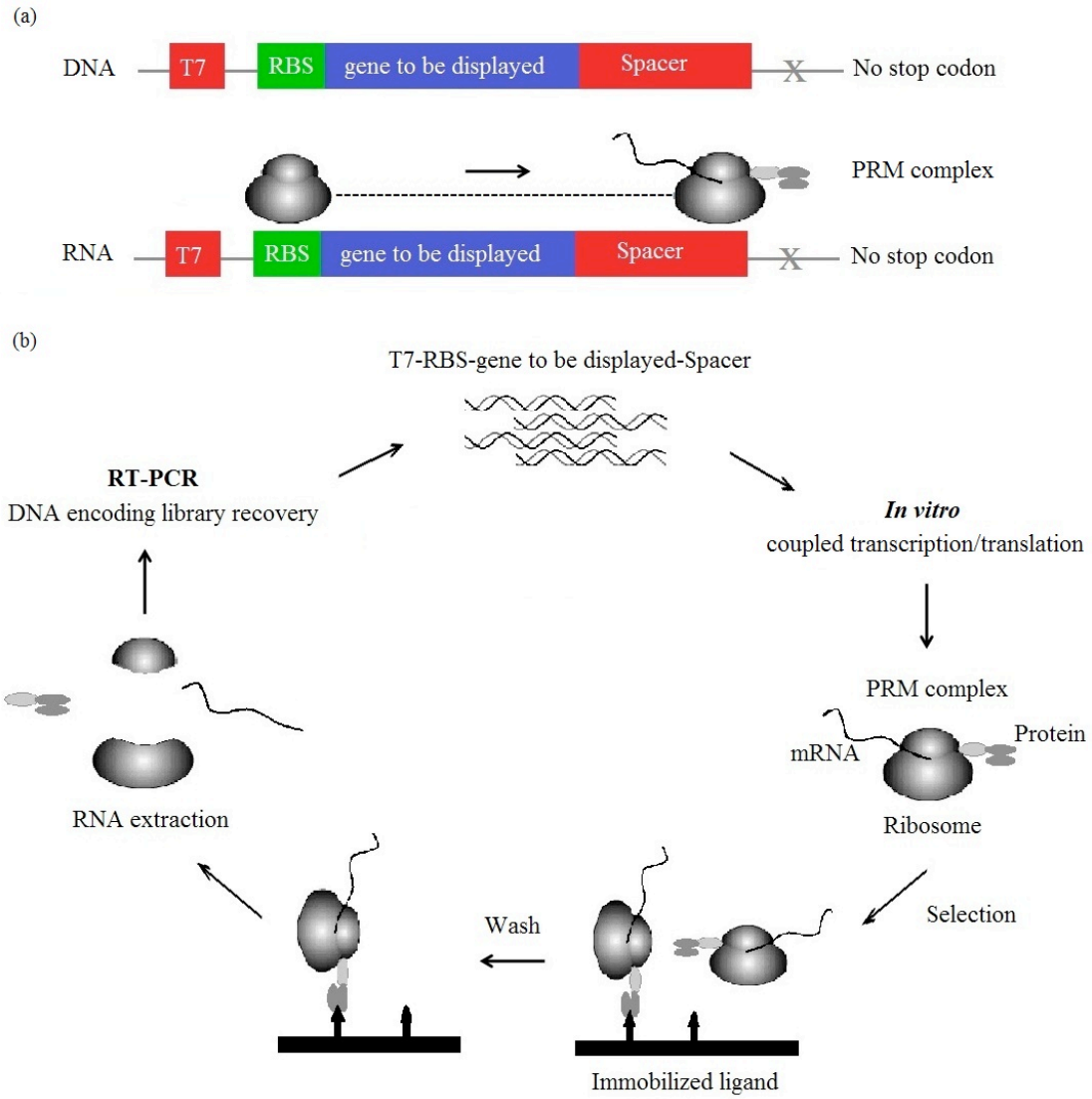

Figure 1.3 Principle of *in vitro* ribosome display. (a) Library construction. DNA library is amplified using PCR and is transcribed to RNA by T7 RNA polymerase. Ribosome binds to the ribosome-binding site (RBS) and translates RNA into a polypeptide. Due to the absence of a stop signal codon the mRNA, protein, the spacer peptide and the ribosome remains bound. (b) Selection cycle. DNA library is first amplified by PCR, as a T7 promoter, ribosome binding site, the gene, the spacer and no stop codon. The amplified DNA library is used in an *in vitro* coupled transcription/translation to form mRNA, the related protein and the ribosome complex (PRM complex). The PRM complexes are affinity selected from the transcription/translation mixture by binding of the immobilized antigen. The bound PRM complexes can then be dissociated and the RNA is isolated from the selected PRM complexes. Isolated mRNA is reverse transcribed to cDNA, and cDNA is then amplified by PCR or One-step RT-PCR to recover the DNA encoding protein sequence. Picture is adapted from (Yan and Xu, 2006).

1. 11 Protein Expression in *Escherichia coli*

1.11.1 Recombinant Protein Expression

A wide range of expression systems (cell-based and cell-free systems) have been developed for recombinant protein expression (e.g. antibody). Cell-based systems are the most commonly used expression systems in which the foreign DNA is introduced to the host cell (bacteria (Baneyx, 1999), yeast (Cregg *et al*., 2000), Baculovirus (Kost *et al*., 2005) and mammalian cells (Rosser *et al*., 2005)) by an expression vector.

One routinely used bacterial expression system is based on *Escherichia coli* (*E. coli*), mainly used for recombinant protein expression due to its well studied genetics, simplicity, fast growth, easy cultivation, low cost, the availability of compatible tools, available varieties of plasmids and bacterial hosts including mutant strains (Sorensen and Mortensen, 2005). However, soluble proteins expression in *E. coli* is still restricted and a couple of empirical rules have been developed to guide the design and selection of an expression system (Makrides, 1996).

To design a recombinant expression system, a plasmid with a compatible genetic background and essential genetic elements (origin of replication (*ori*), antibiotic resistance marker, transcriptional promoter, translation initiation regions (TIRs) and transcriptional and translational terminators) are required.

A strong transcriptional promoter will lead to high level of gene expression. The protein synthesis should be controlled via the presence of the repressor to exhibit a minimal level of basal transcription controlled by a suitable suppressor. In most cases the Lac repressor, encoded by *lacI* gene or its mutants are used as the suppressor. Promoters are normally induced thermally or chemically. One of the most commonly inducers is isopropyl β-D-1-thiogalactopyranoside (IPTG) (Hannig and Makrides, 1998).

Bacterial strains and their genetic backgrounds are also important for protein expression. For an expression strain, several key features such as deficiency in the

most harmful proteases, maintenance of expression plasmid stability and the genetic elements relevant to the expression system (e.g., DE3) (Sorensen and Mortensen, 2005) have been identified. One of the most widely used strains for high-level recombinant protein expression is *E. coli* BL21. This strain is derived from *E. coli* B, a T7 RNA polymerase-based expression systems with a lysogenic host (λDE3) encoding T7 RNA polymerase gene, under the control of *lac*UV5 promoter. Moreover this strain carries two proteases deficiencies, OmpT and Lon, to reduce the possibility of protein degradation and facilitate the intact recombinant proteins isolation.

A modified pET26(+) vector (Novagen, Nottingham, UK), pSANG10-3F vector can be used for recombinant protein and antibody expression. This vector is under control of the T7/lac promoter and the Lac repressor, encoded by the *lacI* gene, to control the basal expression of the protein (Figure 1.4). The T7 promoter requires a DE3 lysogenic strain such as the BL21(DE3) strain, which contains an integrated T7 RNA polymerase in the expression system (Martin *et al*., 2006).

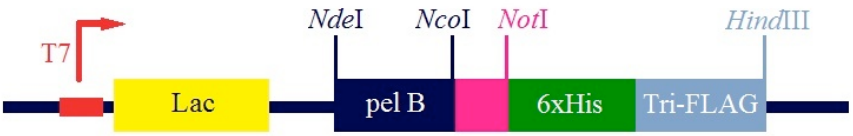

Figure 1.4 Schematic view of the expression cassettes of the pSANG10-3F vector. The protein encoding genes are sub-cloned at the *Nco*I/*Not*I sites. Picture is adapted from (Martin *et al*., 2006)**.**

1.11.2 ScFv Expression in *E. coli*

A single chain antibody (scFv) is a combination of variable light and heavy chains joined via a soluble (serine) and flexible (glycine) polypeptide linker. This small active fragment of approximately 27 kDa in size retains the whole antibody binding site (Huston *et al*., 1991, Sanchez *et al*., 1999, Plueckthun, 1991).

a. Disulfide Bond Formation

Stable disulfide bond formation is required for many proteins such as scFv's to allow

the protein to fold in a proper native conformation to enhance the protein solubility or activity. In the absence of disulfide bonds, these proteins may form inclusion bodies or degrade. Disulfide bond formation normally occurs only when the protein is exported into the periplasmic space, which explains why the first successful expression of the active antibody fragments in *E. coli,* was obtained from the periplasm. This approach is still commonly used to produce scFv antibodies in *E. coli* (Guglielmi and Martineau, 2009, Kipriyanov, 2002).

An alternative approach to produce scFv antibodies in *E. coli* is to express them in the cytoplasm (Martineau and Betton, 1999, Philibert and Martineau, 2004, Guglielmi and Martineau, 2009).

Bacterial strains such as AD494, BL21trxB, Origami, Origami B, Rosetta-gami™, which carry mutations in glutathione reductase (gor) and/or thioredoxin reductase (trxB) can improve disulfide bond formation in *E. coli* cytoplasm (Prinz *et al*., 1997, Bessette *et al*., 1999, Ritz and Beckwith, 2001). Several strains such as Rosetta-gami(DE3) strains carry both trxB and gor mutations. These strains are capable of enhancing the disulfide bond formation and eventually a better solubility and activity compared to strains with only trxB mutation (Bessette *et al*., 1999).

b. Rare Codons

There is more than one codon for most amino acids, and each organism carries its own bias in the usage of 61 available amino acid codons. In *E. coli* codons such as Arginine (Arg), Leucine (Leu), Isoleucine (Ile), Glycine (Gly), and Proline (Pro) are rarely used and therefore this must be taken into account when designing proteins for over-expression.

To improve the expression of eukaryotic proteins in *E. coli*, strains such as Rosetta (derived from the BL21 lacY1 mutant Tuner™ strain) have been designed that contain codons rarely used in *E. coli* (Novy *et al*., 2001, Brinkmann *et al*., 1989, Seidel *et al*., 1992, Kane, 1995, Kurland and Gallant, 1996). These strains also carry a chloramphenicol resistance plasmid that provides tRNAs for the AUA, AGG, AGA, CUA, CCC, GGA codons (Novy *et al*., 2001).

For scFv antibody expression in *E. coli* cytoplasm using the Rossetta gamiB(DE3) strain a designed expression plasmid such as pAHAHis (a modified pET32a(+) plasmid) can be used. This plasmid is under control of the T7/lac promoter and the Lac repressor, encoded by the *lacI* gene, to control the basal expression of the protein in strains such as Rosetta gami B(DE3) (Figure 1.5).

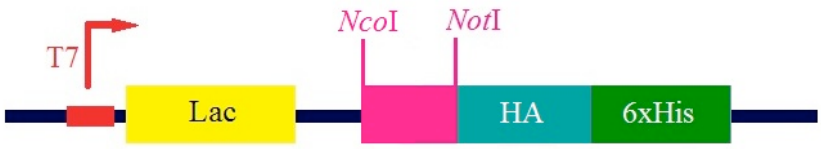

Figure 1.5 Schematic view of the expression cassettes of the pAHAHis vector. pAHAHis vector is constructed using pET32a(+) vector as backbone. The single-chain antibody encoding genes are sub-cloned at the *Nco*I/NotI sites.

1.12 *Pantoea* & Paratransgenesis

Pantoea is a rod-shapped, Gram negative, flagellated, non-capsulated and non-sporulating bacterium belonging to the Enterobacteriaceae family with dimensions of 0.5 to 1.0 to 3.0 μm. This bacterium may or may not produce yellow pigmented colonies (Gavini *et al*., 1989).

1.12.1 *Pantoea agglomerans*

Pantoea agglomerans, also formally known as *Enterobacter agglomerans* (Gavini *et al*., 1989), *Erwinia herbicola*, Ewing and Fife, is a Gram negative plant epiphyte (Lindow and Brandl, 2003, Andrews and Harris, 2000).

This bacterium has been isolated from plant surfaces, seeds, water, animals (Gavini *et al*., 1989) and different insect species (Dillon and Charnley, 2002, Lindow and Brandl, 2003, Loncaric *et al*., 2009, Andrews and Harris, 2000).

Pantoea agglomerans strains are one of the most promising biocontrol agents for a variety of bacterial and fungal plant diseases. This bacterium has been used as a biological control agent for pome and citrus fruits pathogens particularly against fire

blight (Costa *et al*., 2002). Thus it may be an important paratransgenic tool for controlling other diseases such as Pierce's Disease (Pusey, 2002, Rio *et al*., 2004).

Bisi and Lampe (2011) reported a successfully engineered *P. agglomerans* expressing and secreting *Plasmodium* effector proteins to inhibit development of malaria parasites (*Plasmodium* spp.) in the mosquito gut environment. They have reported that two secretion signals, *pelB* and *hlyA,* were used and tested for their ability to secrete anti-*Plasmodium* effector protein in both *E. coli* and *P. agglomerans*. Effector proteins with a HlyA fusion were successfully secreted in *P. agglomerans* and *E. coli*, however neither of these antibodies showed activity in an ELISA assay. The strains that secreted antibodies are under evaluation for anti-*Plasmodium* activity in infected mosquitoes. It was also reported that under laboratory conditions the strains that secreted the effector proteins grew as well as wild-type strains, which may be competitive with the native microbiota in the environment of the mosquito midgut (Bisi and Lampe, 2011).

1.12.2 Alternative Organisms

Recently it has been reported that expression of a recombinant antibody against *Plasmodium falciparum* sporozoite surface protein (Chappel *et al*., 2004a, Chappel *et al*., 2004b) via an engineered entomopathogenic fungus, reduced the levels of *P. falciparum* sporozoite in *Anopheles gambiae* (Fang *et al*., 2011) . In this study a surface accessible highly abundant protein, the circumsporozoite protein was targeted by the recombinant antibody and was designed to agglutinate the parasite. This 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 readily be engineered to agglutinate the bacteria and introduced into the GWSS via paratransgenic organisms such as engineered *Metarhizium spp* (Fang *et al*., 2011) or *Beauvaria bassiana* (Bukhari *et al*., 2011) and an avirulent strain of *Xylella* itself (Miller, 2011).

1.13 Justification and Rationale of the Project

It is more than a decade that *X. fastidiosa* was identified as causal agent of many plant diseases in USA. One of the major agriculture problems in California is Pierce's Disease. This disease, which damages the production of wine, table and raisin grapes, is the result of interaction of the plant host (*Vitis vinifera*), a vector insect (GWSS) and the pathogen (*X. fastidiosa*). Significant efforts have been made to better understand, monitor and control this disease, but until now there is no efficient control measure available for PD.

To minimise the losses of plants by Pierce's Disease, various measures have been developed and classified as cultural, chemical, biological or strain improvement (Hopkins and Purcell, 2002).

A novel avenue towards PD management is to target the causative agent of the disease, *X. fastidiosa*. The main purpose of this strategy would be to disrupt the transmission of the pathogen from the insect vector into the xylem vessels of grapevines. *X. fastidiosa* could be competitively displaced by another manipulated microorganism, which express and secrete an anti-*Xylella* factor in the same niche. The knowledge of genomic information of *X. fastidiosa* may provide efficient candidate targets for anti-*Xylella* factors such as surface exposed components that may potentially play a role in bacterial pathogenicity and will present opportunities for developing diagnostic and paratransgenic applications to track, manage and potentially control PD either in the GWSS or the plant.

1.14 Aim of the Project

The aim of this project is to isolate recombinant single chain Fv fragments against *X. fastidiosa* surface exposed antigenic MopB protein from mice spleens previously immunized with whole heat-killed *X. fastidiosa*.

1.15 Project's Objectives

The main objectives involved in this project are presented as follows:

- To prepare recombinant MopB protein
- To evaluate immune murine sera for anti-MopB activity
- To construct mouse single-chain antibody libraries
- To select specific antibodies using ribosome display technology
- To express selected antibodies in *E. coli*
- To engineer *Pantoea agglomerans* to express selected recombinant antibody

In this project, to isolate recombinant single chain Fv fragments against *X. fastidiosa* surface exposed antigenic MopB protein from mice spleen previously immunized with whole heat killed *X. fastidiosa,* an outer membrane protein MopB (Fjellbirkeland *et al*., 2000, Morano *et al*., 2008), which is partially exposed on the surface of the *X. fastidiosa* bacterium has been chosen as a target protein for creating anti-*Xylella* scFv antibodies. The *X. fastidiosa MopB* gene will be engineered permitting protein expression of two protein constructs (the full length mature and the truncated MopB) in the *E. coli* cytoplasm. The recombinant MopB protein will be used to evaluate mouse sera previously immunized with heat-killed *X. fastidiosa*. Then the antibody library will be assembled from immunoglobulin genes rescued from the same *X. fastidiosa* immune mouse spleens and used in an *in vitro* ribosome display to isolate scFv's against recombinant MopB proteins. The selected scFv's anti-MopB will be expressed in *E. coli*, purified and evaluated by functional analysis (Western blot, ELISA and Immunofluorescence Analysis). Furthermore, *Pantoea agglomerans* will be engineered to express and secrete the selected anti-MopB scFv antibody.

Chapter 2.0
Materials and Methods

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2.1 Molecular Biology Reagents/Kits

MP biomedicals, Illkirch, France

Taq & Go Ready to Use PCR Mix.

New England Biolabs, Hitchin, Herts, UK

10X T4 DNA ligase reaction buffer, 100 base pair (bp) and 1 Kb DNA ladder, Protoscript® First Strand cDNA Synthesis Kit, restriction endonucleases, T4 DNA ligase.

Promega, Southampton, Hampshire, UK

Bright-GLo Luciferase reagent, TNT®T7 Quick Coupled Transcription/Translation System.

Qiagen, Crawley, West Sussex, UK

Ni^{2+}-NTA Spin Columns, QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit, QIAexpress Kit, RNeasy Mini Protocol kit, One-step™ (QIAGEN) RT-PCR.

Roche Diagnostics Ltd., Burgess Hill, West Sussex, UK

DNase I recombinant.

Thermo Fisher Scientific, Loughborough, Leicestershire, UK

TOPO TA Cloning®Kit for Sequencing, TRIZOL®Reagent.

2.2 Chemicals

Becton, Dickinson and Company, Oxford, UK

Bactotryptone, Yeast extract.

BIO-RAD Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK

Precision plus protein dual Xtra standards.

GBIOSCIENCES, Maryland Heights, MO 63043-3202, U.S.A.

Nickel chelating resin.

Merck, Darmstadt, Germany

5-bromo-4-chloro-3-indolyl phosphate (BCIP), Nitro blue tetrazolium (NBT).

Sigma-Aldrich, Poole, Dorset, UK

Agarose, β-mercaptoethanol, dithiothreitol, Diethylaminoethyl (DEAE)-dextran, Mg acetate, Monoclonal anti-polyHistidine-alkaline phosphatase antibody produced in mouse, rabbit anti-mouse antibody, rabbit anti-mouse alkaline phosphatase antibody, goat-anti-rabbit IgG alkaline phosphatase conjugate.

Abcam, Cambridge, UK

Anti-polyHistidine- fluorescein isothiocyanate (FITC) conjugate.

Thermo Fisher Scientific, Loughborough, Leicestershire, UK

5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal), 96 well flat bottom opaque Nunc plate, acetic acid glacial, acrylamide: bis-acrylamide 29:1 solution 40 %, ammonium persulfate, Bovine serum albumin (BSA), Bromophenol blue, $CaCl_2$, carbenicillin, chloramphenicol, Coomassie brilliant blue R-250, Diethylpyrocarbonate (DEPC)-treated H_2O, Dulbecco's modified Eagle medium (DMEM), F96 MaxiSorp Nunc-Immuno plate, glucose, glycerol, Glycine, guanidine hydrochloride, guanidine thiocyanate, Imidazole, Immobilon-P transfer membrane, Isopropyl β-D-1-thiogalactopyranoside (IPTG), isopropyl alcohol, kanamycin, KCl, Luria Bertani (LB) agar, LB broth, methanol, $MgCl_2$, $MgSO_4$, NaCl, p-Nitrophenyl phosphate (pNPP), Sodium dodecyl sulphate (SDS), tetracycline, Tetramethylethylenediamine (TEMED), Tris base, Tween-20.

2.3 Equipment

BIO-RAD Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK

Mini-PROTEAN Tetra Electrophoresis System, Mini Trans-Blot Cell, Gene Pulser.

BMG Labtech, Aylesbury, UK

FLUOstar OPTIMA.

Clare Chemical Research, Dolores, USA

Dark Reader Transilluminator DR-88X.

Thermo Fisher Scientific, Loughborough, Leicestershire, UK

Nanodrop 1000 Spectrophotometer.

Molecular Devices, USA

VersaMax ELISA Microplate Reader.

Cole-Parmer Instrument Company Ltd, UK

Thermo scientific MaxQ 4000 Benchtop shaker.

New Brunswick, USA

Innova®43 incubator shaker series.

DBJ Labcare, UK

Hettich Centrifuge Universal 320/320R rotors: 1617 and 1689-A, SORVALL fresco Jencons pls.

2.4 Oligonucleotide Primers and Plasmids

All of the oligonucleotide primers (Table 2.1) were purchased from Invitrogen Life Technologies (Paisley, UK). pCR®II-TOPO vector (Invitrogen) was used for the cloning of PCR products.

The pSANG10-3F vector for protein expression (Martin *et al*., 2006) was provided by Prof. John McCafferty (Department of Biochemistry, University of Cambridge). The pMALc2x_*mopB* plasmid was kindly provided by Dr. David Lampe. The pA2A10, pET32a(+)RFPHApeptHis, HA-phoAM, pBSK-CA19.9 and pBSKmCit plasmids were kindly provided by Professor Angray Kang.

Table 2.1 Primers (5' to 3' direction) used in PCR. Primers degenerate codons used for synthesising variable regions are: 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; N=A/G/C/T.

	Full length MopB primer
MopB-NdeMat	ATACATATGGCCCAGGAATTCGATGACCGGTGGTATCTCGCTGG
MFNOTR	ATAATGCGGCCGCATTCTGAACGTTCAACTCAGTAC
	Truncated MopB primer
MopB-NdeMat	ATACATATGGCCCAGGAATTCGATGACCGGTGGTATCTCGCTGG
MopB-TrunNot	GCATGCGGCCGCACCAAATGGGATCACGACACC
	First strand cDNA
Oligo-dT29VN	TTTTTTTTTTTTTTTTTTTTTTTTTTTTV
MVHlink2	GGC AGC AGA TCC AGG GGC CAG TGG ATA GAC
	Variable kappa
MVKF1	CGAATTCCACCATGGCAGACRTCMAGATRAYCCAGWCTMCA
MVKF2	CGAATTCCACCATGGCARAMATTKTGCTGACYCARTYTCC
MVKF3	CGAATTCCACCATGGCAGATRYTKTGATGACCCAAACTCCA
MVKF4	CGAATTCCACCATGGCASRAAWTSTTCTCWYMCAGTCTCC
MVKF5	CGAATTCCACCATGGCARRCRTTSWGATGWCACAGTCKCCA
MVKF6	CGAATTCCACCATGGCAGATATTGTGATRACKCAGGMTRMA
MVKF7	CGAATTCCACCATGGCARRYATTGTGATGACCCARWCWC
MVKR1	GAACCACCACCACCACSTWKBABHKYCARYTTKG
	Variable heavy (VH)
MVHF1	GGTGGTGGTGGTTCTMAGCTTCAGGAGTCRGGACC
MVHF2	GGTGGTGGTGGTTCTCAGCTGAAGSASTCAGGACC
MVHF3	GGTGGTGGTGGTTCTMWGSKGGTGGAGTCTGGGGGA
MVHF4	GGTGGTGGTGGTTCTARSSTGGWGGAATCTGGAGGA
MVHF5	GGTGGTGGTGGTTCTARGSTGRTSGAGTCTGGAGG
MVHF6	GGTGGTGGTGGTTCTCARSYGCAGCARYCTGGG
MVHF7	GGTGGTGGTGGTTCTCAGYTGSWGCARTCTGGA
MVHF8	GGTGGTGGTGGTTCTCAGCTGCAGCAGTCWGTG
MVHF9	GGTGGTGGTGGTTCTMASYTGSWGGWGWCTGGAGG
MVHF10	GGTGGTGGTGGTTCTCAGMTSCAGCAGYCTGG
MVHR1	GTAGTCCGCGGCCGCCGMRGARACDGTGASHRDRG
MVHR1.1	GGA AAC TGT CGG TGC GGC CGC CGM RGA RAC DGT GAS HRD RG
	VL-Linkers
MVKFlink	GTAATACGACTCACTATAGGGCGAATTCCACCATGG
MVKRlink$(Gly_4Ser)_3$	GGAGCCGCCGCCGCCAGAACCACCACCACCAGAACCACCACCACCAC
	VH-Linkers
MVHFlink$(Gly_4Ser)_2$	GGCGGCGGCGGCTCCGGTGGTGGTGGTTC

Table 2.1 *Continued on next page*

Table 2.1 Primers used in PCR; *Continued*

Mouse Kappa Constant (MKC)	
MKNotCF	CGGCGGCCGCACCGACAGTTT CC
MKRev	AGAAGCTTGCTCGAGGATTCG
T7 Promoter	
RDT7	CTATAGAAGGGTAATACGACTCACTATAG
Standard Sequencing Primers	
SP6	GATTTAGGTGACACTATAG
M13(-20); M13pUCF	GTAAAACGACGGCCAGT
M13pUCR	CAGGAAACAGCTATGAC

2.5 Cell Culture

Escherichia coli (*E. coli*) XL1-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacI*^{q}*ZΔM15* Tn*10* Tet^{r})] competent cells for plasmid manipulations were purchased from Stratagene (California, USA).

E. coli BL21(DE3) [F^{-} *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)], Rosetta gami B(DE3) [F^{-} *ompT hsdS B*(r_B^- m_B^-) *gal dcm lacY1 aphC(DE3)* gor522::Tn10 trxB pRARE^{2} (Cam ^{R} , Kan ^{R} , Tet ^{R})] *E. coli* strain (Novagen) competent cells were used for recombinant protein expression.

2.6 Chemically Competent Cells

The Cohen method (Cohen *et al*., 1972) was used to prepare *E. coli* chemically competent cells (XL1-Blue, BL21(DE3) and Rosetta gami B(DE3)). Briefly, 10 µL of cells from glycerol stocks or 5 single colonies from an LB agar plate were inoculated into 10 mL of culture media containing the appropriate antibiotic and grown at 37 °C overnight. Pre-warmed LB medium (200 mL) supplemented with the appropriate antibiotic was inoculated with 10 mL of the overnight culture, and grown at 37 °C with vigorous shaking 250 rpm, until the OD_{600} reached between 0.4 - 0.5 and immediately placed on ice for 30 minutes. The cells were pelleted by centrifugation at 1000 *g* and 4 °C for 7 minutes in pre-chilled sterile falcon tubes. The cell pellets were re-suspended in 0.1 M $MgCl_2$ (50 mL for 200 mL of cell culture)

and the pelleting procedure repeated. The resultant cell pellet was re-suspended in 0.1 M $CaCl_2$ (100 mL for 200 mL of cell culture), incubated on ice for 30 minutes and pelleted as described earlier. The cells in the final pellet were gently re-suspended in 4 mL of 0.07 M $CaCl_2$ containing 15 % glycerol (2.8 mL of 0.1 M $CaCl_2$ and 1.2 mL of 50 % glycerol) and 50 μL aliquots stored at –80 °C.

2.7 *E. coli* Transformation

E. coli transformation was performed using the Hanahan method (Hanahan, 1983) with modifications. In brief, competent cells (50 μL) were thawed on ice for 5 minutes. The appropriate ligation mixture or plasmid was then added to the cells and incubated for 5 minutes on ice; the cells were heat shocked at 42 °C in a water bath for 1 minute, and then placed on ice for 5 minutes. Then 250 μL of pre-warmed (37 °C) SOC (20 mg/mL Bactotryptone, 5 mg/mL Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 0.4 % glucose, pH 7.0) was added and further incubated at 37 °C with shaking at 250 rpm for 1 hour. The whole mixture was plated on LB agar plates containing appropriate antibiotic and incubated at 37 °C overnight.

2.8 Colony PCR

After transformation, colony PCR was used to screen the colonies for the desired plasmid. In brief, 2-3 different colonies were randomly picked and each colony was re-suspended in 10 μL of DEPC-treated water (RNase–free). Then 1 μL of each re-suspended colony was used in a PCR reaction with specific primers, which generated the PCR product of known size. Any colony that gave rise to an amplification product of the expected size was likely to contain the correct DNA sequence. The rest of the re-suspended colony with the desired plasmid was used to prepare 10 mL overnight culture for plasmid purification.

2.9 Isolation of Plasmid DNA

Plasmid DNA was isolated from a 10 mL overnight *E. coli* culture using a QIAprep®Spin Miniprep Kit (Qiagen Ltd, UK) according to the manufacturer's

instructions. In brief, a single colony from an LB agar plate or 10 μL of cells from the glycerol stock was inoculated into 10 mL of culture media containing the appropriate antibiotic and grown at 37 °C overnight. Next day a bacterial glycerol stock was prepared using 700 μL of overnight culture with 300 μL of 50 % glycerol and stored at -80 °C. Then, the rest of the culture (~9.3 mL) was centrifuged at 1000 *g* for 10 minutes and the cell pellet re-suspended in 500 μL of P1 buffer (50 mM Tris-HCl, 10 mM EDTA, 50 μg/mL RNase, pH 8.0). Two 250 μL aliquots were transferred into separate 1.5 mL microcentrifuge tubes. Cells in each tube were lysed by the addition of 250 μL of P2 buffer (0.2 M NaOH, 1% SDS) and gentle mixing by inversion 5 times. To neutralise the mixture, 350 μL of N3 buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2) was added to each tube and the tubes were mixed as before. The mixtures were centrifuged at 13,000 *g* for 10 minutes, and the supernatants were carefully transferred onto two QIAprep spin columns and centrifuged at 13,000 *g* for 1 minute. Then the columns were washed with 750 μL of PE buffer (20 mM NaCl, 2 mM Tris-HCl, 80 % ethanol, pH 7.5) and the flow-through was discarded. The columns were centrifuged again at 13,000 *g* for 1 minute to remove residual wash buffer. Purified DNA was eluted by adding 30 μL of DEPC-treated H_2O to each column and centrifuged at 13,000 *g* for 1 minute and stored at -20 °C.

2.10 Gel Electrophoresis

DNA samples (plasmids, digested fragments and PCR products) were resolved by gel electrophoresis with 2 % or 1 % (w/v) agarose depending on the DNA fragment size and 0.5 μg/mL ethidium bromide at 100 V for 1 hour. The gel was visualised with a transilluminator using short wavelength UV light (254 nm) after electrophoresis. The DNA fragment sizes were determined by comparison with 100 bp DNA or 1 kb ladder.

2.11 Gel Extraction

For DNA purification from agarose gels, the piece of gel containing the desired DNA fragment was excised with a clean scalpel using long wavelength UV light (366 nm)

under Dark Reader Transilluminator DR-88X to reduce ultraviolet (UV) DNA damage. The DNA was purified from the gel using QIAquick Gel Extraction Kit according to the manufacturer's instructions. Briefly, excised agarose gel was dissolved in 300 μL of QG buffer (5.5 M guanidine thiocyanate, 20 mM Tris-HCl, pH 6.6) and incubated at 50 °C for 10 minutes. The solution was mixed with 100 μL of isopropyl ethanol before loading on a QIAquick spin column and centrifuge at 13,000 *g* for 1 minute. The column was washed with 750 μL of PE buffer (20 mM NaCl, 2 mM Tris-HCl, 80 % ethanol, pH 7.5) and centrifuged at 13,000 *g* for 1 minute. DNA was eluted in 30 μL of DEPC-treated H_2O and stored at -20 °C.

2.12 Bespoke Protein Marker

The protein markers, which were used to calibrate SDS-gels and Western blot, were based on purified recombinant proteins prepared in the laboratory of Professor Kang. The Bespoke marker was a mixture of four different recombinant proteins with His-tag fusion:

1) Thioredoxin (TRX) protein in pET32a plasmid (20 kDa).
2) Red Fluorescent Protein (RFP) in pSANG (30 kDa).
3) Full length mature MopB protein in pSANG (42 kDa).
4) Heat Shock Protein (HSP) 70 in pET16b (75 kDa).

These protein standards were compared with commercial Precision Plus Protein Dual Xtra standards in SDS-PAGE and Western blot to confirm utility.

2.13 SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using Laemmli system (Laemmli, 1970) with modifications (Markiv *et al*., 2011).

In brief, to analyze the protein a 12 % resolving gel (12 % acrylamide/bis (29:1), 360 mM Tris base, 0.1 % SDS, 0.1 % ammonium persulphate, 0.1 % TEMED, pH 8.8) and 4 % stacking gel (4 % acrylamide/bis (29:1), 60 mM Tris base, 0.1 % SDS, 0.1 % ammonium persulphate, 0.1 % TEMED, pH 6.8) were prepared. The cleaned glass

plates and spacers (0.75 mm thickness) were assembled in a gel holder on a casting stand (Mini-Protean II electrophoresis system). The ingredients for the gels were prepared according to the proportions above, 3.5 mL of resolving gel mixture was poured between the glass plates for each gel, overlaid with water and left to polymerise for 5-10 minutes. The water was poured off and the procedure was repeated for the 4 % stacking gel. The protein samples were prepared by mixing 15 μL of the purified protein fractions with 5 μL of 4X sample loading buffer (62.5 mM Tris base, 2 % SDS, 5 % β-mercaptoethanol, 0.05 % Bromophenol blue, 10 % glycerol, pH 6.8). The un-induced and induced samples were prepared by adding 50 μL 4X sample loading buffer to the cell pellets. The prepared samples were then heated for 5 minutes at 100 °C, centrifuging briefly and loaded on the gel.

Electrophoresis was performed using a Mini-Protean II electrophoresis system, with running buffer (25 mM Tris base, 192 mM glycine, 0.1 % SDS). The gel was run for 45 minutes at 200 V. Resolved proteins were visualised by staining the gel in 0.025 % w/v Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) (Meyer and Lamberts, 1965) in 10 % v/v acetic acid, and heating for approximately 1 minute in a microwave and allowed to cool for 2 minutes followed by destaining by placing in fresh 10 % acetic acid solution and heating as before. The gels were kept in 10 % acetic acid (Markiv *et al*., 2011).

2.14 MopB Protein Expression and Purification

2.14.1 Recombinant MopB Protein

The complete amino acid sequence of *X. fastidiosa* Temecula1 strain (NCBI Reference Sequence: NP_779898.1) MopB was submitted to web based protein prediction programs, SIG-Pred: signal peptide prediction (http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.html) (Bradford, 2001) and to 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/) (Bagos *et al*., 2004a, Bagos *et al*., 2004b, Spyropoulos *et al*., 2004) to identify the signal peptide and the putative

surface exposed domains respectively.

The predicted encoded mature form of full-length MopB (MopB 355) consisting of the outer membrane and the periplasmic domain, without the leader sequence and the N-terminal β-barrel trans membrane domain (MopB 182) without the leader sequence were PCR amplified using primers (MopB-NdeMat and MFNOTR) designed to include *Nde*I (5') site encoding an in-frame methionine start codon and *Not*I (3') site encoding in-frame triple alanine (primers are listed in Table 2.1). The MopB constructs were amplified using Taq & Go polymerase using pMALc2x_*mopB* template. The PCR amplifications were carried out using 16 cycles consisting of 95 °C (30 seconds), 52 °C (30 seconds), and 72 °C (1 minute) after the last cycle a further incubation at 72 °C for 10 minutes, then cooled to 8 °C. To confirm the presence of amplified products, the samples were resolved by gel electrophoresis with 2 % (w/v) agarose and then the desired DNA fragment was excised from the gel and purified using QIAquick Gel Extraction Kit as described in sections 2.10 and 2.11.

The PCR products and the expression vector (pSANG 10-3F) were restricted with *Nde*I and *Not*I using 30 μL of purified DNA from the above step with 5 units of each restriction enzyme *Nde*I and *Not*I in the presence of 1X NEBuffer 3, 100 μg/mL BSA and H_2O to a final volume of 50 μL. The reaction mixture was incubated at 37 °C for 2 hours.

The DNA fragments were subjected to electrophoresis in a standard 1 % agarose gel for the pSANG 10-3F backbone and 2 % agarose gel for the MopB fragments to confirm the presence of the DNA fragments. A 1 kb DNA ladder was used for 1 % agarose gel and 100 bp ladder was used for 2 % agarose gel. The DNA fragments were purified from the gel as described in sections 2.10 and 2.11. The MopB fragments were ligated into pSANG 10-3F vector using T4 ligase, which encodes the protein with an in-frame C-terminal sequence (Ala Ala Ala Ser Ala $(His)_6$ Lys Leu Asp Tyr Lys Asp His Asp Gly Asp Tyr Lys Asp His Asp Ile Asp Tyr Lys $(Asp)_4$ Lys (AAASAHHHHHHKLDYKDHDGDYKDHDIDYKDDDDK).

The ligation reactions were set up by mixing 7 μL of purified insert, 1 μL of purified vector pSANG10-3F, 1 μL containing 20 units of T4 DNA ligase and 1 μL of 10X T4 DNA ligase reaction buffer making up to a final volume of 10 μL. The same transformation was also performed without the plasmid and unligated restricted *Nde*I and *Not*I pSANG 10-3F vector as the ligation controls. The reaction mixture was incubated at room temperature for 2 hours and was transformed into chemically competent XL1-Blue cells (section 2.7) and cultured overnight at 37 °C in LB plates containing 30 μg/mL kanamycin. The colonies were checked by colony PCR and positive colonies for the two MopB constructs were separately inoculated overnight in 10 mL of LB media with 30 μg/mL of kanamycin for plasmid extraction as described in sections 2.8 and 2.9.

The recombinant plasmids were sequenced at the Wolfson Institute for Biomedical Research (WIBR), University College London using Applied Biosystems BigDye3.1 sequencing chemistry, running on an AB3730XL capillary sequencer.

2.14.2 MopB Protein Expression in pSANG 10-3F Vector

The sequence confirmed MopB in pSANG 10-3F plasmids (section 2.14.1) were used to transform BL21(DE3) *E. coli* cells (section 2.7) for protein expression. The molecular weight and isoelectric points was predicted using ExPASy bioinformatics resource portal (http://web.expasy.org/compute_pi/) (Gasteiger *et al*., 2003).

Five fresh colonies were picked and grown in 10 mL LB medium containing 30 μg/mL kanamycin at 37 °C, 250 rpm, for 16 hours and a 700 μL aliquot removed for preparation of a glycerol stock as described earlier in section 2.9. The remaining culture was used to inoculate pre-warmed 200 mL of the same medium, maintained at the same culture conditions until the OD_{600} reached between 0.4 - 0.6. As a control, 1 mL aliquot of culture was collected and centrifuged at 13,000 *g* for 1 minute and the cell pellet was kept at -20 °C as un-induced sample. Expression of recombinant proteins was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM and cultivated for 3 hours at 37 °C with shaking at 250 rpm. Before harvesting the culture, a 1 mL aliquot of the induced

culture was collected and centrifuged at 13,000 *g* for 1 minute and the cell pellet was kept at -20 °C as induced sample for protein analysis. The culture was harvested by centrifuge for 20 minutes at 4000 *g*, 4 °C, and stored for further use at -20 °C.

2.14.3 MopB Protein Purification

a. Purification using Denaturation Condition

The cell pellets obtained from 200 mL liquid cultures (section 2.14.2) were re-suspended in 5 mL 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 (6x30 seconds with 30 seconds rest intervals), in an ultrasonic cell disruptor, and were centrifuged at 16,000 *g* for 45 minutes to remove cellular debris. The supernatant containing soluble cellular material were retained for the purification procedure.

Recombinant protein was purified using Ni^{2+}-NTA agarose affinity resin as described in *The QIAexpressionist*^{TM} *-- A handbook for high-level expression and purification of 6xHis-tagged proteins*, fifth edition. A 10 mL column was packed with 1 mL of nickel chelating agarose resin and equilibrated with 5 mL wash buffer (8 M Urea, 10 mM Tris–HCl, 100 mM NaH_2PO_4 pH 8.0). The supernatants containing the 6xHis-tagged recombinant protein were loaded onto the equilibrated 1 mL Ni^{2+}-NTA column. The flow through was collected and the Ni^{2+}-NTA column was 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 fused proteins were eluted in 5X1 mL fractions using 5 mL of elution buffer (8 M Urea, 10 mM Tris–HCl, 100 mM NaH_2PO_4 pH 4.5). Fractions were assayed for total protein concentration and analyzed by SDS-PAGE (section 2.13).

b. Purification using Native Condition

The cell pellet obtained from 400 mL culture was re-suspended in 5 mL native lysis buffer (20 mM Tris–HCl, 500 mM NaCl, 20 mM Imidazole, 0.1 % Triton X-100 pH

8.0) and 400 μL lysozyme (1 mg/mL) was added. The cells were lysed by sonication on ice (6x30 seconds burst at 30 seconds intervals), in an ultrasonic cell disruptor and then centrifuged at 18,000 *g* for 45 minutes. The supernatant containing soluble cellular material was retained for purification procedure using an ÄKTAprime liquid chromatography system using a 1 mL HisTrap HP column fitted to an ÄKTAprime™ plus liquid chromatography system. The column was equilibrated with 5 mL wash buffer (20 mM Tris–HCl, 500 mM NaCl, 20 mM Imidazole pH 8.0).

The soluble fraction containing the 6xHis-tagged protein was diluted two times with wash buffer (20 mM Tris–HCl, pH 8.0, 500 mM NaCl buffer) and loaded onto the equilibrated 1 mL Ni^{2+}-NTA column. The flow through was collected and the column was washed with 5 column volumes of wash buffer (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 20 mM Imidazole). The bound proteins (full length mature and truncated MopB fusion proteins) were eluted in 1 mL aliquots using 5 column volumes of elution buffer (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 500 mM Imidazole) and analyzed by SDS-PAGE (section 2.13).

2.14.4 Protein Analysis

a. SDS-PAGE

The SDS-PAGE was performed as described in section 2.13

b. Protein Concentration Determination

Recombinant soluble protein concentrations were determined by using a Bradford dye bind protein assay kit.

Protein standards were prepared in buffer ranging from 0.1-1.4 mg/mL using a BSA standard. Using a 96 well plate, 5 μL of the protein standards was added to separate wells and 5 μL of the buffer was added to the blank wells. The recombinant soluble protein was prepared with an approximate concentration between 0.1-1.4 mg/mL. To each well being used, 250 μL of the Bradford Reagent (0.01 % (w/v) Coomassie

Brilliant Blue G-250, 4.7 % (w/v) ethanol, 8.5 % (w/v) phosphoric acid) was added and mixed on a shaker for approximately 30 seconds. The samples were incubated at room temperature for 5 to 45 minutes. Then the absorbance was measured at 595 nm (The protein-dye complex is stable up to 60 minutes). The net absorbance vs. the protein concentration of each standard was plotted.

2.14.5 Mouse Immunization

Preparation of the *Xylella fasitidosa* and initial serum dot-blot analysis was carried out by Arinder Arora in the laboratory of Professor Thoma Miller at the University of California Riverside, USA. Briefly *Xylella fasitidosa* (strain Temecula) was grown in 100 mL PD3 (Davis *et al*., 1980) medium at 28 ºC on an orbital shaker at 180 rpm for 10 days until the OD was 0.5 at 600 nm. The bacteria were pelleted and washed twice by re-suspension in 100 mL PBS and the final pellet wet weight determined. The final pellet was re-suspended in PBS at 10 mg/mL w/v and a 1 mL aliquot prepared. The aliquot was incubated at 28 ºC (viability; positive control) and the remainer at 55 ºC for 1 hour. Samples were removed and plated out on PD3 agar and incubated for 15 days to check for viability, the remainder of the bacterial pellet was frozen at -80 ºC. Once it had been confirmed that no viable bacteria were present in the heat-treated ampules, aliquots were sent to ProSci (Poway, CA USA) for immunization of Balb/c mice (n=5). The initial immunization (0.5 mL/mouse) was followed by boosting at weeks 4 , 8 and 12. Test bleeds were taken a weeks after the third boosting for Dot blot analysis and enzyme-linked immunosorbent assay (ELISA). Following testing, mice were boosted again and three days later underwent a splenectomy, each spleen was placed immediately in 10 mL of TRIZOL for use in RNA isolation.

2.14.6 Bacterial Dot Blot

To carry out the preliminary screening to determine whether the mice had mounted an antibody response to *X. fastidiosa* a Dot blot assay was devised. Thin strips of nitrocellulose ~5 mm wide were prepared and spotted with either 5 μL of 5 % w/v skimmed milk powder in PBS or 5 μL of bacterial suspension of *E. coli* or 5 μL

X. fastidiosa Temecula 1 strain allowed to air dry, blocked with 1% BSA (molecular biology grade Bovine Serum Albumin) in PBS, and incubated in immune sera (bleed 3) diluted 1/20,000 in PBST with 1 % BSA for 1 hour. Following washing with PBS-T, and then incubated with rabbit anti-mouse alkaline phosphatase antibody (diluted 1/20,000 in 1 % BSA in PBST) for 1 hour, then washed as before and developed in substrate solution (0.02 %, BCIP (5-bromo-4-chloro-3-indolylphosphate) and 0.03 % NBT (nitroblue tetrazolium) in 10 mL 100 mM Tris-base, 100 mM NaCl, 5 mM MgCl, at pH 9.5) until the spots were visible.

2.14.7 Recombinant MopB ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) was carried out using Nunc-Immuno plates. The 96 well plates were coated with 100 µL of a 10 µg/mL solution of recombinant purified truncated MopB protein in phosphate buffered saline (PBS) or 100 µL of a solution containing 10 µg/mL of molecular biology grade Bovine Serum Albumin (BSA) in PBS pH 7.4 as negative control at 4 °C overnight. The next day, the wells were washed with PBS containing 0.05 % Tween-20 (PBST), dried and blocked with 200 µL of 2 % BSA/PBS at room temperature for 1 hour. Polyclonal sera from mice (n=5) previously immunized with whole heat-killed *X. fasitidosa* were diluted to 1/200, 1/2000 and 1/20,000 in PBS containing 0.05 % Tween-20 (PBST) added in duplicate to the wells (0.1 µL/well) and incubated at 37 ºC for 2 hours. The wells were washed with PBS containing 0.05 % Tween-20 (PBST) five times and 100 µL of rabbit anti-mouse antibody (diluted 1/20,000 in 1 % BSA in PBST) was added and incubated at 37 °C for 2 hours. The wells were washed as before and goat-anti-rabbit IgG alkaline phosphatase conjugate (diluted 1/40,000 in 1 % BSA in PBS) was added to the wells and incubated at 37 °C for 2 hours. The plates were washed as before with an additional final wash with PBS alone and developed with 1 mg/mL p-nitrophenyl phosphate in 0.2 M Tris buffer pH 8.0 (pNPP) at 37 °C for 30 minutes. The absorbance was measured at 405 nm using a spectrophotometer VersaMax ELISA Microplate Reader. Both sample and control were performed in duplicate. A threshold value of ≥ 0.1 was used as the cut-off value for detection of mouse sera 1-5 binding to the recombinant truncated MopB protein.

2.15 Mouse Immunoglobulin Library Assembly

Mouse single chain antibody libraries were constructed from the five *X. fastidiosa* immunized mice spleens in 10 mL TRIZOL stored at -20 °C in the form of VL-Link-VH-Mouse Kappa Constant (MKC).

Total RNA was first extracted from the corresponding mouse spleen. Then the extracted RNA was used to synthesis the first strand complementary DNA (cDNA) using immunoglobulin specific primers. The synthesized cDNA was used to amplify the immunoglobulin variable regions by PCR. Finally five mice single-chain antibody libraries were assembled individually by linking all individual variable regions (7 light chains and 10 heavy chains) for each mouse by PCR. Detailed procedures are described below.

2.15.1 Total RNA Extraction

The immunized mice spleens (section 2.14.5) were homogenized, disrupted and dissolved in 10 mL TRIZOL®Reagent and stored at -20 °C. Total RNA was isolated using TRIZOL®Reagent according to the manufacture's instruction. In brief, 0.2 mL of chloroform was added for each mL of the homogenized mouse spleen in TRIZOL®Reagent. The sample was shaken vigorously by hand for 15 seconds and then incubated for 15 minutes at room temperature. The mixture was centrifuged at 12,000 *g* for 15 minutes at 4 °C. Without disturbing the pellet the aqueous phase of the sample was transferred to a clean tube. The RNA was precipitated from the aqueous phase by adding 200 μl of 100 % isopropanol alcohol for each 1 mL of the sample, mixed by inversion and incubated for 15 minutes at room temperature. The sample was centrifuged at 12,000 *g* for 10 minutes at 4 °C. The supernatant was removed, leaving only the RNA pellet. The pellet was washed with 1 mL 75 % ethanol per 1 mL of sample, briefly vortexed and incubated for 5 minutes at room temperature. The sample was then centrifuged at 7500 *g* for 5 minutes at 4 °C. The supernatant was removed, the pellet was air dried at room temperature for 10 minutes and re-suspended in 50 μL DEPC-treated water (RNase–free). The dissolved RNA samples were spectrophotometrically analysed at 260 nm/280 nm using a

NanoDrop apparatus to determine the concentration and the purity; the total RNA samples were stored at -20 °C until required.

2.15.2 First Strand cDNA Synthesis

First strand cDNA was synthesised from total RNA using a Protoscript First Strand cDNA Synthesis Kit with Oligo-dT29VN primer for light chain and MVHlink2 primer for heavy chains (all primers are listed in Table 2.1) following the protocol provided with the kit. Briefly, 1 μg of total RNA was incubated at 70 °C for 5 minutes with 25 pmol of primer, 40 nmol of deoxyribonucleotide triphosphate mix (dNTP) and sufficient DEPC-treated H_2O to a final volume of 16 μL. The mixture was placed on ice for at least 30 seconds before adding 2 μL of M-MuLV Reverse Transcriptase Reaction Buffer (10X RT Buffer), 1 μL of M-MuLV Reverse Transcriptase (25 units) and 1 μL of RNase inhibitor (10 units). The mixture was incubated at 42 °C for 1 hour, followed by 95 °C for 5 minutes to inactivate the enzymes. To degrade the remaining RNA, 1 μL of RNase H (2 units) was added to the mixture and incubated for 20 minutes at 37 °C. Finally the mixture was incubated at 95 °C for 5 minutes to inactivate the enzyme. The resulting 20 μL cDNA synthesis reaction was stored at -20 °C until required.

2.15.3 Mouse Single-Chain Antibody Library Construction

A single chain variable fragment antibody library was constructed for ribosome display as VL-link-VH-MKC (Mouse Kappa Constant). The PCR primers for library construction were based on published sequences described by Kettleborough and co-workers (Kettleborough *et al*., 1993), with minor modifications, and ordered from Invitrogen (all primers are listed in Table 2.1). Members of the light chain families were individually amplified by PCR using Oligo-T29VN cDNA template using combinations of MVKF1-7 with MVKR primers. Members of the heavy chain families were individually amplified by PCR using MVHlink2 cDNA template using combinations of MVHF1-10 and MVHR1.1 primers. The primers were designed to introduce *Nco*I restriction site to the 5′ ends of the VL and *Not*I restriction site to the 3’ end of the VH.

The amplified light chain products were purified, pooled and an aliquot was subjected to another round of PCR amplification using MVKFlink and MVKRlink primers to introduce part of the T7 site and Kozak sequence (Kozak, 1987) on the 5' end and an overlap extension to facilitate annealing 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 primers. A synthetic mouse kappa constant domain (optimised for *E. coli* codon usage, synthesised by Epoch Biolabs, Tx USA) was amplified using MKNotCF and MKRev primers. The mouse kappa constant (MKC) was joined to the VL-Link-VH combinations by a PCR overlap extension reaction using MVKFlink and MKRev (Figure 2.1).

Finally the PCR product encoding all the variable light chains and heavy chains combinations were amplified with primers RDT7 and MKRev to produce the DNA encoding the anti-*X. fastidiosa* immunoglobulin scFv libraries. The PCR reactions were performed at 50-62 °C annealing temperatures (depending on the primer pairs) the initial amplification used 30 cycles and subsequent library assembly step used 16 cycles with Taq DNA polymerase and 25 pmol of each primer pair per reaction. DNA fragments were separated by gel electrophoresis on 2 % (w/v) agarose gels (section 2.10). DNA purification from agarose gels was carried out following Qiagen kit instructions (section 2.11). In total, five antibody DNA libraries were constructed.

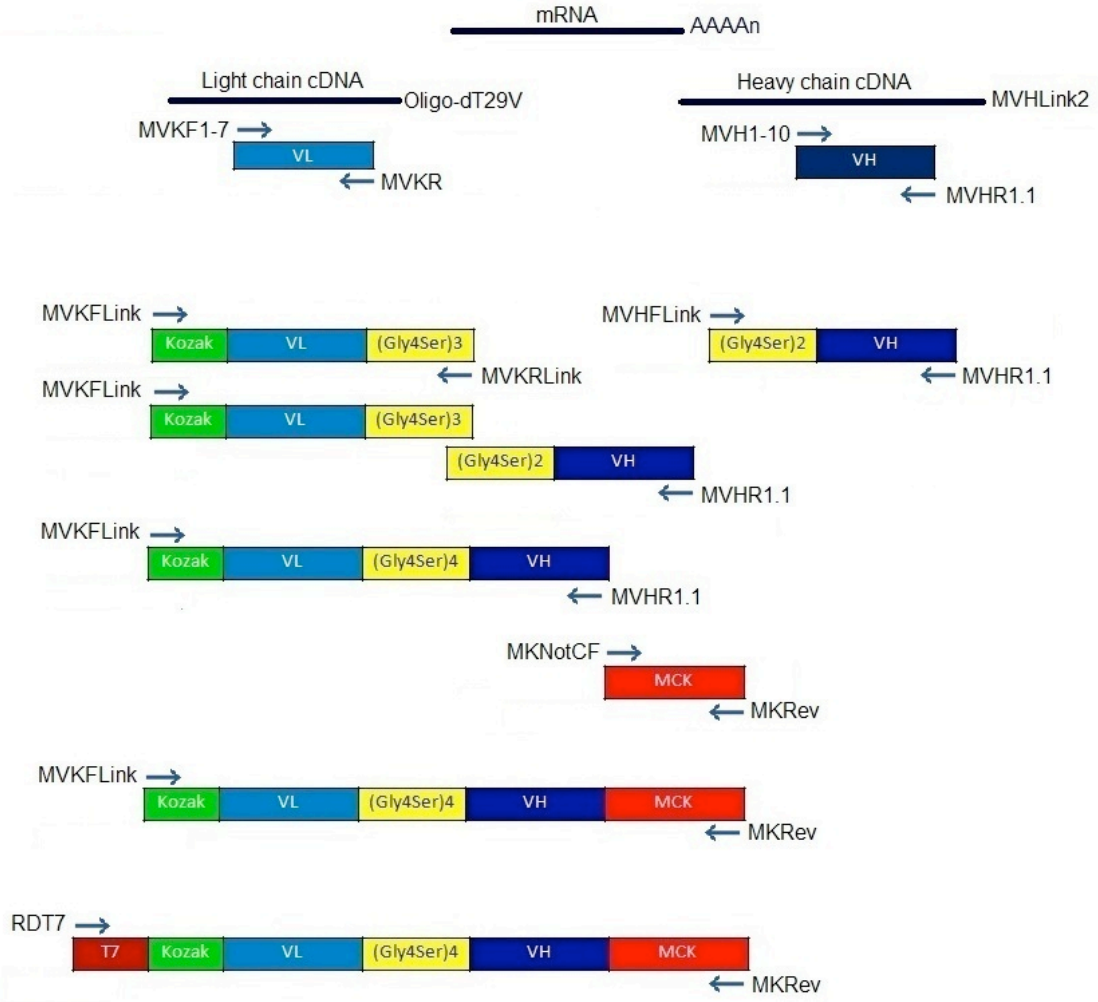


Figure 2.1 An illustration of the mouse immunoglobulin library assembly process showing the PCR amplification and assembly steps using mouse spleen total RNA.

2.15.4 Cloning and DNA Sequencing

The purified single chain antibody library product was cloned into pCR®II-TOPO vector using TOPO TA Cloning® Kit, Invitogen according to the manufacturer's instructions; 4 µL of DNA was ligated into 1 µL of TOPO vector and 1 µL of salt solution (provided in TOPO TA Cloning® Kit for Sequencing) and incubated at room temperature for 1 hour. The ligation mix was used to transform XL1-Blue *E. coli* cells on X-gal plates (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) containing 100 µg/mL of carbenicillin to select for disruption of the β-galactosidase, performing blue/white screening.

Three white colonies were randomly picked and were inoculated in 10 mL of LB

media with 100 μg/mL of carbenicillin; plasmid DNA was isolated from *E. coli* (section 2.9) and sequenced at the Wolfson Institute for Biomedical Research (WIBR), University College London using standard primers, M13 (-20) and SP6 (Table 2.1).

2.16 *In vitro* Ribosome Display

To select single-chain antibodies a modified eukaryotic ribosome display was used as outlined in the schematic in Figure 2.2 (He and Taussig, 2007) with slight modifications.

The PCR generated DNA libraries of antibody coding genes derived from mice (section 2.15.3) were expressed in a rabbit reticulocyte lysate system (Promega's TNT quick coupled transcription/translation system). An *in vitro* coupled transcription/translation was set up in a 0.5 mL PCR tube as follows: 40 μL TNT T7 Quick Master Mix, 1 μL 1 mM methionine (both provided in the kit), 2 μL of DNA library (10 ng-1 μg), 1 μL DNA enhancer and 6 μL DEPC-treated H_2O. The mixture was incubated at 30 °C for 90 minutes. DNase I recombinant, RNase-free (10000 u/mL) was added to the TNT mixture and incubated at 30 °C for 20 minutes to degrade the input DNA.

To select specific antibody fragments the 0.5 mL PCR tubes were coated with 10 μg/mL of the recombinant truncated and full length mature MopB in 100 μL PBS at 4 °C overnight. Protein coated tubes were washed with PBS and blocked with 100 μL of molecular biology grade Bovine Serum Albumin (BSA) in PBS (10 mg/mL) (New England Biolabs) for 1 hour at room temperature.

The translation/transcription mixture (containing the protein-ribosome-mRNA (PRM) complexes) was added to the washed and blocked protein-coated tubes and incubated on ice for 1 hour. The PCR tubes were washed by filling once with PBS (0.5 mL) and decanted. The residual liquid was carefully removed using a sterile pipette tip.

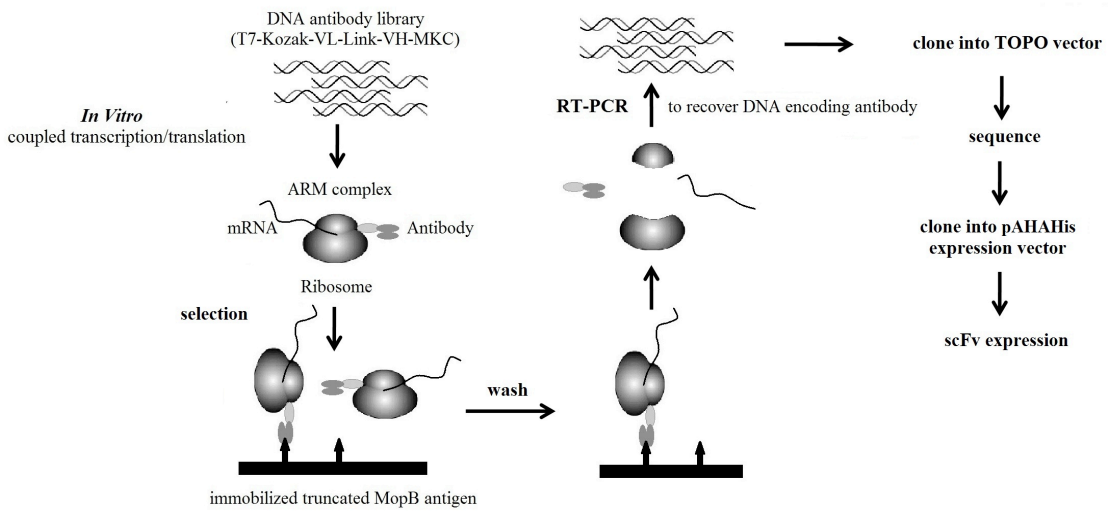


Figure 2.2 A schematic of the steps involved in *in vitro* antibody ribosome display. The scFv antibody library DNA template is prepared flanked with a T7 sequence and mouse kappa constant sequence. The antibody library is transcribed to mRNA and translated to form antibody-ribosome-mRNA complex (ARM). The mixture is incubated with immobilised truncated MopB and the unbound components are removed by washing. The retained complexes are released and the mRNA reverse transcribed and amplified by PCR. The PCR products are cloned into TOPO vectors and recovered plasmids sequenced. Full-length in-frame sequences are sub-cloned into the pAHAHis vector for scFv production.

The retained RNA was recovered using RNeasy Mini Protocol kit according to the manufacturer's instructions. In brief, 350 μL buffer RLT was added to the tubes and mixed by pipetting. The mixture was directly added onto a QIAshredder column and centrifuged for 2 minutes at maximum speed. The flow through was collected and 350 μL of 70 % ethanol was added and mixed well by pipetting. The sample was added onto the RNeasy mini spin column and centrifuged for 15 seconds at 8000 *g*. Then 700 μL buffer RW1 was added onto the RNeasy column, and centrifuged for 15 seconds at 8000 *g* to wash. The flow through was discard and 500 μL buffer RPE was added onto the RNeasy column and centrifuged for 15 seconds at 8000 *g* to wash. The flow through was discarded and another 500 μL buffer RPE was added onto the RNeasy column and centrifuged for 2 minutes at maximum speed to dry the RNeasy membrane. The RNeasy column was transferred into a fresh eppendorf tube and 30 μL of RNase-free water was directly pipetted onto the RNeasy membrane and centrifuged for 1 minute at 8000 *g* to elute. The extracted RNA was stored at -20 °C.

The extracted RNA was used in a One-step^{TM} (QIAGEN) RT-PCR reaction with MVKFlink and MKRD2 primers to amplify the antibody encoding DNA.

One-Step™RT-PCR recovery was carried out using 50 μL RT-PCR mixture as follows: 10 μL 5XQ Solution, 10 μL 5XBuffer, 2 μL dNTP mix, 10 μL DEPC-treated H_2O, 5 μL forward primer, 5 μL reverse primer (25 pmol), 6 μL RNA template and 2 μL enzyme.

The mixture was thermally cycled as follows: 50 °C for 30 minutes, 95 °C for 15 minutes, 35 cycle 94 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for 1 minute, 72 °C for 5 minutes and then kept at 8 °C. Recovered DNA was analyzed by gel electrophoresis as described in section 2.10.

2.16.1 TOPO TA Cloning and DNA Sequencing

The RT-PCR product from a single round of ribosome display was cloned into pCR®II-TOPO® vector (TOPO TA Cloning® Kits, Invitogen) according to the manufacturer's instructions as described in section 2.15.4. Five white colonies were randomly picked, inoculated into 10 mL of LB media with 100 μg/mL of carbenicillin and grown overnight at 37 °C with 250 rpm shaking.

The plasmid DNA was isolated and sequenced as described in section 2.15.4 using M13 (-20) and SP6 standard primers (Table 2.1) at the core facility at WIBR, University College London.

2.17 MopB Selected scFv Cloning and Expression

2.17.1 Expression Plasmid Construction

Since the MopB antigen used in this study was cloned and expressed in a vector that introduced hexa-histidine and Tri-FLAG tag, an alternative antibody expression vector with a different detection tag was constructed to permit evaluation of single chain (scFv) antibody binding. A plasmid was constructed by modifying pET32a(+) to incorporate a carboxyl-terminal-tag peptide sequence derived from influenza virus hemagglutinin, HA tag (Asp Val Pro Asp Tyr Ala Ser: DVPDYAS) followed by a hexa-histidine-tag designated pAHAHis.

The recovered single chain antibodies encoding sequences were ligated into pAHAHis plasmid as *Nco*I/*Not*I fragments for cytoplasmic expression in Rosetta gami B(DE3) *E. coli*.

a. pAHAHis Plasmid Construction

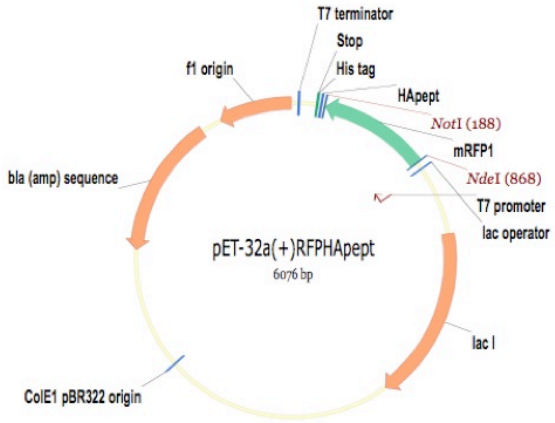
To construct pAHAHis plasmid, pA2A10 (the 2A10 antibody for NPNA malaria antigen) and pET32a (+)RFPHApeptHis (Figure 2.3a) plasmids were digested with *Xba*I and *Not*I restriction sites.

The 2A10 antibody sequence (~750 bp) from pA2A10 plasmid and HA-His (~5 kb) from pET32a(+)RFPHApeptHis plasmid were ligated and transformed into XL1-Blue competent cells using LB agar plates (section 2.7) containing 100 μg/mL of carbenicillin.

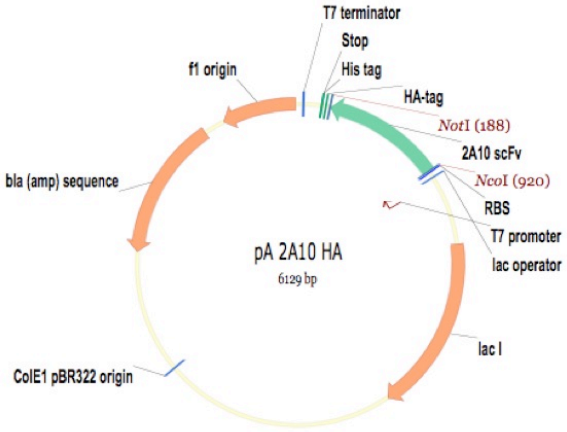
The 2A10HAHis plasmid construct was purified and confirmed by DNA sequencing (Figure 2.3b). To prepare the pAHAHis backbone, the 2A10HAHis plasmid was digested with *Nco*I and *Not*I sites and the fragments were resolved by gel electrophoresis using 1 % (w/v) agarose gel and purified as described in sections 2.10 and 2.11.

The MopB scFv antibodies were digested with *Nco*I and *Not*I sites and were ligated into digested *Nco*I /*Not*I pAHAHis backbone to construct pAMopBscFvHAHis plasmid (Figure 2.3c).

a)



b)



c)

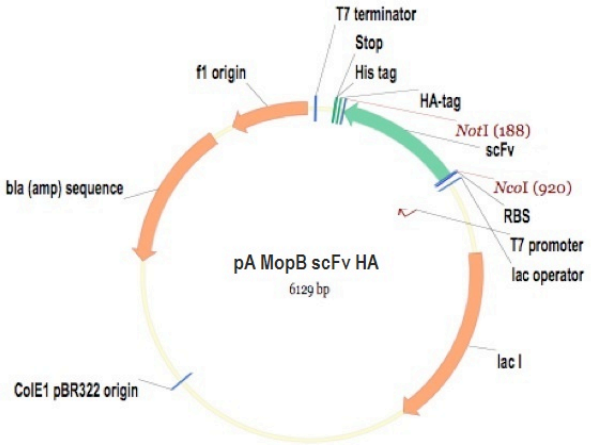


Figure 2.3 pAHAHis expression plasmids constructions. a) pET32a(+)RFPHApeptHis; b) pA2A10HAHis; C) pA MopB scFv HAHis.

b. Anti-HA Antibody (Alkaline Phosphatase), HA-phoAM

The anti-HA (DVPDYAS peptide) scFv was fused to alkaline phosphatase (anti-HA scFv-phoAM) (Figure 2.4).

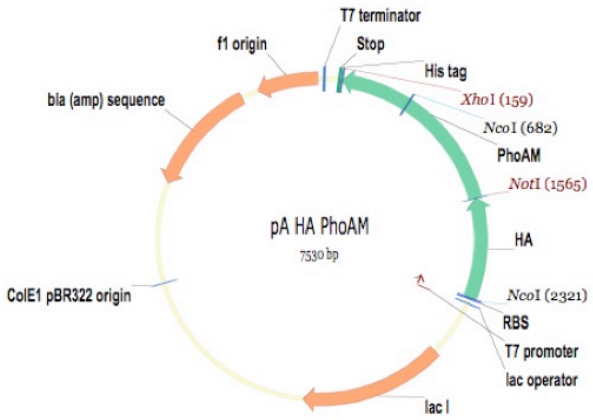


Figure 2.4 pAHAphoAM plasmid construct.

2.17.2 MopB scFv Cloning and Expression in pAHAHis Expression Vector

The recovered single chain antibodies encoding sequences were ligated into pAHAHis plasmid as *Nco*I/*Not*I fragments for cytoplasmic expression in Rosetta gami B(DE3) *E. coli*. The expression plasmid was transformed into XL1-Blue *E. coli* cells as described earlier in section 2.7, and plated onto LB agar plate containing with 100 µg/mL of carbenicillin. A single colony was inoculated into 10 mL of LB medium with 100 µg/mL of carbenicillin and grown at 37 °C with 250 rpm shaking for 16 hours. pAHAHis encoding the scFv plasmid was isolated from *E. coli*, confirmed by sequencing and subsequently transformed into Rosetta gami B(DE3) competent cells (section 2.7), plated onto LB agar plate with 100 µg/mL of carbenicillin and 34 µg/mL of chloramphenicol, and grown at 37 °C for 16 hours. Next day, five colonies were inoculated in 10 mL of LB media with the same antibiotics and grown at 37 °C with 250 rpm shaking for 16-18 hours. The 10 mL overnight culture was used to prepare a glycerol bacterial stock as described

previously in section 2.9 and inoculated into 200 mL of LB medium containing the same antibiotics and grown at 37 °C with shaking at 250 rpm until OD_{600} reached between 0.4-0.6. The cells were then placed on ice for 30 minutes, induced by the addition of IPTG (final concentration 1 mM) and were incubated for 20 hour at 20 °C with shaking. Cells were harvested in 4x50 mL tubes by centrifugation at 4000 *g*, 4 °C for 20 minutes. Cell pellets were frozen and stored at -80 °C before undergoing further processing.

2.17.3 Anti-MopB scFv Purification

The cell pellets from 200 mL culture were used for purification using denaturing and native conditions.

a. Denaturing Conditions

The 50 mL cell pellet from 200 mL culture was re-suspended in 3 mL of lysis buffer (6 M guanidine hydrochloride, 10 mM Tris–HCl, 500mM NaCl, 10 mM Imidazole, pH 8.0). Cells were lysed by sonication on ice (6x30 seconds), and were centrifuged at 14,000 *g* for 15 minutes to remove cellular debris. The supernatant containing the hexa-histidine-tagged recombinant protein were used for the purification procedure. Recombinant protein was purified using 1 mL of nickel chelating resin (GBiosciences) packed in a MicroSpin column. Then the columns were equilibrated with 600 µL lysis buffer (6 M guanidine hydrochloride, 10 mM Tris–HCl, 500 mM NaCl, 10 mM Imidazole, pH 8.0), and centrifuged at 890 *g* for 2 minutes.

The supernatants containing the 6xHis-tagged recombinant protein were loaded onto the equilibrated 1 mL Ni^{2+}-NTA column and centrifuged at 270 *g* for 5 minutes. The flow throughs were collected and the Ni^{2+}-NTA column was washed two times with 600 µL of wash buffer (8 M Urea, 10 mM Tris–HCl, 100 mM NaH_2PO_4 pH 6.3) at 890 *g* for 2 minutes. The bound recombinant fused proteins were eluted into 5X100 µL fractions using 500 µL of elution buffer (8 M Urea, 10 mM Tris–HCl, 100 mM NaH_2PO_4 pH 4.5) at 890 *g* for 2 minutes. Purified proteins were stored at 4 °C until required.

b. Native Conditions

The purification was performed under denaturing conditions as described above; but in this case native lysis buffer (20 mM Tris–HCl, 500 mM NaCl, 20 mM Imidazole, 0.1 % Triton X-100 pH 8.0) with 75 µL lysozyme (1 mg/mL), native wash buffer (20 mM Tris–HCl, 500 mM NaCl, 20 mM Imidazole pH 8.0) and native elution buffer (20 mM Tris–HCl, 500 mM NaCl, 500 mM Imidazole, pH 8.0) were used. The purified protein fractions were kept at 4 °C.

2.17.4 Expression and Purification of 2A10 and HA-phoAM scFvs

The constructed pA2A10HAHis and HA-phoAM (anti-HA scFv antibody) plasmids were transformed into Roseta gamiB(DE3) competent cells and were expressed and purified as described earlier for selected MopB scFv antibody in pAHAHis plasmid (section 2.17.2 and 2.17.3). Purified proteins were stored at 4 °C until required. Fractions were assayed by SDS-PAGE as described before in section 2.13.

2.17.5 Anti-MopB scFv Protein Analysis

a. SDS-PAGE

The SDS-PAGE was performed as described in section 2.13.

b. Western Bloting

Purified selected MopB scFv's were blotted onto polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot Cell (Bio-Rad) at 100 V for 1 hour with Western blot buffer (25 mM Tris base, 192 mM glycine, 20 % methanol) (Towbin *et al*., 1979). PVDF membranes were blocked in 5 % w/v skimmed milk powder in Tris buffered saline (TBS: 50 mM Tris base, 150 mM NaCl pH 7.4) at room temperature for 1 hour, then incubated with monoclonal anti-polyHistidine-alkaline phosphatase antibody (diluted 1/10,000 in 5 % Milk/TBS) at room temperature for 1 hour on a rocking platform, followed by washing with 10 mL TBS-T (50 mM Tris base, 150

mM NaCl, 0.1% Tween-20, pH 7.4) 3x for 5 minutes each, then rinsed once with TBS (5 minutes) and the membrane developed by adding 10 mL of substrate (0.02 %, BCIP (5-bromo-4-chloro-3-indolylphosphate) and 0.03 % NBT (nitroblue tetrazolium) in 10 mL alkaline phosphates buffer (100 mM Tris-base, 100 mM NaCl, 5 mM MgCl, 0.05 % Tween-20, pH 9.5).

2.17.6 Anti-MopB scFv Functional Assays

Antibody binding to recombinant truncated and full length mature MopB was examined by Western blot, ELISA and Immunofluorescence analysis.

The scFv antibody binding to recombinant MopB was detected via the DVPDYAS peptide tag (HA tag). A secondary recombinant antibody was used where the anti-DVPDYAS peptide scFv was fused to alkaline phosphatase (anti-HA scFv-phoAM).

a. Western Blot

Purified recombinant truncated and full length mature MopB were blotted onto PVDF membrane using a Mini Trans-Blot Cell (Bio-Rad) as described in section 2.17.5. PVDF membranes were blocked in 5 % milk in TBS with 0.097 % NaN_3 at room temperature for 1 hour. The selected scFv for MopB after the first round ribosome display was used as the primary antibody (diluted 1/10 in 1 % milk/TBS 0.097 % NaN_3) incubated for 6 hours at 4 ºC, then incubated with secondary antibody, purified anti-HA-phoAM antibody alkaline phosphatase conjugate (diluted 1/10 in 1 % milk/TBS NaN_3) incubated at 4 ºC overnight and detected using BCIP/NBT substrate solution as described earlier in section 2.17.5.

b. ELISA

Ninety-six well Nunc-Immuno plates were coated with either full-length mature or truncated MopB as described earlier with modification using 5 % w/v skimmed milk powder in TBS containing 0.097 % NaN_3 instead of BSA as a negative control.

Purified antibodies, selected antibody for MopB, and negative control (2A10 monoclonal antibody for NPNA repeat malaria antigen), were diluted 1/10 using TBS/NaN_3 and incubated in the wells at 4 °C for 6 hours. The plate was washed with TBST for 5 times before adding the secondary antibody, purified anti-HA-phoAM antibody (1/10 diluted in 1 % milk/TBS NaN_3) at 4 °C overnight. The assay was developed with p-nitrophenyl phosphate (pNPP) as described in section 2.14.6. A threshold value of ≥ 0.1 was used as the cut-off value for detection of 4XfMopB3 HA scFv binding to mature full and truncated MopB protiens.

c. Immunofluorscence Analysis

The pellet from a 1 mL culture of heat-killed whole *X. fasitidosa* was re-suspended in 50 μL PBS and 10 μL of the spread on 3 glass slides, air dried and heat fixed by passing over a flame and blocked with 2 % w/v milk in PBS for 30 minutes. Purified scFv antibodies (selected antibody for MopB and 2A10 antibody as control) were diluted 1 in 10 with 1 % w/v skimmed milk powder in PBS. The slides were incubated with either the anti-MopB antibody, anti-NPNA antibody (negative control), or without scFv for 1 hour. After washing with PBS, anti-polyHis-fluorescein isothiocyanate (FITC) conjugate (1/10,000 dilution in 1 % milk/PBS) was added. The slides were incubated for 1 hour at ambient temperature in the dark. Slides were washed with PBS and viewed under oil immersion on an Axioscop 50 fluorescence microscope (Zeiss) equipped with appropriate filter set for the detection of FITC (excitation at 450-480nm and emission at 510–550nm). Images were captured using a charge-coupled-device (CCD) camera (PowerShot digital camera; Canon) and AxioVision software (Zeiss).

2.18 Engineering *Pantoea agglomerans* to Express Selected scFv Antibody

2.18.1 *Pantoea agglomerans* Electro-Competent Cells

The preparation of the competent cells was performed as described in section 2.6 with slight modifications. The *Pantoea agglomerans* cells were grown at 30 °C until the OD_{600} was 0.4 - 0.5. Cells were harvested by centrifugation for 5 minutes at

5000 *g* at 4 °C in chilled sterile falcon tubes. Cells were re-suspended 2 times (2 washes) in chilled MilliQ water and centrifuged for 10 minutes at 5000 *g* at 4 °C. The cells were then re-suspended in 10 % glycerol and centrifuged for 10 minutes at 4000 *g* at 4 °C. Cells were re-suspended again in 10 % glycerol (final volume 2 mL). The cells were stored in 80 μL aliquots at –80 °C.

2.18.2 Antibiogram

Susceptibility of *Pantoea agglomerans* was performed using a modified Kirby-Bauer disc diffusion method (Bauer *et al*., 1966). Briefly, 5 single colonies of *Pantoea agglomerans* from the LB agar plate were inoculated in 10 mL of fresh LB media grown at 37 ºC overnight. Next day, 100 μl of the overnight culture was spread onto agar plates using glass beads. The plate was allowed to dry for about 5 minutes.

Individual antibiotic disks (OXIOD antimicrobial susceptibility test discs: Ampicillin 10 μg, Tetracycline 50 μg, Neomycin 10 μg and Chloramphenicol 50 μg) were placed on the surface of the LB agar plate using flame-sterilized forceps and incubated for 16-18 hours at 37 ºC. Then the diameters of the inhibition zones were measured using a ruler.

2.18.3 Electro-Transformation

Eighty μL of electrocompetent cells were mixed with 7 μL DNA plasmid in a 1-mm gapped electrocuvette (Bio-Rad), and subjected to a 1.8 kV pulse via the pulse controller (25 μF capacitor, 200 Ω external resistance) (Gene Pulser Xcell™, Biorad). Pulsed cells were mixed with 250 μL SOC (20 mg/mL Bactotryptone, 5 mg/mL Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 0.4 % glucose, pH 7.0) and were incubated for 2 hours at 37 ºC with 200 rpm. Then the cells were plated to LB agar supplemented with the appropriate antibiotic.

2.18.4 pARM4 Plasmid

Pantoea agglomerans is not resistant to ampicillin, tetracylin or kanamycin. To express selected recombinant MopB single chain antibody (scFv) in *Pantoea agglomerans* a plasmid with a constitutive promoter (lac promoter), pelB signal peptide and an in-frame C-terminal 6xHis-tag was assembled.

a. pARM4 Plasmid Construction

The anti-MopB selected single chain antibody sequence (anti-MopB scFv) was digested with *Nco*I and *Not*I restriction enzymes and ligated into *Nco*I and *Not*I restiction sites of previously digested pSANG10-3F plasmid to make the pSANG-MopB scFv plasmid. The pSANG10-3F plasmid carries an in-frame *pelB* signal peptide sequence. The fragment including the *pelB* leader and the MopB scFv (Figure 2.5b) was ligated into the digested pBSKCA19.9 plasmid (Figure 2.5c) as a *Nde*I and *Not*I fragment and transformed into XL1-Blue competent cells. The plasmid was isolated and confirmed by DNA sequencing (section 2.7).

The plasmid pBlueScriptII mCit (pBSKmCit) contains a constitutive promoter (lac promoter) to drive the expression of monomer fluorescent protein citrine. *Pantoea agglomerans* transformed with this vector were observed as green/yellow fluorescent bacteria. The plasmid has suitable *Nde*I and *Hind*III insertion sites (Figure 2.5e) to accept the pelB signal peptide-MopB scFv-6xHis-tag encoding fragment (Figure 2.5d) resulting in pARM4 plasmid. This expression cassette starts from a constitutive promoter with a pelB signal peptide sequence-MopB scFv-6xHis-tag to engineer *Pantoea agglomerans* to express selected recombinant antibody. The sequence was confirmed by DNA sequencing (section 2.14.1).

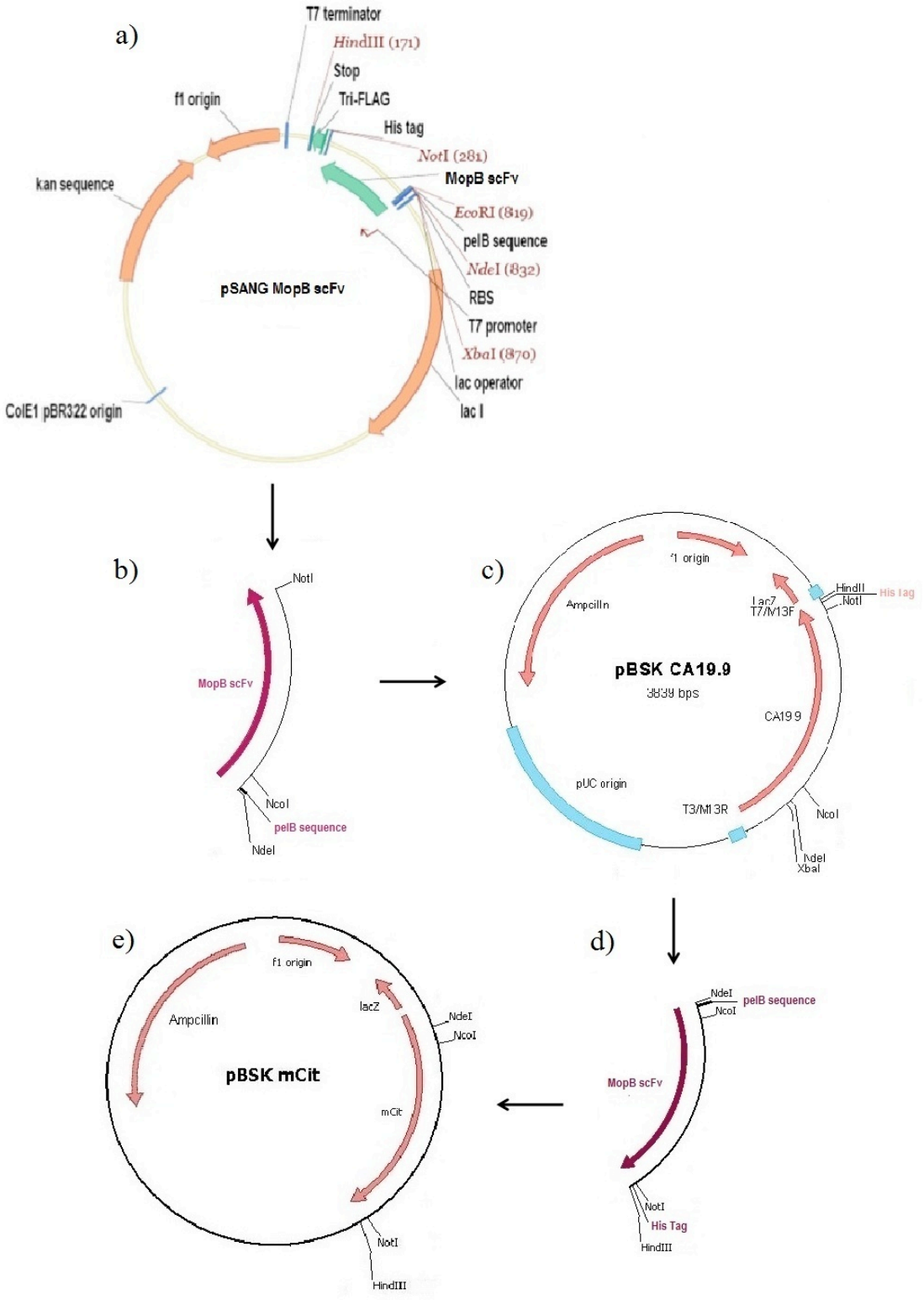


Figure 2.5 pARM4 plasmid construction. a) The MopB scFv was ligated into pSANG10-3F plasmid to construct pSANGMopBscFv plasmid. b) This plasmid was digested with *Nde*I/*Not*I to add *pelB* sequence to MopB scFv. c) This fragment was ligated into pBSKCA19.9 plasmid to construct pBSKCA19.9 *pelB* sequence- MopB scFv. d) This plasmid was digested with *Nde*I/*Hind*III to add 6xHis-tag to the *pelB* sequence-MopB scFv. e) The *pelB* sequence- MopB scFv-6xHis fragment was ligated into pBSKmCit plasmid to add a constitutive promoter to the *pelB* sequence- MopB scFv-6xHis fragment.

2.18.5 Anti-MopB scFv Expression and Detection in *Pantoea agglomerans*

a. Electro-Transformation

Pantoea agglomerans was electro-transformed with 7 µL pARM4 plasmid using Bio-RAD electroporation as described in section 2.18.3. A transformation without the plasmid was also performed. In parallel, XL1-Blue cells were transformed (section 2.7) with pARM4. The transformed cells were plated onto LB agar plates containing 100 µg/mL of carbenicillin and grown overnight at 37 °C.

Two colonies from each plate were used in colony PCR (section 2.8) and inoculated into 10 mL LB medium containing the same antibiotic and grown overnight at 37 °C. Purified plasmid from the XL1-Blue *E. coli* overnight culture was confirmed by sequencing (section 2.14.1).

b. Overnight anti-MopB scFv Expression and Purification

Five colonies from *Pantoea agglomerans* and XL1-Blue cells transformed with pARM4 plasmid (section 2.18.5) were inoculated in to 50 mL LB medium containing 100 µg/mL of carbenicillin and grown overnight at 37 °C. The cultures were allowed to reach the same OD_{600} absorbance of 1.3.

The expression was checked using the following steps; first 1 mL of the overnight cultures were centrifuged at 13,000 *g* for 1 minute and kept as a cell pellet sample. Next, to check the presence of the anti-MopB scFv in the media ethanol precipitation method was performed. Briefly, the supernatant of 1 mL of overnight culture was mixed with 9 mL 100 % ethanol and kept at -20 °C at least for 1 hour. The 10 mL mixture was centrifuged at 14,000 *g* for 15 minutes and the supernatant was discarded and tubes dried by inversion. The cell pellet was washed with 10 mL 90 % cold ethanol and centrifuged at 14,000 *g* for 5 minutes. The cell pellet was air dried and kept as precipitated protein in the media.

The rest of the overnight cultures were centrifuged at 4000 *g* for 10 minutes and the cell pellets were used in periplasm (osmotic shock) protein purification as described in *The QIAexpressionist*^{TM} *-- A handbook for high-level expression and purification of 6xHis-tagged proteins*, fifth edition with slight modification.

The periplasmic fractions were collected by re-suspending the cell pellet in 3 mL of buffer PA (30 mM Tris base, 1 mM Ethylenediaminetetraacetic acid (EDTA), 20 % sucrose pH 8.0) followed by 5 minutes incubation at 4 °C on ice. The bacterial cells were collected by centrifugation at 8000 *g* for 15 minutes at 4 °C; the supernatant (periplasmic fraction 1) was collected and stored on ice, while the cell pellet was re-suspended in 7 mL of 5 mM $MgCl_2$. After incubating for 10 minutes on ice, the suspension was centrifuged at 8000 *g* for 15 minutes at 4 °C. The supernatant (periplasmic fraction 2) was collected and pooled with periplasmic fraction 1 (20 µL of both periplasmic fractions were stored on ice for further analysis). All the samples and the remaining cell pellets for periplamic purification (for the non-secreted recombinant protein expression) were analysed by SDS-PAGE as described in section 2.13.

2.18.6 MopB scFv Protein Expression Analysis

a. SDS-PAGE

The SDS-PAGE was performed as described in section 2.13.

b. Western Blot

The Western blot was performed as described in section 2.17.5 using monoclonal anti- polyHistidine-alkaline phosphatase antibody (diluted 1/10,000 in 5 % Milk/TBS) to detect the MopB scFv expression in *Pantoea agglomerans*.

Chapter 3.0
Results

Chapter 3: Results

3.1 Calibration of Bespoke Protein Marker

In order to facilitate the detection and monitoring of protein size, especially in a Western blot probed by anti-His-tag antibody, His-tagged protein standards were prepared. Four known recombinant proteins [Thioredoxin TRX protein (20 kDa), RFP (30 kDa), MopB protein (42 kDa) and HSP 70 (75 kDa)] were fused to 6xHis-tag and produced in *E. coli*, followed by affinity purification (section 2.14.3a). These His-tagged proteins were mixed for use as the markers in SDS-PAGE and Western blots, as described in section 2.5.5. A comparison of this bespoke protein marker and commercial protein marker is shown in Figure 3.1.

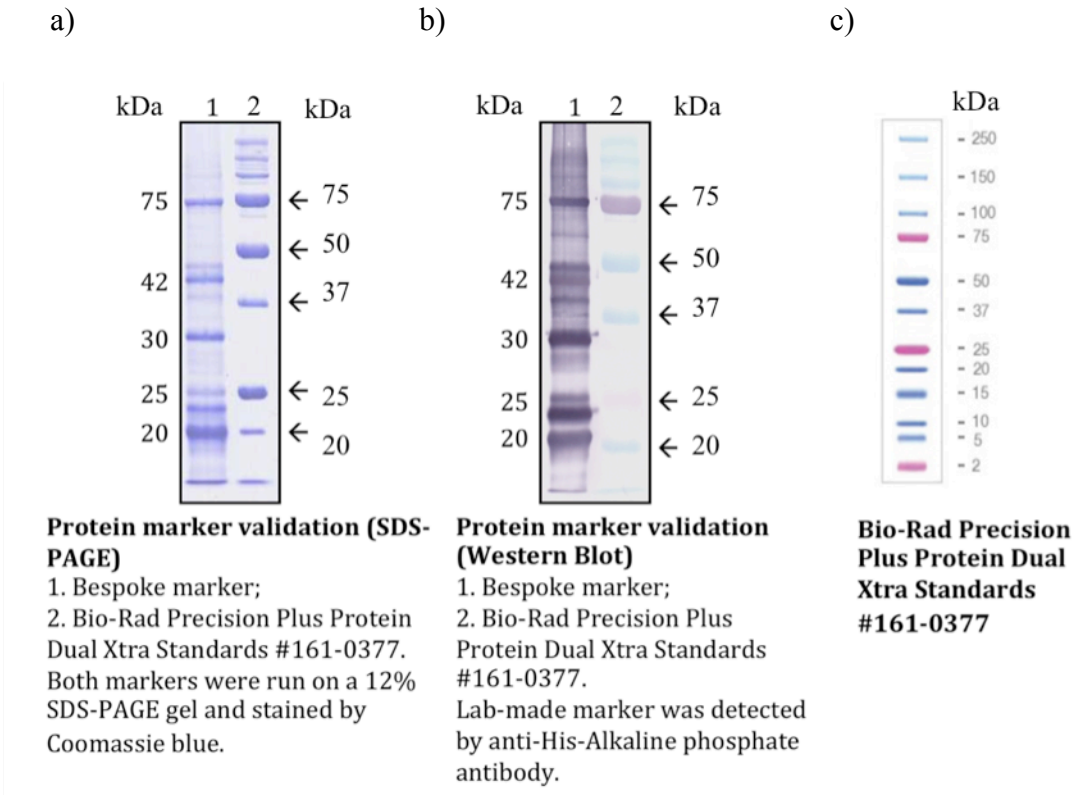


Figure 3.1 Comparison of bespoke protein marker with Bio-Rad standards. Bespoke protein marker (5 μL) and Bio-Rad Precision plus protein dual Xtra standards (10 μL) were run on a 12% SDS-PAGE gel. a) The size comparison on SDS-PAGE stained by Coomassie blue; b) The comparison on Western blot, detected by monoclonal anti-polyHistidine-alkaline phosphatase antibody produced in mouse; c) The Bio-Rad Precision plus protein dual Xtra standards.

Furthermore, a size calibration graph was also plotted for both SDS-PAGE gels and Western blots results to show the correlation between two sets of protein markers (the size calibration graphs are presented in appendix 1.1, page 141). The comparison results for the bespoke protein marker and the Bio-Rad Precision plus protein dual Xtra standards reveals a good correlation between the observed sizes according to the SDS-PAGE gels and its related size calibration plot. However the observed sizes obtained from the Western blots and its related size calibration plot were slightly different.

3.2 MopB Protein

The aim of this project was to develop anti-*Xylella* proteins. According to previous studies (Fjellbirkeland *et al*., 2000, Bruening, 2003, Bruening, 2005, Morano *et al*., 2008) and *X. fastidiosa* genomic information, an outer membrane protein (MopB) was chosen as a target to develop anti-*Xylella* MopB scFv antibodies.

3.2.1 MopB Bioinformatic Analysis

The *X. fastidiosa* Temecula 1 MopB protein sequence downloaded from NCBI data base (NCBI Reference Sequence: NP_779898.1) was analyzed using SIG-PRED to predict the putative signal sequences (section 2.14.1). Accordingly, Met1-Ala36 and/or possibly Met15-Ala36 were identified by the programme as putative signal sequences (Figure 3.2).

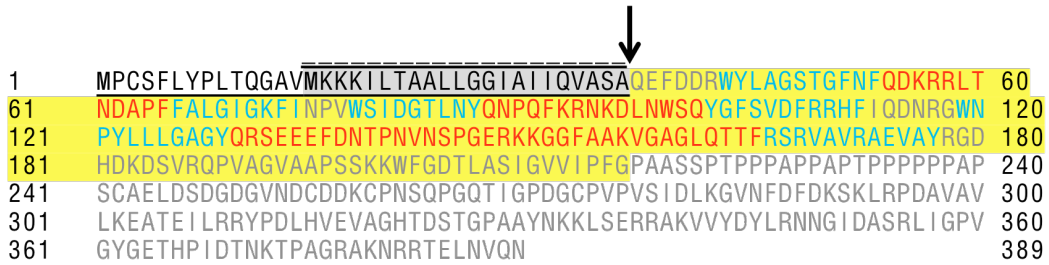


Figure 3.2 The amino acid sequence of *Xylella fastidiosa* Temecula 1 MopB and the predicted model for the β-barrel outer membrane domain. The putative leader sequence starting at Met1 is indicated with black underlining; a shorter potential leader sequence starting at Met15 (previously identified by (Bruening, 2002) is indicated with a black line over the sequence and gray highlighting.

The SIG-PRED and PRED TMBB programmes (section 2.14.1) were used to predict the signal peptide cleavage site (Figure 3.2). The arrow between Ala36 and Gln37 denotes signal peptide cleavage site, periplasmic regions (grey), membrane spanning regions (blue) and surface exposed loops (red). The yellow highlighted region corresponds to the truncated MopB (the surface exposed loops, which is the predicted antigenic part of the protein presented on the cell surface).

Sequence scored a value of 2.929, which is lower than the threshold value of 2.965. The difference between the value and the threshold indicates the possibility of the protein being an outer membrane protein. The well characterised *E. coli* OmpA sequence analysed using PRED TMBB scored 2.861.

The β-barrel trans-membrane domain identification using PRED TMBB (section 2.14.1) was predicted to be between Trp43-Tyr177 using three methods: Viterbi algorithm (Rabiner, 1989), N-best methods (Krogh, 1997) and the posterior decoding method. The Viterbi, N-best methods had an identical output in predicting the trans-membrane regions and exposed loops. The posterior decoding method differed slightly from the other two methods in its prediction of the final exposed loop amino acid 130-160 and a trans-membrane region 161-177 (as opposed to 130-164 and 165-177 predicted by the Viterbi and N-best methods). A two dimensional representation of the Viterbi method show the exposed loops with the last loop being the longest (Figure 3.3).

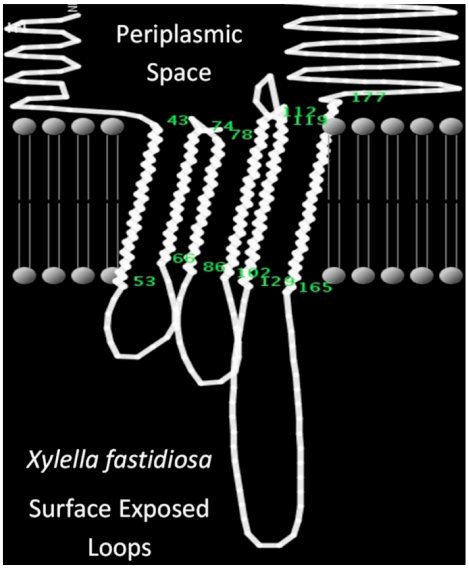


Figure 3.3 A two-dimensional (2D) figure of the predicted MopB transmembrane β-barrel with the protruding surface exposed loops generated using PRED TMBB. The amino acid numbers of the beginning and end of the β-strand traversing the outer membrane are in green.

3.2.2 Expression and Purification of the MopB Protein

The gene encoding full length mature Met-Ala36-Asn389 (1084 bp) and a truncated (564 bp) Met-Ala36-Gly252 MopB (the surface exposed portion of MopB) from *X. fastidiosa MopB* were PCR amplified from pMALc2x_*mopB* plasmid as described in section 2.14.1 (Figure 3.4).

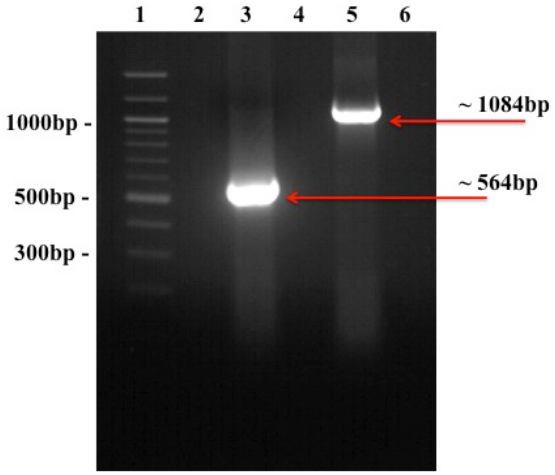


Figure 3.4 Gel electrophoresis for MopB PCR products using pMALc2x_*mopB* as a template. Lane 1, 100 bp DNA ladder; Lane 2, blank; Lane 3, Truncated MopB PCR product with expected size of ~564 bp; Lane 4, blank; Lane 5, full-length MopB PCR product with expected size of ~1084 bp; Lane 6, blank.

The amplified PCR products were cloned into the pSANG10-3F vector and were used to transform XL-Blue *E. coli* competent cells. The purified plasmids (Figure 3.5a and b) were verified by DNA sequencing (Appendix 1.2, pages 142-143).

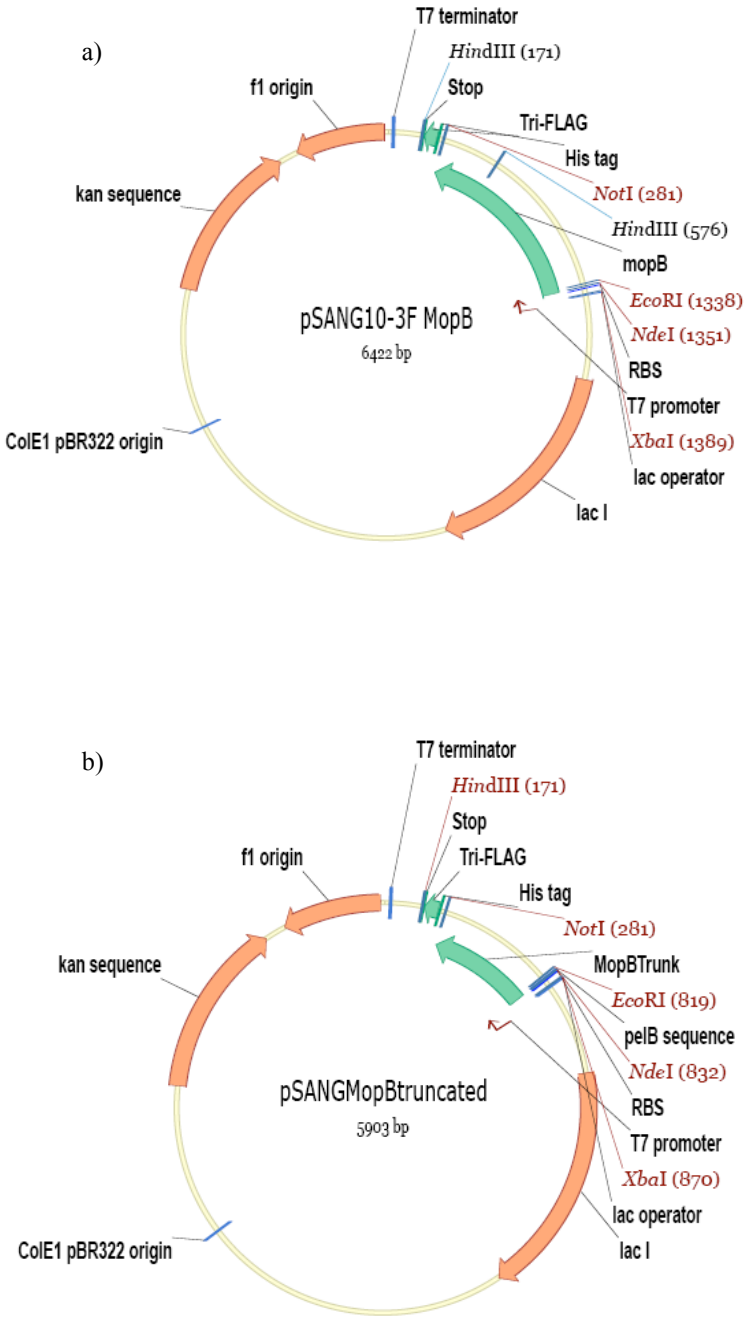


Figure 3.5 Plasmid map of *MopB* contructs in pSANG10-3F vector. a) Full length mature *MopB*; b) Truncated *MopB*.

The plasmids were transformed into BL21(DE3) *E. coli* strain to express the cloned MopB proteins. Proteins analysis by SDS–PAGE revealed abundant full length mature and truncated MopB at 3 hours at 37 °C post induction with IPTG (section 2.14.2).

The recombinant full length mature and truncated MopB were expressed in pSANG10-3F vector as fusion with hexa-histidine followed by the Tri-FLAG tag.

Expressed recombinant mature MopB was predicted to have a theoretical iso-electric point (pI) (Gasteiger *et al*., 2003) of 6.36 and a molecular weight (Mw) of 42.98 kDa. Expressed truncated form of recombinant MopB, the exposed loops, which is the predicted antigenic part of the protein presented on the cell surface, was predicted to have a pI of 6.79 and Mw of 24.64 kDa.

The SDS–PAGE results indicated proteins with an apparent molecular weight of about 43 kDa for full length mature MopB and 25 kDa for truncated MopB (Figure 3.6a and b). Using the method described in section 2.14.3a the recoveries of recombinant mature and truncated MopB proteins using denaturing conditions were estimated to be approximately 50 mg/mL of culture.

Native non-denaturing conditions during protein purification (section 2.14.3b) failed to recover either of the recombinant proteins in the soluble fraction (Figure 3.6 c and d), with the expressed proteins being retained in the cell pellets.

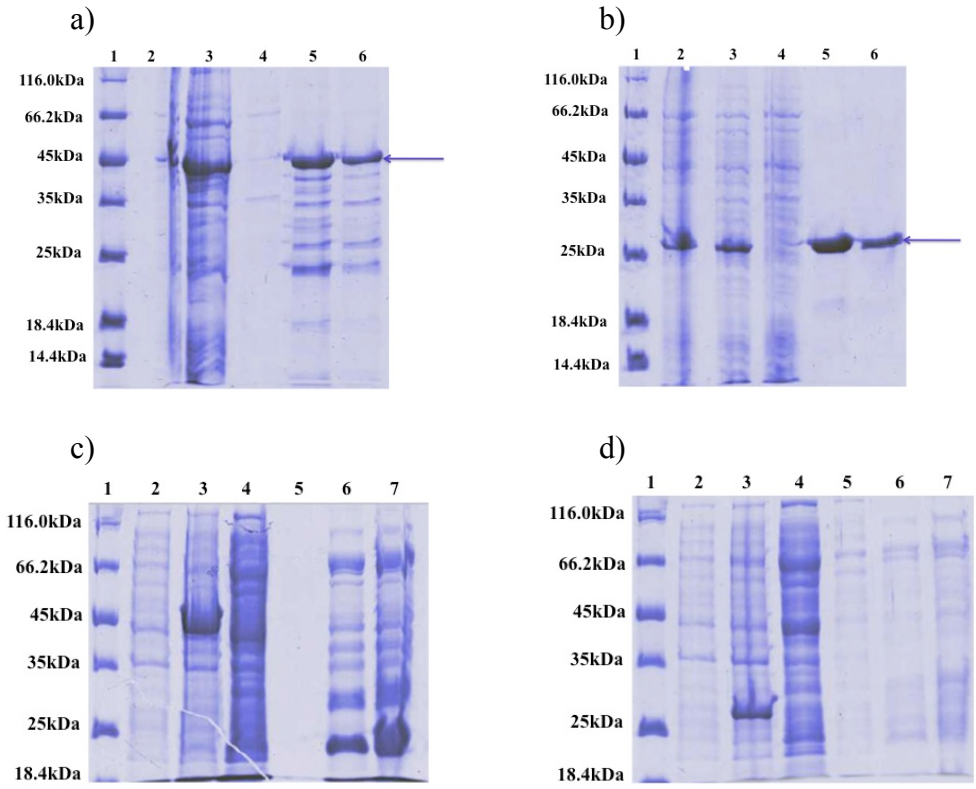


Figure 3.6 SDS-PAGE analysis of recombinant MopB proteins. a) Denaturing conditions full-length mature MopB expression and purification. Lane 1, EZ- RUN protein molecular weight marker; Lane 2, non induced; Lane 3, induced; Lane 4, final wash; Lanes 5-6, elutions 2-3. The expected size of full length mature MopB protein (42.98 kDa) is indicated by arrow. b) Denaturing conditions truncated MopB expression and purification. Lane 1, EZ- RUN protein molecular weight marker; Lane 2, induced; Lane 3, flow through; Lane 4, final wash; Lanes 5-6, elutions 2-3. The expected size of truncated MopB protein (24.64 kDa) is indicated by arrow. c) Native conditions full length mature MopB expression and purification. Lane 1, EZ- RUN protein molecular weight marker; Lane 2, non induced; Lane 3, induced; Lane 4, soluble fraction; Lane 5, flow through; Lanes 6 and 7 elutions 2 and 3. d) Native conditions truncated MopB expression and purification. Lane 1, EZ- RUN protein molecular weight marker; Lane 2, non induced; Lane 3, induced; Lane 4, soluble fraction; Lane 5, flow through; Lanes 6 and 7 elutions 2 and 3.

3.3 Mouse Immunization

Whole heat-killed *X. fastidiosa* (section 2.14.5) was used to immunize 5 Balb/c mice at ProSci (Poway, CA USA). Initial immunization was followed by boosting at week 4, 8 and 12. Two weeks after boosting the test bleeds were taken and used in Dot blot analysis. The mice were boosted again and underwent a splenectomy after three days. Each mouse spleen was placed immediately in 10 mL of TRIZOL for RNA isolation (section 2.14.5).

3.3.1 Bacterial Dot Blot

The immune sera from bleed 3 (section 2.14.6) were evaluated in a Dot blot assay against milk protein, whole intact *E. coli* and *X. fastidiosa* Temecula 1 strain (Figure 3.7). Mouse sera 1, 2, 4 and 5 had clear antibody binding to *X. fastidiosa* at a dilution of 1/20,000 whereas mouse 3 sera did not. The antibody activity against *E. coli* was not observed and the activity against milk proteins was absent or very weak in mouse sera 2 and 4.

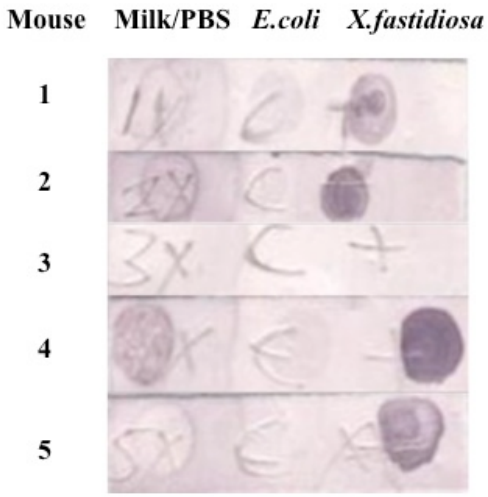


Figure 3.7 Mouse immune sera evaluation by immuno-dot blot. Immuno-dot blot screen with 1/20,000 dilution of bleed 3 mouse 1-5 sera on 5 % milk protein control, or 5 μL of *E. coli* or *X. fastidiosa* spotted directly onto nitrocellulose strips and probed with rabbit anti-mouse alkaline phosphatase and developed with NBT/BCIP.

3.3.2 Recombinant MopB ELISA

An ELISA assay was performed using recombinant truncated MopB protein and immune sera (bleed 3) from mice immunized with whole heat-killed *X. fastidious* (section 2.14.7). The ELISA results indicated that 4 out of 5 mice produced antibodies that recognise the recombinant truncated MopB protein with little or no cross-reactivity with the control protein BSA (Figure 3.8). The study was carried out with immune mouse sera dilutions of 1/20,000 and a threshold value of ≥ 0.1 was used as the cut-off value for detection of mouse sera 1-5 binding to the recombinant truncated MopB protein.

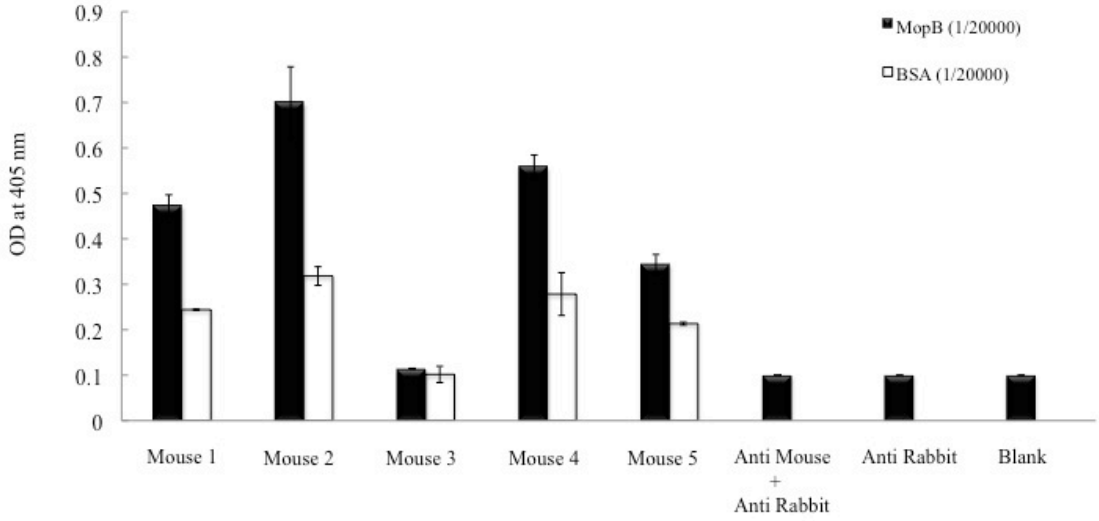


Figure 3.8 Mouse immune sera evaluation by ELISA. Mouse 1-5 sera from bleed 3 diluted 1/20,000 in the ELISA on truncated MopB (10 μg/mL) and BSA (10 μg/mL), blank (all the buffers without the antigen), rabbit anti-mouse (1/20,000) + anti-rabbit alkaline phosphatase 1/40,000) and anti-rabbit alkaline phosphatase (1/40,000) without anti-mouse. A threshold value of ≥ 0.1 was used as the cut-off value for detection of mouse sera 1-5 binding to the recombinant truncated MopB protein.

3.4 Mouse Immunoglobulin Library Assembly

The mice spleens (n=5) previously immunized with whole heat-killed *X. fastidiosa* kept in TRIZOL were used to construct five individual mouse single chain antibody libraries (section 2.15) in the form of VL-Link-VH.

Total RNA was isolated from each mouse spleen (n=5) and were used as the template to generate five required cDNA synthesis followed by PCR amplification of individual variable light chain (VL) and heavy chain (VH) using specific designed primers (Table 2.1).

3.4.1 RNA Extraction and cDNA Synthesis

The RNA isolation followed the instructions provided with the TRIZOL reagent and the final volume was 50 μL (section 2.15.1). The concentration and purity of RNA recovered from the mouse spleens (n=5) previously immunized with whole heat-killed *X. fastidiosa* is presented in Table 3.1.

Table 3.1 Total RNA extraction concentrations and $A_{260/280}$ and $A_{260/230}$ ratios obtained from 5 mice spleens previously immunized with whole heat-killed *X. fastidiosa*.

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

The individual (n=5) total RNA samples obtained from five immunized mice spleens provided ample material for the subsequent five cDNA reactions (section 2.15.2). The Oligo-dT29VN primer was used to synthesis cDNA for light chains and MVHlink2 primer for heavy chains (all primers are listed in Table 2.1).

3.4.2 Construction of Single-Chain Antibody Libraries

Five immunoglobulin libraries were assembled individually using their corresponding cDNAs as outlined in the schematic (Figure 2.1) section 2.15.3.

Oligo-dT29VN and MVHlink2 generated cDNAs were used as template with antibody family-specific primers to amplify individual PCR products of 7 variable light chains fragments (VL) and 10 variable heavy chain fragments (VH).

The PCR amplification results and steps of the library construction performed using mouse 4 spleen derived cDNA are shown in figures 3.9 and 3.10. All 5 mice immunoglobulin libraries were assembled using the same procedure.

Figure 3.9a and b present individual PCR products of seven light chain fragments (VL) and 10 heavy chain fragments (VH) amplified from mouse spleen 4. DNA bands with expected size of ~350 bp for VL and ~400 bp for VH (indicated by arrow) were excised and recovered.

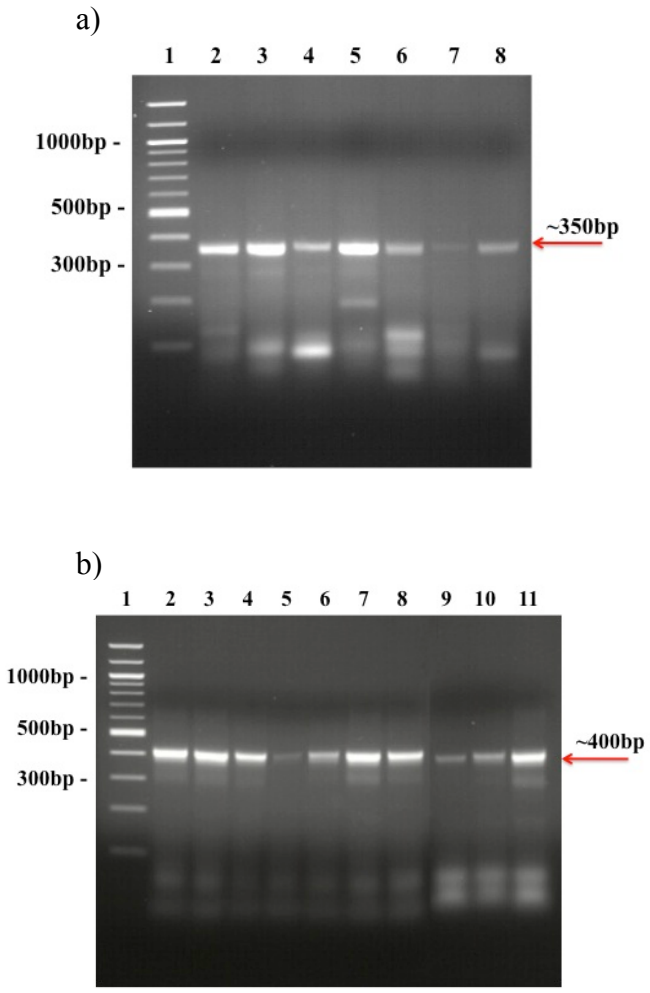


Figure 3.9 PCR products of 7 light chains and 10 heavy chains. a) PCR product of 7 light chains. Lane 1, 100 bp ladder; Lane 2, Vκ1; Lane 3, Vκ2; Lane 4, Vκ3; Lane 5, Vκ4; Lane 6, Vκ5; Lane 7, Vκ6; Lane 8, Vκ7. The expected size of products (~350 bp) is indicated by arrow. b) PCR product of 10 heavy chains. Lane 1, 100 bp ladder; Lane 2, VH1; Lane 3, VH2; Lane 4, VH3; Lane 5, VH4; Lane 6, VH5; Lane 7, VH6; Lane 8, VH7; Lane 9, VH8; Lane 10, VH9; Lane 11, VH10. The expected size of VH products (~400 bp) is indicated by arrow.

In all five mice library constructions, mouse Vκ6, VH4 and 8 were amplified at lower levels; all other families were readily amplified. This amplification pattern for mouse variable light chains and heavy chains was likely due to the primer design.

Variable light chains and heavy chains were assembled by adding linkers to both purified mixtures of VL and VH families separately generating a ~410 bp PCR product (Figure 3.10a); Then the VL-Link and VH-Link were assembled in the form of VL-Link-VH PCR product of ~750 bp (Figure 3.10b).

The Mouse Kappa Constant (MKC) was amplified using pUC57NPNAMKC plasmid (GenScript) as template, and MKNotCF and MKRev primers (Table 2.1). The expected size for MKC was ~310 bp (Figure 3.10c).

The MKC was joined to the VL-Link-VH combinations by a PCR overlap extension reaction using MVKFlink and MKRev with expected size of ~1100 bp indicated by the arrow (Figure 3.10d).

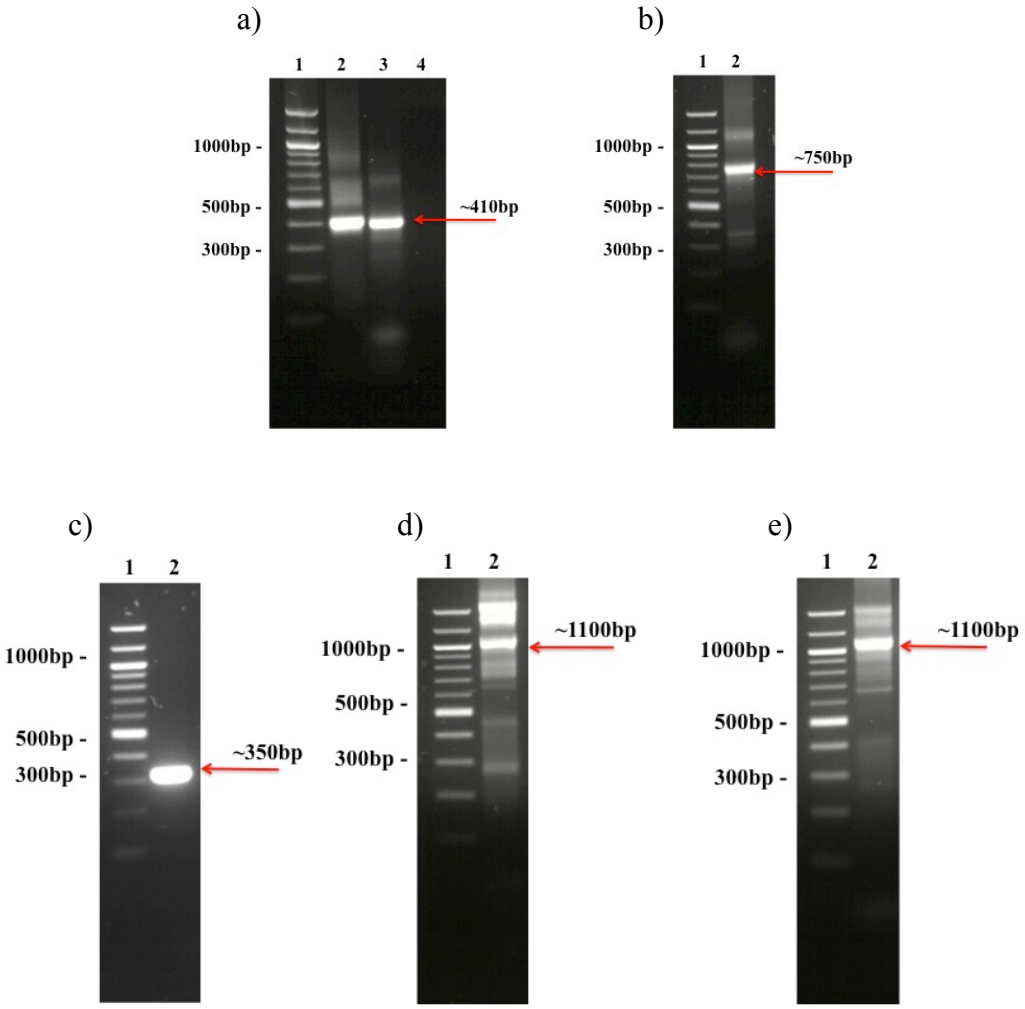


Figure 3.10 PCR products of mouse library construction. a) VL-Link & VH-Link, Lane 1, 100 bp ladder; Lane 2, VL-Link; Lane 3, VH-Link. The expected size of VL-Link and VH-Link products (~410 bp) is indicated by arrow. b) VL-Link-VH. Lane 1, 100 bp ladder; Lane 2, VL-Link-VH. The expected size of VL-Link-VH products (~750 bp) is indicated by arrow. c) Mouse Kappa Constant (MKC). Lane 1, 100 bp ladder; Lane 2, MKC 3.The expected size of MKC products (~350 bp) is indicated by arrow. d) VL-Link-VH-MKC. Lane 1, 100 bp ladder; Lane 2, VL-Link-VH-MKC; The expected size of VL-Link and VH-Link products (~1100 bp) is indicated by arrow. e) T7-VL-Link-VH-MKC. Lane 1, 100 bp ladder; Lane 2, T7-VL-Link-VH-MKC. The expected size of T7-VL-Link-VH-MKC products (~1100 bp) is indicated by arrow.

Finally, the T7 site was added to the PCR product encoding all the variable light chains, variable heavy chains and MKC combinations using primers RDT7 and MKRev to produce the DNA encoding the anti-*X. fastidiosa* immunoglobulin scFv libraries with expected size of ~1100 bp indicated by arrow (Figure 3.10e) (all primers are listed in Table 2.1).

The final DNA template encoding the antibody library flanked by a T7 site and a synthetic mouse kappa constant (MKC) chain was used in an *in vitro* ribosome display (Figure 3.10e).

3.5 *In vitro* Ribosome Display and Antibody Selection

Monoclonal antibodies were traditionally produced using rodent immunization followed by hybridoma screening. Recently to generate high affinity, specific and stable monoclonal antibodies, display technologies are used (Kohler and Milstein, 1975, He and Khan, 2005).

In this project, specific antibodies were selected against recombinant MopB protein by the use of a powerful display technology, *in vitro* ribosome display (section 2.16).

Five mice antibody libraries were assembled and extended at the 5' with T7 site and in the 3' end with a spacer (MKC) (Figure 3.10e) and were used individually in an *in vitro* ribosome display as described in section 2.16.

The full-length antibody library (T7-VL-Link-VH-MKC) PCR products (about 1100 bp) were added into TNT coupled reticulocyte lysate and the mixture were incubated at 30 °C for 90 minutes to generate (antibody-ribosome-mRNA) ARM complexes as described in section 1.10. The translation mixture was then added into wells coated with 10 μg/mL of recombinant truncated and full length MopB antigen for an additional one hour. After washing with 1X PBS, the bound ARM complexes on wells were directly subjected to one step® RT-PCR using MVKFlink and MKRD2 primers as described in section 2.16 (All primers are listed in Table 2.1) to recover

the selected genetic information. Recovered PCR products of ~900 bp was analysed by agarose gel electrophoresis (Figure 3.11).

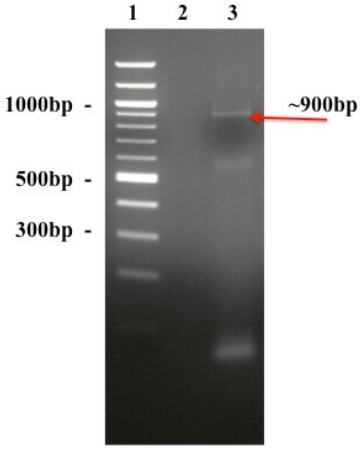


Figure 3.11 PCR products of first round of ribosome display. The encoding antibody DNA after the first round of ribosome display was recovered using MVKFlink and MKRD2 primers. Lane 1, 100 bp ladder; Lane 2, PCR product after first round of ribosome display against BSA (negative control); Lane 3, PCR product after first round of ribosome display against truncated MopB. The expected size of the recovered encoding antibody PCR product was ~900 bp (shown arrowed)

To select antibodies against recombinant MopB, one round of ribosome display was initially performed using the mouse libraries (n=5) individually. The affinity enriched mRNA was amplified by RT-PCR, TOPO cloned and transformed into XL1-Blue *E. coli* to screen for blue/white colonies on an X-gal plate.

In this chapter, only results obtained from mouse 4 antibody library are presented. From approximately one hundred white colonies on the X-gal plate after the first round of *in vitro* ribosome display using mouse 4 antibody library, four colonies were randomly selected and used to prepare plasmids for sequencing.

The four recovered antibody-encoding DNA sequences (4XfMopB1-4) provided from 4 random colonies are presented in appendix 1.3 (pages 144-147) [The nomenclature for numbering the scFv is as follows: mouse number (#1-5), bacterial strain antigen (*X. fastidiosa*), bait (MopB) and clone in order of isolation i.e.,]. DNA sequencing analysis results revealed that from 4 recovered encoding antibody sequences, three antibodies sequences were confirmed intact in-frame (4XfMopB1, 4XfMopB2 and 4XfMopB3). The 4XfMopB4 scFv antibody sequence showed a stop codon (tag) in the variable heavy chain.

The selected antibodies sequences (4XfMopB1, 4XfMopB2, 4XfMopB3 and 4XfMopB4) were analysed at the IMGT website (Giudicelli *et al*., 2004). The variable region sequence analysis (Table 3.2) shows that different antibody gene segment sequences are present in the selected recombinant antibodies clones. The sequences were aligned with the online database and all of the Vκ and VH domains showed 99-100 % similarities to published mouse VL and VH domains(Altschul *et al*., 1990).

Table 3.2 Junction analysis and alignment of the VL and VH CDR3 sequences of the selected anti-MopB scFv's (4XfMopB1-4). The sequences of the scFv's were analysed using IMGT®, the international ImMunoGeneTics information system®. (Lefranc *et al*., 2009, Yousfi Monod *et al*., 2004).

ScFv	Mouse IGVK	Mouse IGVKJ	VL CDR3	Mouse IGVH	Mouse IGVHJ	Mouse IGVHD	VH CDR3
4XfMopB1	17-127*01F	2*01F	LQSDNMPYT	2-9-1*01F	4*01F	1-1*01F	ARDQVYGSSYYAMDY
4XfMopB2	12-46*01F	5*01F	LQHGESPLT	5-6-5*01F	1*01F	-	ARYYYGTSFYWYFDV
4XfMopB3	12-44*01F	1*01F	QHHYGTPWT	14-3*02F	2*02F	-	AFPHY
4XfMopB4	2-59*01F	5*01F	QQRSSYPLT	1-82*01F	4*01F	2-1*01F	ARSRDGNYGYYYAMDY

3.6 MopB scFv Expression and Purification

3.6.1 Analysis of MopB Antibody Expression and Solubility in *E. coli*

The confirmed 4XfMopB1, 4XfMopB2 and 4XfMopB3 intact in-frame VL and VH combinations were sub-cloned into pAHAHis expression vector (incorporating a C-terminal peptide sequence HA tag (DVPDYAS) followed by a hexa-histidine-tag; this allowed for his-tagged antibodies to be purified for cytoplasmic protein expression in Rosetta gami B(DE3) *E. coli* and subsequent characterization) (section 2.17).

Rosetta gami B(DE3) strains were used due to their ability to enhance the disulfide bond formation and improve eukaryotic proteins expression in *E. coli* by carrying a plasmid for codons rarely used in *E. coli*. DNA sequencing results for selected MopB

antibodies (4XfMopB1-3) in pAHAHis expression vector are presented in appendix 1.4 (page 148).

To determine whether or not the antibodies were expressed in the Rosetta gami B(DE3) *E. coli* strain, the expressed antibody fragments were first examined by purifying the scFvs by Ni^{2+}-NTA column from total bacterial extracts under denaturing conditions with 8 M Urea as described in section 2.17.3a. The His-tagged scFv proteins were eluted from Ni^{2+}-NTA columns by decreasing the pH from 8.0 to 4.5. The eluted fractions were analysed by SDS-PAGE (Figure 3.12a). The SDS-PAGE analysis revealed that dominant proteins with expected scFv size were isolated. To determine whether the antibodies were soluble and correctly folded, the expressed antibody fragments were purified from the total cell extract under native conditions as described in section 2.17.3b. Under native conditions, 8 M Urea was replaced by 500 mM NaCl and the pH was maintained at 8.0 in all buffers. The scFvs were eluted from the Ni^{2+}-NTA column by increasing the imidazole concentration (Figure 3.12b).

Analysis of the recombinant antibodies by SDS-PAGE show that they were successfully expressed and purified using Rosetta gami B(DE3) *E. coli.* Figure 3.12 shows the SDS-PAGE gel for 4XfMopB3 scFv antibody (the SDS-PAGE gels for 4XfMopB1 and 2 are not presented). Comparing the purified antibodies expected size (~27 kDa) upon IPTG induction with the non-induced (lane 2) and induced (lane 3) protein samples, revels the correct expected protein size. The scFv recovery under the denaturing condition shows large quantity of eluted proteins (Figure 3.12a; Lane 7, 8, 9 and 10). However small amount of the scFv was eluted under the native condition (Figure 3.12b), which indicates that although recombinant scFv was expressed in a large quantity, but only a small fraction was soluble. The native condition purification also revels a number of contaminating co-purifying proteins, detected in the eluted samples (Figure 3.12b), which may reflect the inefficiency of immobilised metal ion affinity chromatography (IMAC) purification under the native condition.

The presence of the successful selected scFv antibodies expression was confirmed by Western blot using soluble scFv proteins fractions and monoclonal anti-polyHistidine-alkaline phosphatase antibody. A ~27 kDa protein of the expected size

of the expressed anti-MopB-scFv protein was observed by Western blotting (lane 3) and eluted fractions (lane 7-10) from total cell extract purification under the native condition. Figure 3.13 shows the Western blot result for anti-MopB scFv antibody 4XfMopB3; Western blot results for 4XfMopB1 and 2 are not shown.

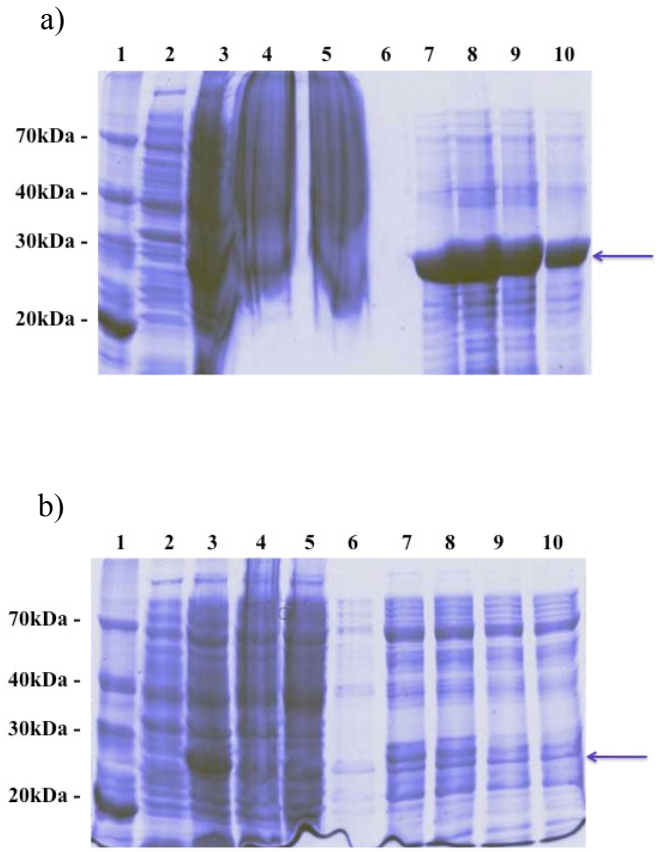


Figure 3.12 SDS-PAGE analysis of anti-MopB scFv antibody (4XfMopB3). a) MopB scFv antibody expression and purification under denaturing conditions. Lane 1, Bespoke protein marker; Lane 2, non induced; Lane 3, induced; Lane 4, soluble fraction, Lane 5, flow through; Lane 6, final wash; Lane 7-10, elutions 1-4. b) MopB scFv antibody expression and purification under native conditions. Lane 1, Bespoke protein marker; Lane 2, non induced; Lane 3, induced; Lane 4, soluble fraction; Lane 5, flow through; Lanes 6, final wash; Lane 7-10 elutions 1-4. Immunoreactive bands of approximately 27 kDa are indicated by the arrow.

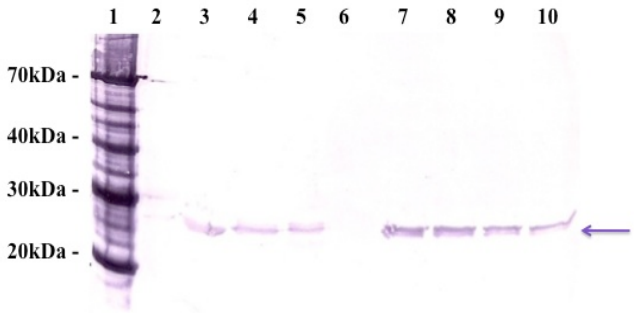


Figure 3.13 Western blot analysis of anti-MopB scFv antibody (4XfMopB3) expression and purification (Native conditions). Lane 1, Bespoke protein marker, Lane 2, non induced, Lane 3, induced, Lane 4, soluble fraction, Lane 5, flow through, Lanes 6, final wash; Lane 7-10 elutions 1-4. Immunoreactive bands of approximately 27 kDa are indicated by the arrow.

3.6.2 MopB scFv Functional Assays

To examine antibody specificity and binding, the selected anti-MopB scFv antibodies (4XfMopB1-3) expressed in pAHAHis vector were tested first by Western blot. After confirming the binding to the recombinant MopB proteins on the Western blot the candidate scFvs were used in ELISA and Immunofluorescence assays against recombinant full length mature, truncated MopB and heat-killed *X. fastidiosa.*

Binding of the scFv antibodies to recombinant MopB were detected via a peptide sequence derived from influenza hemagglutinin, HA tag (section 2.17.1). A secondary recombinant antibody was used where the anti-HA peptide scFv was fused to alkaline phosphatase (anti-HA scFv-phoAM) as described in section 2.17.1b. To confirm the MopB scFv binding, a non-specific antibody for MopB protein 2A10 scFv (2A10 scFv antibody for NPNA malaria antigen) was expressed and purified as negative control (section 2.17.1a).

The scFvs were expressed using pAHAHis plasmid and purified under native conditions (section 2.17.4). Analysis of the purified anti-HA-phoAM and 2A10 purified scFv antibodies by SDS-PAGE are presented in Figures 3.14a and b for anti-HA-phoAM and 3.15a-c for 2A10 scFv.

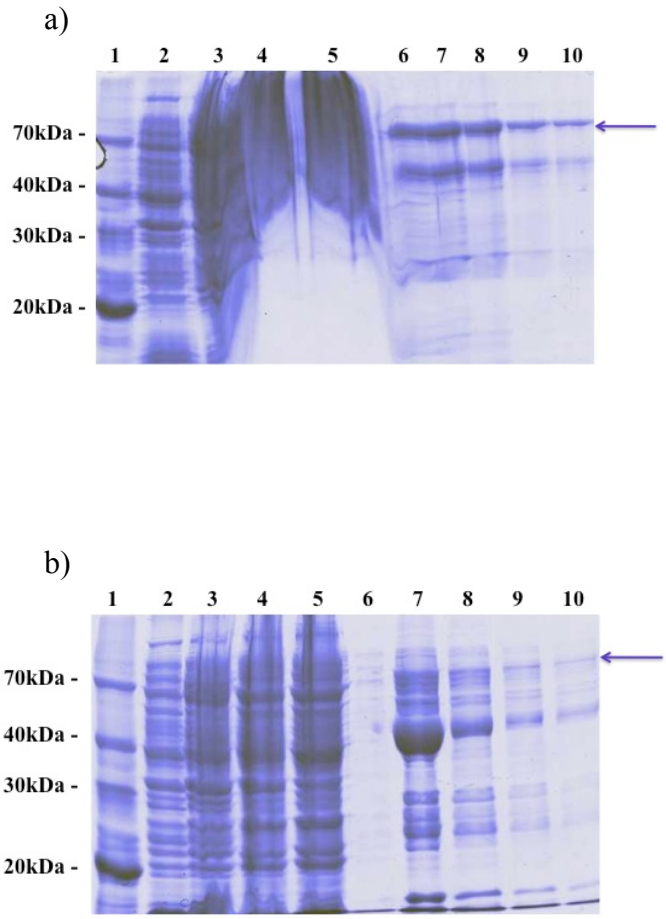


Figure 3.14 SDS-PAGE analysis of anti-HA-phoAM scFv antibody expression and purification using pAHAHis plasmid. a) Denaturing conditions. Lane 1, Bespoke protein marker; Lane 2, non induced; Lane 3, induced; Lane 4, soluble fraction, Lane 5, flow through; Lane 6, final wash; Lane 7-10, elutions 1-4. b) Native conditions. Lane 1, Bespoke protein marker, Lane 2, non induced, Lane 3, induced, Lane 4, soluble fraction, Lane 5, flow through, Lanes 6, final wash; Lane 7-10 elutions 1-4. Immunoreactive bands of approximately 75 kDa are indicated by the arrow.

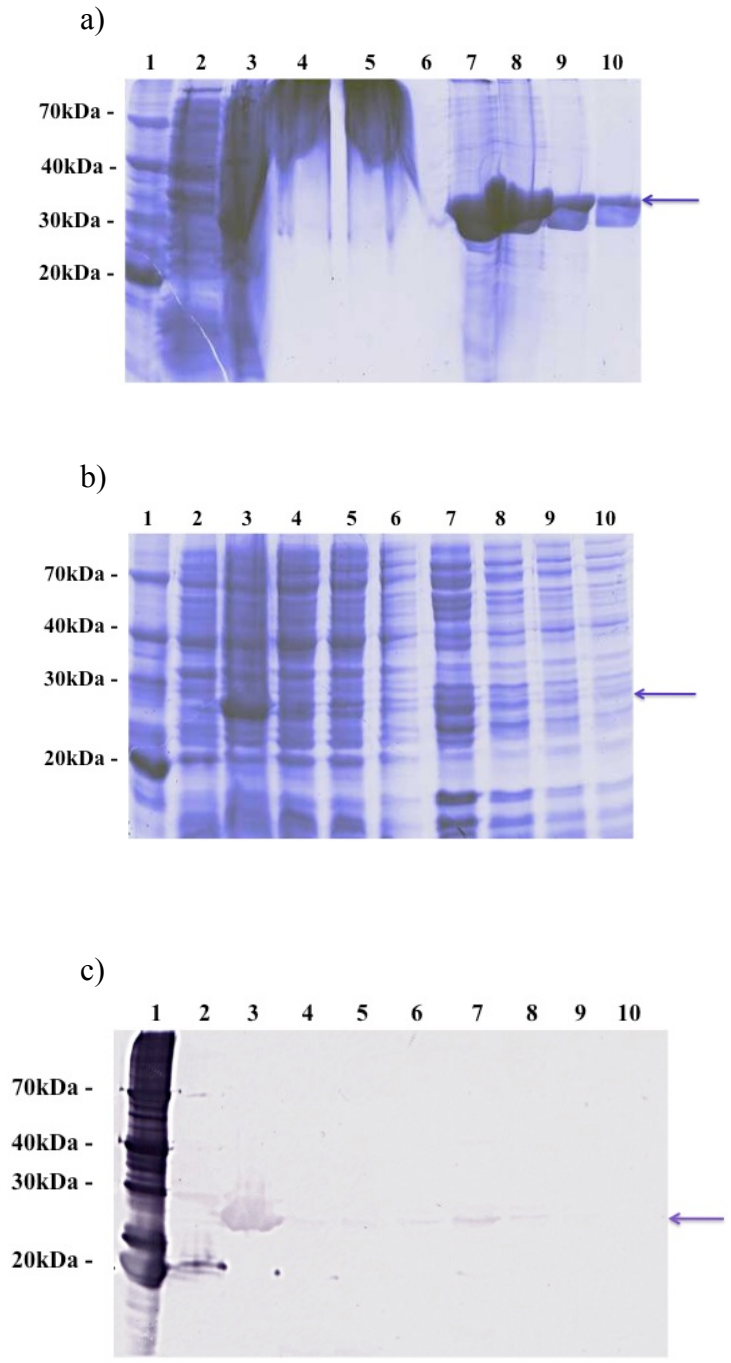


Figure 3.15 SDS-PAGE and Western blot analysis of 2A10 scFv antibody expression and purification using pAHAHis plasmid. a) Denaturing conditions. Lane 1, Bespoke protein marker; Lane 2, non induced; Lane 3, induced; Lane 4, soluble fraction, Lane 5, flow through; Lane 6, final wash; Lane 7-10, elutions 1-4. b) Native conditions. Lane 1, Bespoke protein marker, Lane 2, non induced, Lane 3, induced, Lane 4, soluble fraction, Lane 5, flow through, Lanes 6, final wash; Lane 7-10 elutions 1-4. c) Western blot analysis (Native conditions). Lane 1, Bespoke protein marker, Lane 2, non induced, Lane 3, induced, Lane 4, soluble fraction, Lane 5, flow through, Lanes 6, final wash; Lane 7-10 elutions 1-4. Immunoreactive bands of approximately 27 kDa are indicated by the arrow.

a. Western Blot

Western blot analysis was performed to examine anti-MopB scFv antibodies (4XfMopB1-3) binding to the recombinant MopB proteins. Recombinant truncated and full length mature MopB proteins were transferred to PVDF membrane and purified diluted (1/10) anti-MopB scFv antibody elutions (primary antibody) were added to the membrane and incubated. To identify the antibody binding to the MopB proteins, the purified HA-phoAM scFv conjugate with alkaline phosphatase was added to the membrane (secondary antibody) and incubated. The HA-phoAM scFv detects the HA peptide fused to the purified anti-MopB scFv antibody.

Western blot results showed that anti-MopB scFv 4XfMopB1 (result not shown) and 4XfMopB3 detected recombinant full length mature and truncated MopB. Results for 4XfMopB3 are shown in Figure 3.16.

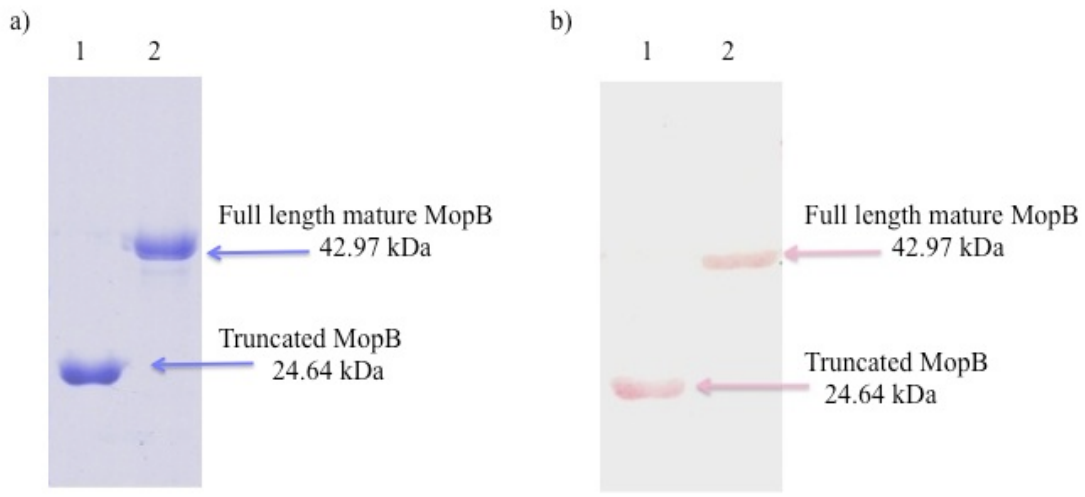


Figure 3.16 Functional Analysis of 4XfMopB3 HA scFv by Western blot. a) SDS-PAGE analysis of the mature full-length and truncated MopB proteins. Lane 1, truncated MopB; Lane 2, full-length mature MopB. b) Western blot using 4XfMopB3 HA scFv and HA-PhoAM scFv to detect full-length mature and truncated MopB on PVDF membrane. Lane 1, truncated MopB; Lane 2, full-length mature MopB.

b. ELISA

To examine the antibody specificity and binding, the selected antibodies 4XfMopB1 and 4XfMopB3 expressed in pAHAHis vector were tested in ELISA against recombinant full length mature and truncated MopB. The ELISA was performed on a

96-well Nunc plate, with milk and 2A10 scFv were used as the negative controls. The purified bacterially expressed anti-MopB antibody (diluted 1/10 elutions) was incubated in each well. The secondary antibody, the purified HA-phoAM scFv conjugate with alkaline phosphatase (diluted 1/10 elutions), was added and incubated. A threshold value of ≥ 0.1 was used as the cut-off value for detection of 4XfMopB3 scFv antibody binding to mature full and truncated MopB proreins. After detection, the anti-MopB (4XfMopB3) scFv antibody showed binding activity to recombinant full length mature and truncated MopB in ELISA. Figure 3.17 shows the ELISA result for anti-MopB scFv 4XfMopB3 and recombinant MopB proteins.

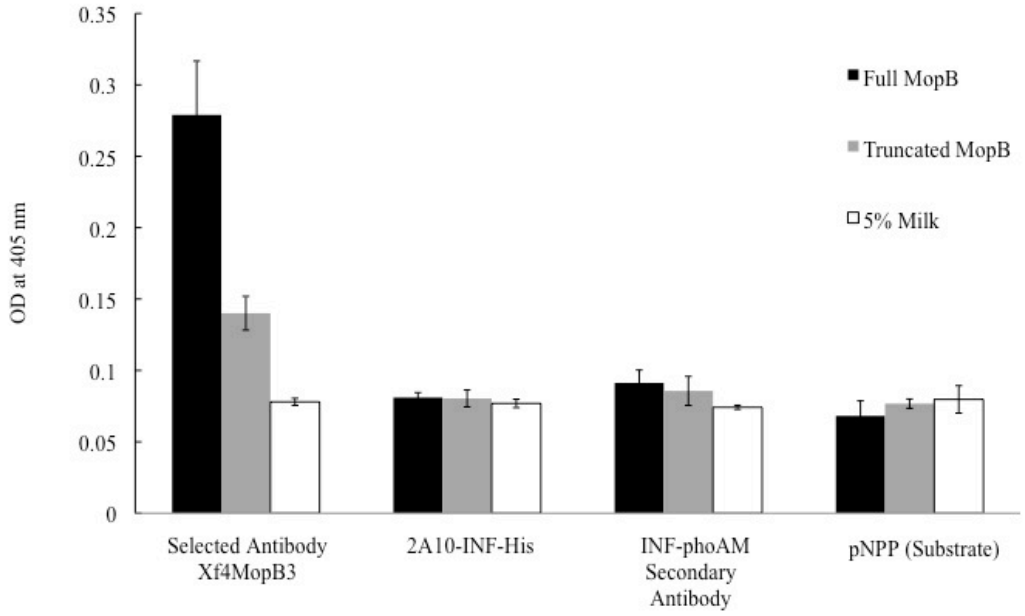


Figure 3.17 Functional analysis of 4XfMopB3 HA scFv by ELISA. The antibody was tested against 5% milk protein, mature full-length and truncated MopB. 2A10 scFv expressed in pAHAHis control antibody NPNA repeat (against Asn-Ala-Asn-Pro repeat) was used as an irrelevant negative control scFv. The secondary conjugate without a first antibody, and substrate alone, were used as additional controls. A threshold value of ≥ 0.1 was used as the cut-off value for detection of 4XfMopB3 HA scFv binding to mature full and truncated MopB protiens.

The ELISA results show that 4XfMopB3 scFv antibody binds to truncated and full length mature MopB, whereas 4XfMopB1 detected MopB in Western blots only (data not shown). The ELISA result for 4XfMopB3 agrees with the Western blot result, confirming the binding activity of 4XfMopB3 antibody in both assays. The 4XfMopB3 scFv was consequently used to image *X. fasitidosa* by immunofluorescence microscopy.

c. Immunofluorescence Analysis

The functionality of recombinant anti-MopB scFv (4XfMopB3) was tested by immunofluorescence (IF) as described in section 2.17.6c.

In this assay, slides of heat-killed whole *X. fasitidosa* were individually probed with purified 6xHis-tag antibodies. One *X. fasitidosa* slide was probed with anti-MopB 4XfMopB3 scFv antibody (specific antibody to *X. fasitidosa* MopB) as primary antibody followed by secondary anti-polyHistidine-FITC antibody.

Another *X. fasitidosa* slide was probed with primary antibody 2A10 scFv (antibody for NPNA malaria antigen) as negative control, followed by secondary anti-polyHistidine-FITC antibody.

In addition, to check for non-specific binding of the anti-polyHistidine-FITC antibody to *X. fasitidosa*, this antibody was used as a primary antibody only on an individual heat-killed whole *X. fasitidosa* slide as a control (section 2.17.6c).

The *X. fasitidosa* slide probed with 4XfMopB3 MopB scFv specific antibody was first viewed with bright-field microscopy and then with fluorescence microscopy. *X. fasitidosa* were green immunofluorescent stained with anti-polyHistidine-FITC antibody using fluorescence microscopy, confirming that 4XfMopB3 scFv antibody binds to *X. fasitidosa* surface exposed MopB protein (Figure 3.18A and B).

Negative control slides with *X. fasitidosa* and 2A10 scFv antibody (antibody for NPNA malaria antigen) and *X. fasitidosa* with the anti-polyHistidine-FITC antibody only, were also viewed with both bright-field microscopy and fluorescence microscopy. On both slides, *X. fasitidosa* did not show any green immunofluorescent staining using fluorescence microscopy (Figure 3.18C-F), which indicate that there was no non-specific binding to *X. fasitidosa* of control antibody or FITC conjugate.

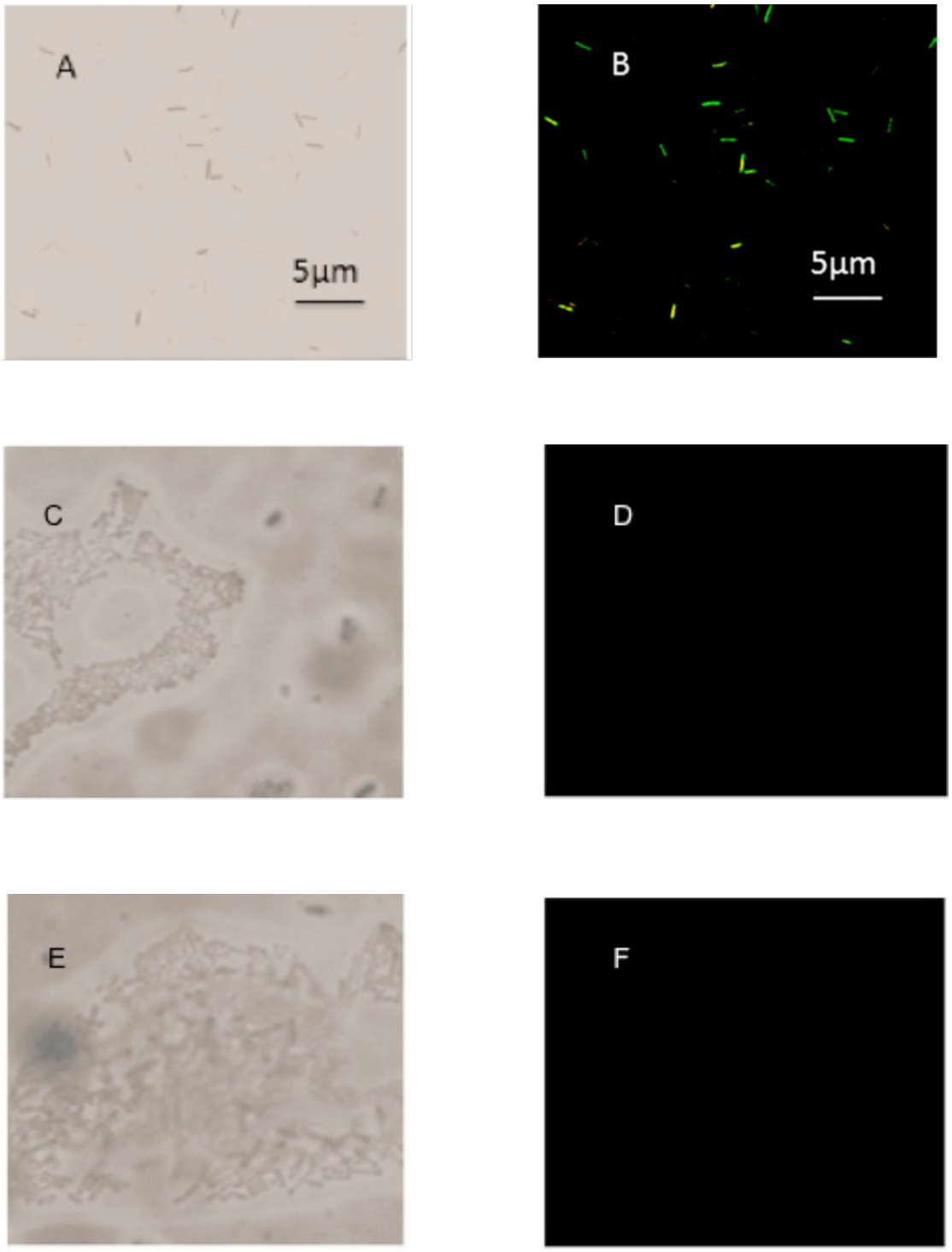


Figure 3.18 Immunofluorescence detection of *X. fastidiosa* with 4XfMopB3 scFv. Panels A, C and E were viewed with bright-field microscopy and B, D and F with fluorescence microscopy. *X. fastidiosa* was probed with: A and B anti-MopB (4XfMopB3) HA scFv and secondary anti-polyHistidine-FITC; C and D 2A10HA scFv and secondary anti-polyHistidine-FITC (negative control); E and F probed with primary anti-polyHistidine-FITC only (negative control). The size bar shown in panels A and B are 5 µm.

3.7 *Pantoea agglomerans*

Pantoea agglomerans (*P. agglomerans*), a member of the Enterobacteriaceae family, has been isolated from plants and insects. This bacterium is one of the important paratransgenic candidate species for controlling diseases such as Pierce's Disease (PD).

In this study, *P. agglomerans* was engineered to express selected recombinant antibody for paratransgenesis control of PD. Therefore an expression cassette was designed for *P. agglomerans* to express and secrete the selected anti-MopB 4XfMopB3 scFv antibody.

The MopB scFv antibody expression cassette (section 2.18.4) for *P. agglomerans* is presented in figure 2.5. This expression cassette contains a constitutive promoter (lac promoter) from pBSKmCit plasmid, *pelB* sequence from pSANG10-3F plasmid and 6xHis-tag from pBSKCA19.9 plasmid. The anti-MopB 4XfMopB3 scFv antibody-encoding gene was cloned into the *Nco*I/ *Not*I sites between the *pelB* sequence and 6xHis-tag.

To check for a suitable plasmid for *P. agglomerans* with a constitutive promoter, a pBlueScriptII mCit (pBSKmCit) plasmid was used. The pBSKmCit plasmid contains a constitutive promoter to drive the expression of monomeric fluorescent protein citrine. This plasmid was used to transform *P. agglomerans* and as the result green/yellow fluorescent bacteria were observed (Figure 3.20).

The pBSKmCit plasmid has suitable *Nde*I and *Hind*III insertion sites (Figure 2.5d) to accept the *pelB* signal peptide sequence-MopB scFv-6xHis-tag encoding fragment (Figure 2.5e) resulting in pARM4 plasmid. This expression cassette starts from the constitutive promoter with a *pelB* signal peptide sequence-anti-MopB scFv 4XfMopB3-6xHis-tag to engineer *P. agglomerans* to express selected recombinant antibody. The pARM4 plasmid sequence was confirmed by DNA sequencing (section 2.14.1). The pARM4 plasmid sequence is presented in appendix 1.5 (page 149).

3.7.1 *Pantoea agglomerans* Antibiotic Susceptibility (Antibiogram)

In order to find a compatible plasmid vector for *P. agglomerans,* it was necessary to identify a suitable antibiotic resistance marker to select the cells. To determine the antibiotic susceptibility of *P. agglomerans,* the disc diffusion (Kirby–Bauer) technique was performed.

P. agglomerans were subjected to 4 common, antibiotics (Tetracycline, Ampicillin, Neomycin and Chloramphenicol) on LB agar plates (Figure 3.19). The results presented in Table 3.3 and Figure 3.19 show that *P. agglomerans* was sensitive to all antibiotics.

Figure 3.19 *Pantoea agglomerans* antibiogram plate. Growth inhibition around antibiotic-impregnated disks indicates sensitivity of the bacterium to these antibiotics. *Pantoea agglomerans* was sensitive to all antibiotics (Tetracycline > Ampicillin> Neomycin > Chloramphenicol).

Table 3.3 *Pantoea agglomerans* antibiogram zones of inhibition.

Antibiotics	Disc potency	Diameter of zone of inhibition (cm)
Tetracycline	50µg	1.5
Ampicillin	10µg	0.7
Neomycin	10µg	0.4
Chloramphenicol	50µg	0.1

According to the *Pantoea agglomerans* antibiogram zones of inhibition, ampicillin was chosen as the selection marker. However, in this study ampicillin was replaced

with an analogue antibiotic, carbenicillin, due to its lower sensitivity to low pH and greater stability. For this reason *Pantoea agglomerans* was transformed with a plasmid containing carbenicillin resistance.

Figure 3.20 shows the electro-transformed *P. agglomerans* fluorescence colonies under UV with pBSKmCit plasmid containing a constitutive promoter to drive the expression of monomer fluorescent protein citrine and carbenicillin resistance.

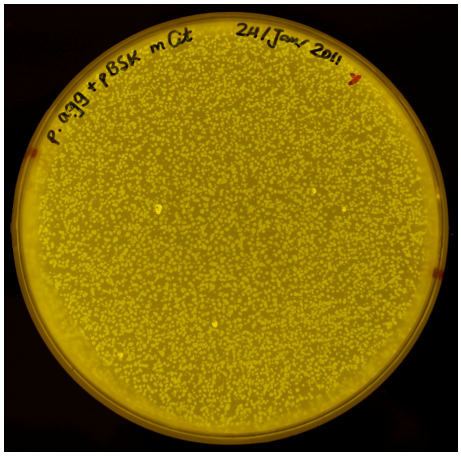


Figure 3.20 Electro-transformation of *P. agglomerans* with pBSKmCit plasmid. *P. agglomerans* fluorescence colonies under UV on LB agar plates containing 100 µg/mL of carbenicillin.

3.7.2 Electro-Transformation of *Pantoea agglomerans* with pARM4 Plasmid

To express the anti-MopB scFv antibody in *Pantoea agglomerans,* the pARM4 plasmid containing the expression cassette (constitutive promoter-*pelB* signal peptide sequence-anti-MopB scFv (4XfMopB3)-6xHis-tag) was electro-transformed into *P. agglomerans* as described in section 2.18.5a. Figure 3.21 shows the successful transformation of *P. agglomerans* with the pARM4 plasmid on LB agar plates containing 100 µg/mL of carbenicillin.

a) b)

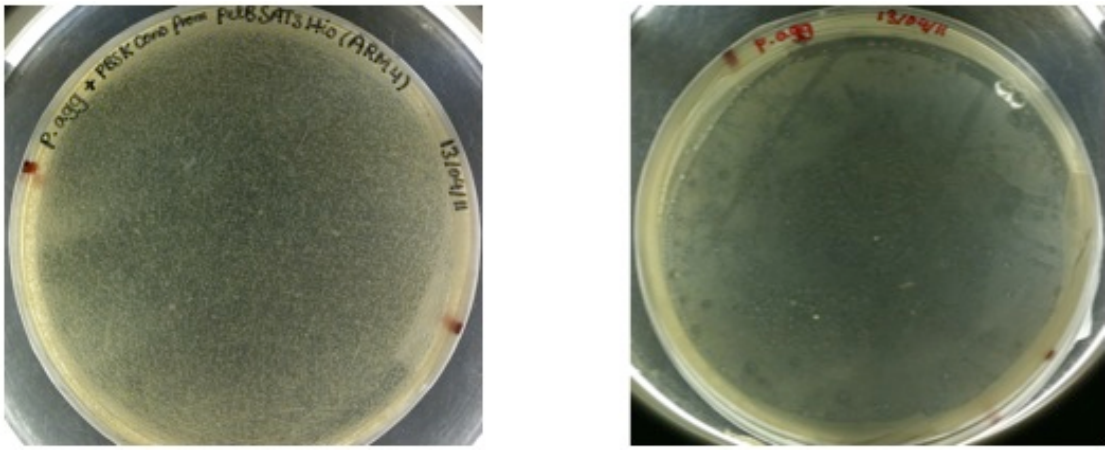


Figure 3.21 Electro-transformation of *Pantoea agglomerans* with pARM4 plasmid. *P. agglomerans* colonies on LB agar plates containing 100 µg/mL of carbenicillin. a) *P. agglomerans* with pARM4. b) *P. agglomerans* without the plasmid (control).

Colony PCR was performed to confirm the presence of the plasmid in the transformed *P. agglomerans* (Figure 3.22) (all primers are listed in Table 2.1). The DNA sequencing result for pARM4 plasmid with 4XfMopB3 anti-MopB antibody from XL1-Blue *E. coli* is presented in appendix 1.5 (page 149)

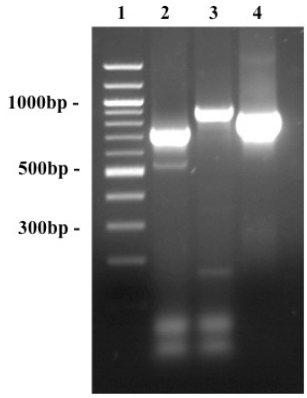


Figure 3.22 Colony PCR results of *P. agglomerans* with the pARM4 plasmid. Lane 1, 100 bp ladder; Lane 2, PCR product of MopB scFv using MVKF1 & MVHR1.1 primers; Lane 3, PCR product using pUC M13F & MVHR1.1 primers. Lane 4, PCR product using MVKF1 & pUC M13R primers.

3.7.3 Expression and Secretion of anti-MopB 4XfMopB3 scFv in *P. agglomerans*

The pARM4 vector with the constitutive promoter followed by *pelB* sequence anti-MopB scFv (4XfMopB3) and 6xHis-tag was overnight expressed (section 2.18.5b) in both *P. agglomerans* and *E. coli.* The Western blot analysis for anti-MopB scFv secretion showed that the anti-MopB scFv was expressed in *P. agglomerans* but not secreted, while the results for *E. coli* showed that the anti-MopB scFv was expressed and secreted (Figures 3.23 and 3.24).

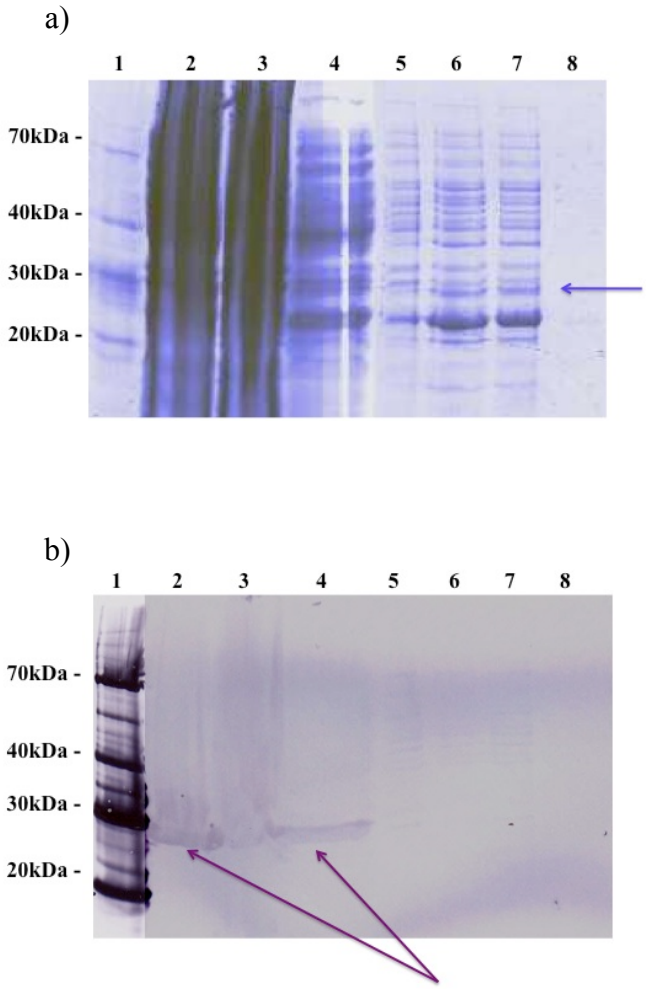


Figure 3.23 SDS-PAGE and Western blot analysis of anti-MopB (4XfMopB3) scFv overnight expression and detection in *E. coli* using pARM4 plasmid. a) SDS-PAGE. Lane 1, Bespoke protein marker; Lane 2, cell pellet; Lane 3, cell pellet after periplasmic purification; Lane 4, media ethanol precipitate, Lane 5, perplasmic fraction 1; Lane 6, perplasmic fraction 2; Lane 7, perplasmic fractions 1 and 2; Lane 8, blank. b) Western blot. Lane 1, Bespoke protein marker; Lane 2, cell pellet; Lane 3, cell pellet after periplasmic purification; Lane 4, media ethanol precipitate, Lane 5, perplasmic fraction 1; Lane 6, perplasmic fraction 2; Lane 7, perplasmic fractions 1 and 2, Lane 8, blank. The arrows indicate the expressed and secreted scFv (4XfMopB3) in *E. coli* with the predicted size of ~27kDa for the immunoreactive bands (lanes 2 and 4).

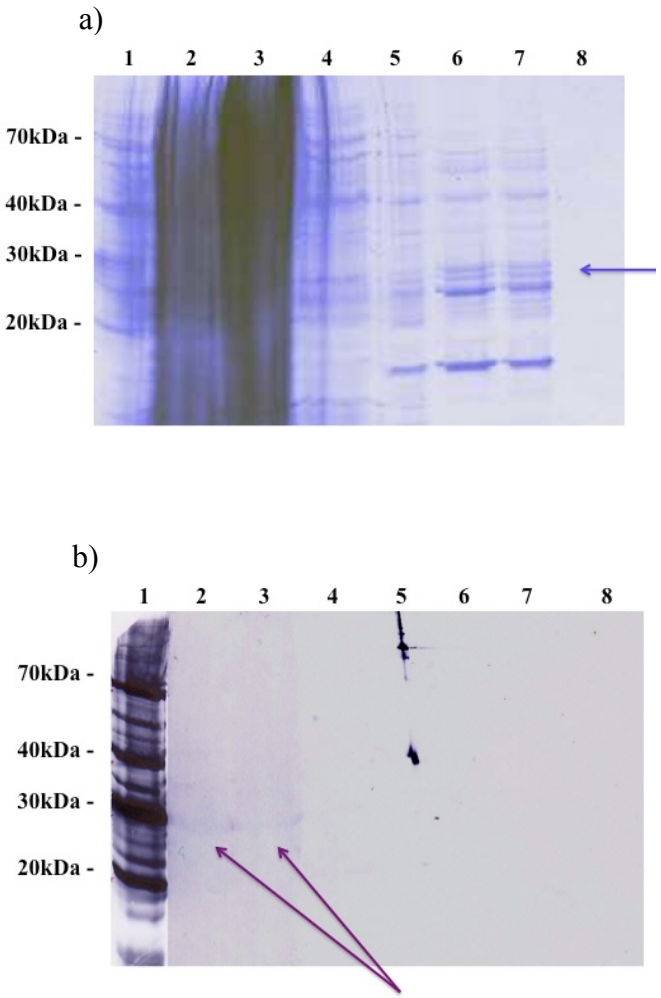


Figure 3.24 SDS-PAGE and Western blot analysis of anti-MopB (4XfMopB3) scFv overnight expression and detection in *P. agglomerans* using pARM4 plasmid. a) SDS-PAGE. Lane 1, Bespoke protein marker; Lane 2, cell pellet; Lane 3, cell pellet after periplasmic purification; Lane 4, media ethanol precipitate, Lane 5, perplasmic fraction 1; Lane 6, perplasmic fraction 2; Lane 7, perplasmic fractions 1 and 2; Lane 8, blank. b) Western blot. Lane 1, Bespoke protein marker; Lane 2, cell pellet; Lane 3, cell pellet after periplasmic purification; Lane 4, media ethanol precipitate, Lane 5, perplasmic fraction 1; Lane 6, perplasmic fraction 2; Lane 7, perplasmic fractions 1 and 2, Lane 8, blank. The arrows indicate the expressed scFv (4XfMopB3) in *P. agglomerans* with the predicted size ~27kDa for immunoreactive bands.

Chapter 4.0
Discussion

Chapter 4: Discussion

An emerging problem for the grape industry in California is Pierce's Disease (PD). A Gram negative xylem limited bacterium *Xylella fastidiosa (X. fastidiosa)* is the causative agent of PD (Davis *et al*., 1978). This is a vector-borne disease, in which xylem-feeding insects such as glassy-winged sharpshooter (GWSS) transmit *X. fastidiosa* from infected plants to others. These insects pick up the bacterium from the infected plants while they are feeding on them (Backus *et al*., 2009, Morano *et al*., 2008, Ramirez *et al*., 2008, Myers *et al*., 2007). *X. fastidiosa* attaches itself to the buccal cavity in the foregut of the xylem sap-sucking insect and the insects transmit the bacterium from one plant to another (Myers *et al*., 2007, Ramirez *et al*., 2008, Jackson *et al*., 2008). From the site of inoculation, *X. fastidiosa* multiplies and spreads to colonize the xylem, blocking the water transport network, causing scorch-like symptoms. One approach to control PD is to suppress transmission of the GWSS insect vector. Understanding the plant–pathogen–insect interface and molecular interactions may provide important information to prevent or reduce pathogen transmission.

Release of the *X. fastidiosa* genome sequence (Simpson *et al*., 2000) facilitated the study of the surface proteins of *X. fastidiosa,* which may provide targets for intervention of Pierce's Disease. MopB is an outer membrane protein, which has been identified on the coat surface of *X. fastidiosa* (Fjellbirkeland *et al*., 2000, Morano *et al*., 2008). The role of this protein (MopB) is not known, but it is well established that, in Gram negative bacteria, outer membrane proteins (OMPs) play important roles such as: 1) maintaining structural integrity of the outer membrane (OM), 2) transportation 3) membrane pores, 4) recognition proteins, 5) membrane bound enzymes or components of signal cascades (Bishop, 2008, Koebnik *et al*., 2000, Kostakioti *et al*., 2005, Chen *et al*., 2010), 6) resistance to stressful conditions (OprF in *Pseudomonas aeruginosa* and OmpA in *Escherichia coli* are implicated in this role) (Gotoh *et al*., 1989, Woodruff and Hancock, 1989, Wang, 2002), 7) pathogenesis (for instance, OspC in *Borrelia burgdorferi* and OmpA in *Escherichia coli*) (Khan *et al*., 2003, Prasadarao *et al*., 1996, Chen *et al*., 2010), and 8) agglutination.

The aim of this project was to generate recombinant antibodies to investigate the interaction of MopB with the plant and GWSS. Towards this goal, recombinant MopB has been produced. Mice spleens (n=5) previously immunized with whole heat-killed *X. fastidiosa* were used to construct five mouse DNA libraries. These libraries were used to generate MopB specific recombinant scFv antibodies by exploiting *in vitro* ribosome display technology. The selected recombinant scFvs were characterized by Western blot, Enzyme-Linked Immunosorbent assay (ELISA) and Immunofluorescence assay (IFA) analysis of recombinant MopB proteins and heat-killed *X. fastidiosa*.

Furthermore, to prevent PD in the insect or plant, *Pantoea agglomerans*, a partrangenenic candidate, has been engineered to express selected recombinant antibodies against MopB protein.

4.1 Surface Exposed Protein, MopB

The *X. fastidiosa* genome sequence has provided valuable information about genes, metabolic pathways and potential virulence factors that may be involved in pathogenicity (Simpson *et al*., 2000).

The *X. fastidiosa* genome sequence has been annotated, analysed and compared with other species genomes (Simpson *et al*., 2000, Van Sluys *et al*., 2003). *X. fastidiosa* is closely related to various *Xanthomonas* species (a Gram negative bacterium that colonizes the plant xylem). Genomic comparison analysis revealed similarities and differences between *X. fastidiosa* and *Xanthomonas* species. The Xanthomonad pathogen genome has been widely investigated and many genes have been implicated in the virulence of *Xanthomonas* species. Likewise, many of these homologous sequences are present in *X. fastidiosa* (Simpson *et al*., 2000, Hopkins and Purcell, 2002, Chatterjee *et al*., 2008). According to this information it is possible to predict components that may have key roles in bacterial virulence and bacterial attachment to both plant and insect hosts.

The outer membrane protein (MopB) has been studied in *Xanthomonas*. Chen *et*

al.,(2010) reported that mutation in *Xanthomonas* MopB (outer membrane protein) encoding gene results in: 1) changes in cell surface construction, 2) cell aggregation formation, 3) reductions in adhesion, motility and EPS xanthan gum productivity and 4) sensitivity to stress conditions and loss of pathogenicity (Chen *et al*., 2010).

A putative surface exposed molecule MopB (Fjellbirkeland *et al*., 2000, Morano *et al*., 2008) was selected from the completed genome sequence of *X. fastidiosa* Temecula1. The selected MopB protein sequence comparison indicated that the sequence was closely related to other *X. fastidiosa* strains and to *Xanthomonas* strains (about 96% identity). The sequence analysis showed the *X. fastidiosa* and *Xanthomonas* strain MopB proteins belong to the OmpA family.

MopB protein is an appropriate target, because: 1) MopB is the most abundant protein in membrane preparations, 2) the sequence of MopB was identical in all *X. fastidiosa* subspecies and 3) MopB is accessible on the surface of *X. fastidiosa* (Dandekar *et al*., 2012). 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 (section 2.14.1). *X. fastidiosa* 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 as described earlier in section 3.2.1.

4.2 MopB Protein Expression in *E. coli*

MopB is an outer membrane protein belonging to OmpA family (Fjellbirkeland *et al*., 2000, Morano *et al*., 2008). In previous attempts to express recombinant MopB protein, the protein was inserted into the outer membrane using its natural leader sequence or an alternative such as *ompA*, which may be toxic to *E. coli* and resulted in limited success (Bruening, 2003, Bruening, 2005). A leaderless construct results in proteins not traversing or inserting into the inner membrane but being retained and aggregated in the bacterial cytoplasm due to the hydrophobic nature of the transmembrane β-sheets.

In this project, leaderless *MopB* gene constructs, cloned into the pSANG10-3F expression vector (Martin *et al*., 2006) result in proteins not traversing or inserting in the inner membrane but being retained in the bacterial cytoplasm and aggregated due to the hydrophobic nature of the transmembrane β-sheets (section 2.14).

SDS-PAGE analysis showed that the leaderless full length mature (42.97 kDa) and truncated MopB (25.65 kDa) recombinant proteins were both efficiently expressed with the expected size. Extraction using denaturing conditions released the recombinant MopB protein 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 forms of the MopB protein, with yield of ~50 mg/L of bacterial culture (section 3.2.2).

Attempts to extract the proteins under native conditions using bacterial pellets from liquid cultures were unsuccessful, despite proteins of the predicted size being present in the induced cultures. The inability to release the recombinant MopB using the milder purification conditions (native conditions without 8 M urea) may be due to protein aggregation due to hydrophobicity of the β-barrel domain and or the formation of insoluble inclusion bodies.

Using 6 M guanidine hydrochloride and 8 M urea in denaturing purification condition facilitated the solubilisation of the recombinant protein and permitted subsequent affinity enrichment, and the major product eluting for the affinity resin with the expected size. To remove the urea from the protein samples different methods such as dialysis (specially step-wise dialysis), buffer-exchange by gel filtration and dilution (Tsumoto *et al*., 2003) can be used. Attempts to remove the urea by buffer exchange using Sephadex G25 PD 10 column resulted in on column protein aggregation (data not shown), thus a dilution method was performed. The soluble truncated MopB protein was diluted in PBS to reduce the urea concentration and permit the protein to be immobilized on microtitre plate wells to determine whether immune sera raised against whole heat-killed *X. fastidiosa* would recognize elements of the transmembrane β-barrel component of MopB.

4.3 Mouse Immunoglobulin Library Assembly

The ELISA results for recombinant truncated MopB protein and five mice immune sera previously immunized with whole heat-killed *X. fastidious* (section 3.3.2) revealed the presence of antibodies against the recombinant MopB protein. Five antibody libraries were assembled using the corresponding immune mice spleens in Trizol. The immune mice spleens were used to extract total RNA for 1st strand cDNA synthesis. These cDNA templates were used for PCR amplification of variable light and heavy chain families to construct five individual mouse immunoglobulin libraries.

A set of degenerate primers was designed in this project to construct mouse immunoglobulin libraries. Using degenerate primers facilitates the diversity of the amplified product and introduces a complex pool of many oligonucleotide primers to achieve all possible rearranged variable regions.

Based on information in the murine variable gene databases, 5' primers were designed to amplify mouse variable light chain (VL) and heavy chain (VH), as described by Kettleborough *et al.,* (1993). This set of primers enables recognition of all functional mouse variable genes. The degenerate 5' primers for the variable light chain integrated an *Nco*I restriction site and the 3' primers were designed to anneal at the end of the light chains to use elbow between VL and serine-glycine linker to join to the N-terminus linker of heavy chains. The 3' primers for heavy chain application annealed at the end of gamma chain. This primer incorporated a *Not*I restriction site and as these DNA antibody libraries were used in the subsequent selection procedure (*in vitro* ribosome display) the primer was also designed to remove the stop codon from the DNA library as described earlier in section 1.10.

Five individual mouse antibody libraries were assembled following the steps outlined in figure 2.1 by PCR to contain 7 variable light chain families and 10 variable heavy chain families. The gel electrophoresis analysis for variable light and heavy chains revealed that the mouse Vκ6, VH4 and 8 were present at lower levels of amplification while all other families were readily amplified. Light chains and heavy

chains were combined in the form of VL-Link-VH by linking each VL and VH fragment separately.

In order to use the antibody DNA library in an *in vitro* ribosome display without requiring a cloning step, a T7 promoter and Kozak sequence were introduced upstream of the VL-Link-VH construct to allow the protein synthesis in an *in vitro* rabbit reticulocyte lysate as described in section 3.4.2.

4.4 *In vitro* Ribosome Display

Five individual mouse DNA libraries were constructed in the form of T7-VL-Link-VH-MKC and used in an *in vitro* ribosome display to select specific functional antibodies against MopB protein.

In vitro ribosome display is fully *in vitro* cell free technology for synthesising, screening and selecting polypeptides from large libraries. This technology, which is based on coupling the phenotype (protein) and genotype (genetic information DNA or RNA), is an alternative method to other display methods as described in section 1.10 (Hanes and Pluckthun, 1997, He and Taussig, 2002, He and Khan, 2005).

The advantage of *in vitro* ribosome display over other display technology methods (section 1.10) such as cell based systems (phage display (Winter *et al*., 1994)) are: 1) a large library can be displayed without bacterial transformation limitations; 2) toxic, proteolytical sensitive and unstable proteins can be generated using this technology (He and Khan, 2005, He and Taussig, 2002).

The principal of this technology is based on *in vitro* transcription and translation of DNA library encoding open reading frames that lack stop codons at the 3′ ends. Due to the absence of the stop codon the ribosome will remain on the mRNA encoding protein to retain the genotype and phenotype, forming stable protein-ribosome-mRNA (PRM) complexes. These complexes are used for affinity selection by immobilized target or ligand. The retained complexes, after removal of the

unbounded ones, will be reverse transcribed to cDNA and PCR amplified (He and Taussig, 2002, He and Khan, 2005, Douthwaite *et al*., 2006).

Five mice DNA libraries encoding antibodies were individually used in the selection procedure. The *in vitro* ribosome display steps (He and Taussig, 2007) were optimized to facilitate the selection of affinity antibodies against the MopB protein.

After a first round of selection (a single enrichment step) using recombinant truncated MopB as bait and mouse 4 DNA library in an *in vitro* ribosome display, single chain antibodies against MopB were selected and isolated (section 3.5). Only a single round of selection was performed due to using antibody libraries constructed from intentionally immunized sources, which may be a common feature of this rapid enrichment.

DNA sequencing results for the four randomly selected anti-MopB scFv antibodies (4XfMopB1-4) (Appendix 1.3, pages 144-147), confirmed three intact in-frame VL-VH combination antibodies (4XfMopB1-3). These antibodies (4XfMopB1-3) were analysed using the BLAST network service at the National Center for Biotechnology Information (NCBI, http:// www.ncbi.nlm.nih.gov), which indicated similarity to immunoglobulin superfamilies. BLASTX (for putative amino acid sequence) were used for sequence similarity searches for the light chains and the heavy chains. The sequence comparisons indicated that the 4XfMopB1, 4XfMopB2, 4XfMopB3 light chains were 96%, 98% and 97% identical respectively to immunoglobulin light chain variable region [*Mus musculus*]; and 4XfMopB1, 4XfMopB2, 4XfMopB3 heavy chains showed 99%, 98% and 100% identity respectively to immunoglobulin μ heavy chain variable regions [*Mus musculus*] (Altschul *et al*., 1990) .

Furthermore, the sequences of the scFv's were analysed using the International ImMunoGeneTics (IMGT®) information system® (Li *et al*., 2007, Zhang *et al*., 2011) to assign the mouse variable genes and alleles. For each isolated scFv (4XfMopB1-3) the light chain variable kappa region (Vκ), variable J region (VκJ) and complementarity determining region 3 (VL CDR3) and for the heavy chain variable region (VH), variable J region (VHJ), variable D segment (VHD) and

complementarity determining region 3 (VH CDR3) were analysed (Table 3.2). Analysis of the variable region sequences showed that different gene segments of the antibody sequences were present in the selected recombinant antibody clones.

4.5 Anti-MopB scFv Expression in *E. coli*

After the first round of ribosome display using mouse 4 DNA antibody library, four randomly selected anti-MopB scFv antibodies (4XfMopB1-4) were sequenced (Appendix 1.3). Three of the four confirmed intact in-frame anti-MopB antibodies (4XfMopB1-3) were digested with *Nco*I and *Not*I and sub-cloned into pAHAHis expression vector for scFv expression in the Rosetta gami B(DE3) *E. coli* cytoplasm to overcome the expression of rare codons in *E. coli* (Novy *et al*., 2001). The pAHAHis plasmid was designed to add a C-terminal 6xHis-tag to the antibody to facilitate both purification and detection of expressed anti-MopB antibodies by Western blot using monoclonal anti-polyHistidine-alkaline phosphatase antibody (section 2.17).

Rosetta gami B(DE3) is DE3 lysogen for λ prophage, which contains a chromosomal copy of an IPTG inducible T7 RNA polymerase under *lacUV5* promoter control as described earlier in section 1.11.2a and b (Novy *et al*., 2001). This strain facilitates the eukaryotic protein expression by carrying a plasmid, which provides the tRNA for the codons (AUA, AGG, AGA, CUA, CCC, GGA) rarely used in *E. coli* (Novy *et al*., 2001) and enables the disulfide bond formation of the target protein in the bacterial cytoplasm (Bessette *et al*., 1999, Prinz *et al*., 1997, Ritz and Beckwith, 2001).

The anti-MopB scFv antibodies were expressed by using a routine antibody production method. The standard condition for antibody (protein) expression was set at 20 °C for 20 hours to reduce the cellular protein concentration and improve protein folding.

The expression of the anti-MopB scFv's was confirmed by purifying the expressed anti-MopB scFv's from the cytoplasm under denaturing condition using a Ni^{2+}-NTA column. The presence of 6 M guanidine hydrochloride and 8 M urea solubilised the

anti-MopB scFv's recombinant protein and allowed subsequent affinity enrichment using affinity resin. Protein gel electrophoresis analysis revealed that the recombinant anti-MopB scFv's were expressed, enriched and eluted with the expected size of ~27 kDa for selected antibodies. After confirming the expression of the anti-MopB scFv antibodies in the Rosetta gami B(DE3) *E. coli* strain, it was necessary to purify soluble anti-MopB antibodies from the cytoplasm under the native condition to preserve antibody activity, increase the likelihood of correct folding and being functional.

SDS-PAGE gel and Western blot results for purified 4XfMopB1-3 antibodies under native condition showed that the anti-MopB antibodies were purified with the expected size of ~27 kDa. However, this gel also showed contaminating proteins in the fractions.

The presence of the contaminating proteins could be related to the inefficiency of immobilised metal ion affinity chromatography (IMAC) purification under the native condition. To eliminate the contaminated proteins, different concentrations of imidazole could be tested in the wash buffer followed by a few more washes. The purified anti-MopB scFvs from the cytoplasm under native conditions could be used directly for functional analysis.

4.6 Anti-MopB scFv Functional Analysis

The expressed selected anti-MopB scFv antibodies using pAHAHis expression vector (as described earlier in section 4.6 and 2.17.1) were tested for their functional properties against MopB protein using Western bolt, ELISA and Immunofluorescence Assay (IFA).

In the pAHAHis vector, the C-terminal HA peptide (section 2.17.1) was designed to facilitate the detection of scFv and antigen binding. To detect the HA peptide, a secondary recombinant scFv antibody (anti-HA-phoAM scFv) against HA peptide was expressed, purified (section 3.6.2) and used in Western blot and ELISA. Moreover, a C-terminal 6xHis-tag was added to this construct to facilitate both

purification and detection of anti-MopB antibodies by IFA using anti-polyHistidine-FITC antibody.

Western bolt results show that of three selected anti-MopB scFv (4XfMopB1-3) antibodies two (4XfMopB1 and 4XfMopB3) recognized the recombinant truncated and mature full length MopB protein.

The 4XfMopB3 anti-MopB scFv antibody was used in ELISA assays. The ELISA results indicated binding of the 4XfMopB3 antibody to both the recombinant truncated and mature full length MopB proteins. Although the signals are not strong in comparison with the negative controls they do however demonstrate binding.

The weak binding signal could possibly be due to several reasons, including the low concentration of the recombinant scFvs (4XfMopB3 and HA-phoAM) used in the assay, using a recombinant scFv (HA-phoAM) to detect the binding of anti-MopB scFv with the recombinant MopB proteins. Despite the low binding, the ELISA result agrees with the Western blot results confirming the binding activity of 4XfMopB3 antibody.

Immunofluorescence assays (section 3.6.2c) demonstrated binding of the selected anti-MopB scFv (4XfMopB3) to the *X. fasitidosa* surface exposed MopB protein. The binding was specific sience the negative controls [*X. fastidiosa* probed with 2A10 scFv antibody for NPNA malaria antigen (a non-specific scFv antibody for MopB protein) followed by the secondary anti-polyHistidine-FITC antibody and for *X. fastidiosa* with the anti-polyHistidine-FITC antibodies only] did not fluoresce. This results show that there was no non-specific binding between *X. fastidiosa* and the controls.

Of the four selected scFv's antibodies (4XfMopB1-4), one recognized the mature full length and truncated recombinant MopB proteins in both the Western blot and ELISA, and heat-killed *X. fastidiosa* by IF, suggesting that the epitope is nonconformational and possibly one of the three predicted loops exposed on the surface of the bacteria. The results of this study not only indicate the successful selection of functional scFv antibodies from mouse spleens previously immunized

with whole heat-killed *X. fastidiosa* by *in vitro* ribosome display technology, but also show the binding and interaction of the selected anti-MopB scFv (4XfMopB3) with the recombinant MopB proteins and the *X. fastidiosa* surface protein. The anti-MopB scFv (4XfMopB3) may also have a great potential in basic research and for easy, rapid, sensitive and specific early diagnosis of infected grapevines. Moreover, 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.

4.7 Engineered *Pantoea agglomerans*

New control strategies are required for successful control and prevention of vector-borne diseases. One strategy is paratransgenesis, a novel strategy that use genetically modified insect symbionts to express molecules within the vector, which blocks the pathogen development or vector transmission. The aim of this strategy is to reduce the insect vectorial capacity and block the pathogen in the insect gut as described earlier insection 1.7.1 (Coutinho-Abreu *et al*., 2010).

Advantages of paratransgenesis over the direct transgenic approach are: 1) bacterial genetic manipulation is much easier; 2) bacteria can be made to secrete or carry very specific agents such as single chain antibodies. This strategy has a wide application in disrupting vector-borne infectious diseases such as Chagas' disease (Beard *et al*., 2001), and also HIV (Chang *et al*., 2003); *Candida* infections (Beninati *et al*., 2000), and has also been applied to the delivery of cytokines in mammalian guts to relieve colitis (Steidler *et al*., 2000, Steidler, 2001).

There are many symbionts that are able to express an array of molecules, which block pathogen development and have the potential to reduce pathogen transmission, but still there are many difficulties that need to be overcome. For example Chagas disease vector*, Rhodnius prolixus,* has been transformed by the symbiont *Rhodococcus rhodnii* to express a lethal peptide (cecropin A) to *Trypanosoma cruzi* parasite (Durvasula *et al*., 1997). *Trypanosoma cruzi* were reduced in the gut of *R. prolixus* without interfering with insect fitness (Durvasula *et al*., 1997). Furthermore,

functional antibodies have also been expressed without the use of antibiotic selection in the guts of *R. prolixus* (Durvasula *et al*., 1999) and *Triatoma infestans* (Durvasula *et al*., 1997, Durvasula *et al*., 2008).

Paratransgenesis approaches, which control the spread of pathogens, are promising methods for controlling PD. As described earlier in section 1.7.2, many probable bacterial candidates have been suggested for this approach, including *Alcaligenes xylosoxidans* (Axd) and *Pantoea agglomerans* (Lacaval *et al*., 2009).

In this project an anti-MopB scFv antibody expression cassette for *Pantoea agglomerans*, a promising paratransgenic bacterium (Pusey, 2002, Rio *et al*., 2004) was constructed using a constitutive promoter (lac promoter), *pelB* leader sequence, the selected functional anti-MopB scFv antibody (4XfMopB3) and a C-terminal 6xHis-tag (pARM4 plasmid) as described in section 2.18.4. This construct was successfully transformed into *P. agglomerans*. The pelB signal peptide from the pectate lyase-encoding gene of a related species, *Erwinia carotovora* (Sherald and Lei, 1991, Lei *et al*., 1987), was used due to its ability to lead the newly synthesised protein (scFv) to the periplasm by using the type II secretion pathway. The pelB signal peptide will be removed in the periplasm, from which the mature protein can leak out from the periplasm into the growth medium (Sandkvist, 2001).

This expression cassette was expressed in both XL1-Blue *E. coli* and the *P. agglomerans* strains at 37 ºC. Western blot results for the overnight expression indicated that 4XfMopB3 scFv antibody was expressed in both strains but only *E. coli* was able to secrete the 4XfMopB3 scFv antibody. It is possible that the anti-MopB scFv antibody was secreted by *P. agglomerans* but in low amounts undetectable by colorimetric detection in a Western blot and/or the secreted anti-MopB scFv antibody (4XfMopB3) was bound to the *P. agglomerans* surface proteins. These two hypotheses can be checked using a more sensitive Western blot detection system such as chemiluminescence detection and immunofluorescence assays (IFA). For immunofluorescence assays (IFA), a slide of *P. agglomerans* probing with anti-MopB 4XfMopB3 scFv antibody as primary antibody followed by secondary anti-polyHistidine-FITC antibody can be used.

To improve *P. agglomerans* scFv antibody expression and secretion, it is necessary to identify the optimal expression and secretion conditions. The anti-MopB scFv antibody was expressed in *P. agglomerans* and *E. coli* using the designed expression cassette at 37 ºC, while *P. agglomerans* is normally incubated at 30 ºC (Gavini *et al*., 1989, Bisi and Lampe, 2011). It is important to reduce the temperature to 30 ºC and to optimise the codon usage to improve protein expression and secretion.

In addition, the pelB signal peptide allows the passage of the protein (scFv) through the periplasm. Many problems such as periplasmic inclusion bodies, errors in folding or disulfide bond formation and degradation (Schlapschy *et al*., 2006, Kolaj *et al*., 2009) may occur in the periplasm, which could explain the reasons for detection of the expressed scFv in the cell pellet but not in the culture media for *P. agglomerans*.

4.8 Conclusion

In this project the main goal was to generate recombinant scFv antibodies to be used to investigate the interaction of MopB with the plant and GWSS. Towards this goal, recombinant MopB has been produced and used with sera from five mice immunized with whole heat-killed *X. fastidiosa* in an ELISA. The ELISA results revealed the presence of antibodies against the recombinant MopB protein. The corresponding immune mouse spleens were used for 1st strand cDNA synthesis and these cDNA templates were used for PCR amplifying members of immunoglobulin variable region, 7 light chains and 10 heavy chains, families. The combinatorial libraries of single-chain fragment variable (scFv) antibodies were assembled in the VL-Link-VH orientation. These libraries were accessed for MopB specific recombinant scFv antibodies by *in vitro* ribosome display. Using the pAHAHis expression plasmid and Rosetta gami B(DE3) strain, selected anti-MopB scFv (4XfMopB3) was succefully expressed and purified from the *E. coli* cytoplasm and characterized by Western blot and ELISA, and immunofluorescence assay binding to heat-killed *X. fastidiosa*.

The results reveal that recombinant MopB proteins that are immunoreactive with sera from mice immunized with heat-killed *X. fastidiosa* had been produced. Such antibodies could have potential use in *X. fastidiosa* diagnostic applications. The recombinant anti-MopB could readily be engineered to agglutinate the bacteria and

introduced into the GWSS via paratransgenic organisms such as engineered *Pantoea agglomerans*. In this study, *P. agglomerans* has been engineered with pARM4 (anti-MopB scFv expression plasmid) to express and secrete the anti-MopB (4XfMopB3) scFv antibody. Despite this, *P. agglomerans* was not able to secrete the anti-MopB antibody but it was successfully expressed.

Note:

Parts of the results presented in this thesis have been published in *Applied and Environmental Microbiology* (AEM) (Appendix 2).

AZIZI, A., ARORA, A., MARKIV, A., LAMPE, D. J., MILLER, T. A. & KANG, A. S. 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 and Environmental Microbiology,* 78**,** 2638-2647.

4.9 Future work

Various approaches could be attempted to continue this work. The results presented provide evidence that immunization with whole heat-killed *X. fastidiosa* clearly induces antibodies against surface exposed antigenic molecules exemplified by MopB. It is likely that the immune sera may also contain antibodies against other putative surface exposed molecules, some of which could have a role in virulence involving molecules intimately associated with attachment in either the GWSS or the grapevine xylem (Koide *et al*., 2006). One approach could focus on isolating more recombinant antibodies against MopB and other abundant surface exposed molecules on *X. fastidiosa* by using the *in vitro* anti- *X. fastidiosa* scFv libraries generated in this study, and the strategy for preparing recombinant putative membrane proteins provide a unique approach for discovering additional recombinant antibodies against surface components of *X. fastidiosa*. An alternative application for the antibody could be to engineer the selected antibodies for diagnostic applications by assembling the recombinant antibody molecules with in-built fluorophores (Markiv *et al*., 2011) for use in hand-held field-based microfluidic devices.

The genetically encoded molecules that target the surface of *X. fastidiosa* will facilitate the bacteria–vector interactions studies to develop intervention strategies such as paratransgenesis. *Rhodococcus rhodnii* bacterium was the first engineered symbiotic bacterium, which expressed and secreted cecropin A in *Rhodnius prolixus*, the Chagas disease vector (Durvasula *et al*., 1997), and subsequently to express a scFv antibody (Durvasula *et al*., 1999). Furthermore, a recombinant antibody against *Plasmodium falciparum* sporozoite surface protein (Chappel *et al*., 2004a, Chappel *et al*., 2004b) has been used to reduce *P. falciparum* sporozoite levels in *Anopheles gambiae* via an engineered entomopathogenic fungi (Fang *et al*., 2011). In the malaria study, the recombinant antibody targeted a surface accessible highly abundant target, the circumsporozoite protein, and was designed to agglutinate the parasite. 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.

The next attempt will be to improve the expression and secretion of the anti-MopB scFv in *P. agglomerans* by identifying the optimal conditions, reducing the temperature to 30 °C and checking the codon usage. Then the newly engineered *P. agglomerans,* exhibiting the selected anti-MopB scFv generated will be tested at the University California Riverside in view of counteracting Pierce's Disease. A possible avenue to take might be to infuse the genetically modified *P. agglomerans* into a known sample of grapevines and allow GWSS to feed on them. It would be most probable that the GWSS will easily pick up the modified *P. agglomerans* into its buccal cavity. Upon the subsequent feeding on infected (or even non-infected) grapevines, the genetically altered *P. agglomerans* might be able to displace or disrupt *X. fastidiosa*.

Paratransgenesis (symbiotic control) offers a long-term solution to existent agricultural and infection disease problems. However, progress might be slow owing to the novelty of this particular application, the risk assessment, which is one of the most important concerns in using genetically modified symbiont, proving that the symbiont will not cause unexpected consequences, the regulatory process and approval requirements (Miller, 2011). Symbiotic control also demands for an

extensive background research on a particular symbiont and its niche should be exactly as that of the pathogen in question (both pathogen and symbiont should have evolved together over a defined period of time). It is important to remember the overall success of any paratransgenic strategy depends on the similar growth characteristics of the engineered strains and the wild type parent strain.

Moreover, recombinant antibodies against MopB and other abundant surface exposed molecules on *X. fastidiosa* could be readily engineered to agglutinate the bacteria and introduced into the GWSS via paratransgenic organisms such as engineered *Pantoea agglomerans, Metarhizium spp* (Fang *et al*., 2011) or *Beauvaria bassiana* (Bukhari *et al*., 2011), or an avirulent strain of *Xylella* itself (Miller, 2011), providing new platforms to investigate controlling PD.

References

References

ALMEIDA, R. P. P., MANN, R. & PURCELL, A. H. 2004. *Xylella fastidiosa* cultivation on a minimal solid defined medium. *Current Microbiology,* 48, 368-372.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology,* 215, 403-410.

ANDREWS, J. H. & HARRIS, R. F. 2000. The ecology and biogeography of microorganisms of plant surfaces. *Annual Review of Phytopathology,* 38, 145-180.

BACCARI, C. & LINDOW, S. E. 2011. Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape cultivars. *Phytopathology,* 101, 77-84.

BACKUS, E. A., HOLMES, W. J., SCHREIBER, F., REARDON, B. J. & WALKER, G. P. 2009. Sharpshooter X Wave: Correlation of an electrical penetration graph waveform with xylem penetration supports a hypothesized mechanism for *Xylella fastidiosa* Inoculation. *Annals of the Entomological Society of America,* 102, 847-867.

BACKUS, E. A. & MORGAN, D. J. W. 2011. Spatiotemporal colonization of *Xylella fastidiosa* in its vector supports the role of egestion in the inoculation mechanism of foregut-borne plant pathogens. *Phytopathology,* 101, 912-922.

BAGOS, P. G., LIAKOPOULOS, T. D., SPYROPOULOS, I. C. & HAMODRAKAS, S. J. 2004a. A Hidden Markov Model method, capable of predicting and discriminating beta-barrel outer membrane proteins. *BMC Bioinformatics,* 5, 29-41.

BAGOS, P. G., LIAKOPOULOS, T. D., SPYROPOULOS, I. C. & HAMODRAKAS, S. J. 2004b. PRED-TMBB: a web server for predicting the topology of beta-barrel outer membrane proteins. *Nucleic Acids Research,* 32, W400-W404.

BANEYX, F. 1999. Recombinant protein expression in *Escherichia coli. Current Opinion in Biotechnology,* 10, 411-21.

BAUER, A. W., KIRBY, W. M. M., SHERRIS, J. C. & TURCK, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology,* 45, 493-496.

BAZZI, C., STEFANI, E. & ZACCARDELLI, M. 1994. SDS-PAGE: a tool to discriminate *Xylella fastidiosa* from other endophytic grapevine bacteria. *Bulletin OEPP/EPPO Bulletin,* 24, 121-127.

BEARD, C. B., MASON, P. W., AKSOY, S., TESH, R. B. & Richards, F. F. 1992. Transformation of an insect symbiont and expression of a foreign gene in the Chagas disease vector *Rhodnius prolixus*. *American Journal of Tropical Medicine and Hygiene,* 46, 195-200.

BEARD, C. B., DOTSON, E. M., PENNINGTON, P. M., EICHLER, S., CORDON-ROSALES, C. & DURVASULA, R. V. 2001. Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. *International Journal for Parasitology,* 31, 621-627.

BEN BEARD, C., CORDON-ROSALES, C. & DURVASULA, R. V. 2002. Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. *Annual Review of Entomology,* 47, 123-141.

BENINATI, C., OGGIONI, M. R., BOCCANERA, M., SPINOSA, M. R., MAGGI, T., CONTI, S., MAGLIANI, W., DE BERNARDIS, F., TETI, G., CASSONE, A., POZZI, G. & POLONELLI, L. 2000. Therapy of mucosal candidiasis by expression of an anti-idiotype in human commensal bacteria. *Nature Biotechnology,* 18, 1060-1064.

BESSETTE, P. H., ASLUND, F., BECKWITH, J. & GEORGIOU, G. 1999. Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm. *Proceedings of the National Academy of Sciences of the United States of America,* 96, 13703-13708.

BEXTINE, B., LAMPE, D., LAUZON, C., JACKSON, B. & MILLER, T. A. 2005. Establishment of a genetically marked insect-derived symbiont in multiple host plants. *Current Microbiology,* 50, 1-7.

BEXTINE, B., LAUZON, C., POTTER, S., LAMPE, D. & MILLER, T. A. 2004. Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Current Microbiology,* 48, 327-331.

BISHOP, R. E. 2008. Structural biology of membrane-intrinsic beta-barrel enzymes: Sentinels of the bacterial outer membrane. *Biochimica et Biophysica Acta-Biomembranes,* 1778, 1881-1896.

BISI, D. C. & LAMPE, D. J. 2011. Secretion of Anti-Plasmodium Effector Proteins from a Natural *Pantoea agglomerans* Isolate by Using PelB and HlyA Secretion Signals. *Applied and Environmental Microbiology,* 77, 4669-4675.

BLUA, M. J., PHILLIPS, P. A. & REDAK, R. A. 1999. A new sharpshooter threatens both crops and ornamentals. *California Agriculture,* 53, 22-25.

BOUCIAS, D. G., SCHARF, D. W., BREAUX, S. E., PURCELL, D. H. & MIZELL, R. E. 2007. Studies on the fungi associated with the glassy-winged sharpshooter Homalodisca coagulata with emphasis on a new species Hirsutella homalodiscae nom. prov. *Biocontrol*, 52, 231-258.

BRADFORD, J. R. 2001. In silico methods for prediction of signal peptides and their cleavage sites, and linear epitopes M. Res. thesis. University of Leeds, Leeds, United Kingdom.

BRADY, J. A., FASKE, J. B., CASTANEDA-GILL, J. M., KING, J. L. & MITCHELL, F. L. 2011. High-throughput DNA isolation method for detection of *Xylella fastidiosa* in plant and insect samples. *Journal of Microbiological Methods,* 86, 310-312.

BRINKMANN, U., MATTES, R. E. & BUCKEL, P. 1989. High-level expression of recombinant genes in *Escherichia coli* is dependent on the availability of the dnay gene-product. *Gene,* 85, 109-114.

BRUENING, G., CIVEROLO, E. AND GILCHRIST, D. 2002. Virulence analysis of the Pierce's Disease agent *Xylella fastidiosa.* [Report from *November 1, 2001 to October 31, 2002, 3-4*].

BRUENING, G., CIVEROLO, E., DANDEKAR, A. M. AND GUPTA, G. 2003. Roles of *Xylella fastidiosa* proteins in virulence. [Report from *November 1, 2002 to October 13, 2003*, 135-137].

BRUENING, G., DANDEKAR, A. M., CIVEROLO, E. AND GUPTA, G. 2004. Exploiting *Xylella fastidiosa* proteins for Pierce's Disease control. [Report from *October 15, 2003 to October 11, 2004*, 286-289].

BRUENING, G., DANDEKAR, A. M., CIVEROLO, E. AND GUPTA, G. 2005. Exploiting *Xylella fastidiosa* proteins for Pierce's Disease control. [Report from *October 15, 2004 to Setember 30, 2005*, 221-224].

BUKHARI, T., TAKKEN, W. & KOENRAADT, C. J. M. 2011. Development of *Metarhizium anisopliae and Beauveria bassiana* formulations for control of malaria mosquito larvae. *Parasites & Vectors,* 4, 23-36.

CARBAJAL, D., MORANO, K. A. & MORANO, L. D. 2004. Indirect immunofluorescence microscopy for direct detection of *Xylella fastidiosa* in xylem sap. *Current Microbiology,* 49, 372-375.

CASERTA, R., TAKITA, M. A., TARGON, M. L., ROSSELLI-MURAI, L. K., DE SOUZA, A. P., PERONI, L., STACH-MACHADO, D. R., ANDRADE, A., LABATE, C. A., KITAJIMA, E. W., MACHADO, M. A. & DE SOUZA, A. A. 2010. Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. *Applied and Environmental Microbiology,* 76, 4250-4259.

CHANG, C. J. & DONALDSON, R. C. 2000. Nutritional requirements of *Xylella fastidiosa*, which causes Pierce's Disease in grapes. *Canadian Journal of Microbiology,* 46, 291-293.

CHANG, C. J., GARNIER, M., ZREIK, L., ROSSETTI, V. & BOVE, J. M. 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa. Current Microbiology,* 27, 137-142.

CHANG, T. L. Y., CHANG, C. H., SIMPSON, D. A., XU, Q., MARTIN, P. K., LAGENAUR, L. A., SCHOOLNIK, G. K., HO, D. D., HILLIER, S. L., HOLODNIY, M., LEWICKI, J. A. & LEE, P. P. 2003. Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4. *Proceedings of the National Academy of Sciences of the United States of America,* 100, 11672-11677.

CHAPPEL, J. A., HOLLINGDALE, M. R. & KANG, A. S. 2004a. IgG(4) Pf NPNA-1 a human anti-*Plasmodium falciparum* sporozoite monoclonal antibody cloned from a protected individual inhibits parasite invasion of hepatocytes. *Human Antibodies,* 13, 91-96.

CHAPPEL, J. A., ROGERS, W. O., HOFFMAN, S. L. & KANG, A. S. 2004b. Molecular dissection of the human antibody response to the structural repeat epitope of *Plasmodium falciparum* sporozoite from a protected donor. *Malaria Journal,* 3, 28-39.

CHATTERJEE, S., NEWMAN, K. L. & LINDOW, S. E. 2008. Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. *Molecular Plant-Microbe Interactions,* 21, 1309-1315.

CHEN, Y. Y., WU, C. H., LIN, J. W., WENG, S. F. & TSENG, Y. H. 2010. Mutation of the gene encoding a major outer-membrane protein in *Xanthomonas campestris* pv. *campestris* causes pleiotropic effects, including loss of pathogenicity. *Microbiology,* 156, 2842-2854.

CHENG, D. W., LIN, H., WALKER, M. A., STENGER, D. C. & CIVEROLO, E. L. 2009. Effects of grape xylem sap and cell wall constituents on *in vitro* growth, biofilm formation and cellular aggregation of *Xylella fastidiosa*. *European Journal of Plant Pathology,* 125, 213-222.

CHOAT, B., BALL, M., LULY, J. & HOLTUM, J. 2003. Pit membrane porosity and water stress-induced cavitation in four co-existing dry rainforest tree species. *Plant Physiology,* 131, 41-48.

COHEN, S. N., CHANG, A. C. & HSU, L. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proceedings of the National Academy of Sciences of the United States of America,* 69, 2110-2114.

COSTA, E., USALL, J., TEIXIDO, N., DELGADO, J. & VINAS, I. 2002. Water activity, temperature, and pH effects on growth of the biocontrol agent *Pantoea agglomerans* CPA-2. *Canadian Journal of Microbiology,* 48, 1082-1088.

COSTA, H. S., RAETZ, E., PINCKARD, T. R., GISPERT, C., HERNANDEZ-MARTINEZ, R., DUMENYO, C. K. & COOKSEY, D. A. 2004. Plant hosts of *Xylella fastidiosa* in and near southern California vineyards. *Plant Disease,* 88, 1255-1261.

COUTINHO-ABREU, I. V., ZHU, K. Y. & RAMALHO-ORTIGAO, M. 2010. Transgenesis and paratransgenesis to control insect-borne diseases: Current status and future challenges. *Parasitology International,* 59, 1-8.

CREGG, J. M., CEREGHINO, J. L., SHI, J. Y. & HIGGINS, D. R. 2000. Recombinant protein expression in *Pichia pastoris*. *Molecular Biotechnology,* 16, 23-52.

DANDEKAR, A. M., GOURAN, H., IBANEZ, A. M., URATSU, S. L., AGUERO, C. B., MCFARLAND, S., BORHANI, Y., FELDSTEIN, P. A., BRUENING, G., NASCIMENTO, R., GOULART, L. R., PARDINGTON, P. E., CHAUDHARY, A., NORVELL, M., CIVEROLO, E. & GUPTA, G. 2012. An engineered innate immune defense protects grapevines from Pierce disease. *Proceedings of the National Academy of Sciences of the United States of America,* 109, 3721-3725.

DAVIS, M. J., PURCELL, A. H. & THOMSON, S. V. 1978. Pierces Disease of grapevines - isolation of causal bacterium. *Science,* 199, 75-77.

DAVIS, M. J., PURCELL, A. H. & THOMSON, S. V. 1980. Isolation media for the pierces disease bacterium. *Phytopathology,* 70, 425-429.

DAVIS, M. J., RAJU, B. C., BRLANSKY, R. H., LEE, R. F., TIMMER, L. W., NORRIS, R. C. & MCCOY, R. E. 1983. Periwinkle wilt bacterium - axenic culture, pathogenicity, and relationships to other gram-negative, xylem-inhabiting bacteria. *Phytopathology,* 73, 1510-1515.

DE LA FUENTE, L., MONTANES, E., MENG, Y. Z., LI, Y. X., BURR, T. J., HOCH, H. C. & WU, M. M. 2007. Assessing adhesion forces of type I and type IV pili of *Xylella fastidiosa* bacteria by use of a microfluidic flow chamber. *Applied and Environmental Microbiology,* 73, 2690-2696.

DE SOUZA, A. A., TAKITA, M. A., COLETTA, H. D., CALDANA, C., GOLDMAN, G. H., YANAI, G. M., MUTO, N. H., DE OLIVEIRA, R. C., NUNES, L. R. & MACHADO, M. A. 2003. Analysis of gene expression in two growth states of *Xylella fastidiosa* and its relationship with pathogenicity. *Molecular Plant-Microbe Interactions,* 16, 867-875.

DILLON, R. & CHARNLEY, K. 2002. Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. *Research in Microbiology,* 153, 503-509.

DOUTHWAITE, J. A., GROVES, M. A., DUFNER, P. & JERMUTUS, L. 2006. An improved method for an efficient and easily accessible eukaryotic ribosome display technology. *Protein Engineering Design & Selection,* 19, 85-90.

DURVASULA, R. V., GUMBS, A., PANACKAL, A., KRUGLOV, O., AKSOY, S., MERRIFIELD, R. B., RICHARDS, F. F. & BEARD, C. B. 1997. Prevention of insect-borne disease: An approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 94, 3274-3278.

DURVASULA, R. V., GUMBS, A., PANACKAL, A., KRUGLOV, O., TANEJA, J., KANG, A. S., CORDON-ROSALES, C., RICHARDS, F. F., WHITHAM, R. G. & BEARD, C. B. 1999. Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via transgenic bacterial symbiont *Rhodococcus rhodnii. Medical and Veterinary Entomology,* 13, 115-119.

DURVASULA, R. V., SUNDARAM, R. K., KIRSCH, P., HURWITZ, I., CRAWFORD, C. V., DOTSON, E. & BEARD, C. B. 2008. Genetic transformation of a Corynebacterial symbiont from the Chagas disease vector *Triatoma infestans. Experimental Parasitology,* 119, 94-98.

FANG, W. G., VEGA-RODRIGUEZ, J., GHOSH, A. K., JACOBS-LORENA, M., KANG, A. & ST LEGER, R. J. 2011. Development of transgenic fungi that kill human Malaria Parasites in mosquitoes. *Science,* 331, 1074-1077.

FIECK, A., HURWITZ, I., KANG, A. S. & DURVASULA, R. 2010. *Trypanosoma cruzi*: Synergistic cytotoxicity of multiple amphipathic anti-microbial peptides to *T. cruzi* and potential bacterial hosts. *Experimental Parasitology,* 125, 342-347.

FJELLBIRKELAND, A., BEMANIAN, V., MCDONALD, I. R., MURRELL, J. C. & JENSEN, H. B. 2000. Molecular analysis of an outer membrane protein, MopB, of *Methylococcus capsulatus* (Bath) and structural comparisons with proteins of the OmpA family. *Archives of Microbiology,* 173, 346-351.

FRENCH, W. J., CHRISTIS, R. G. & STASSI, D. L. 1977. Recovery of rickettsia-like bacteria by vacuum infiltration of peach tissues affected with phony disease. *Phytopathology,* 67, 945-948.

FRENCH, W. J., STASSI, D. L. & SCHAAD, N. W. 1978. Use of immunofluorescence for the identification of phony peach bacterium. *Phytopathology,* 68, 1106-1108.

FRY, S. M. & MILHOLLAND, R. D. 1990. Multiplication and translocation of *Xylella fastidiosa* in petioles and stems of grapevine resistant, tolerant, and susceptible to Pierces Disease. *Phytopathology,* 80, 61-65.

GASTEIGER, E., GATTIKER, A., HOOGLAND, C., IVANYI, I., APPEL, R. D. & BAIROCH, A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research,* 31, 3784-3788.

GAVINI, F., MERGAERT, J., BEJI, A., MIELCAREK, C., IZARD, D., KERSTERS, K. & DELEY, J. 1989. Transfer of *enterobacter-agglomerans* (beijerinck 1888) ewing and fife 1972 to *pantoea* gen-nov as *pantoea*

agglomerans comb nov and description of *pantoea-dispersa* sp-nov. *International Journal of Systematic Bacteriology,* 39, 337-345.

GEORGIOU, G., STATHOPOULOS, C., DAUGHERTY, P. S., NAYAK, A. R., IVERSON, B. L. & CURTISS, R. 1997. Display of heterologous proteins on the surface of microorganisms: From the screening of combinatorial libraries to live recombinant vaccines. *Nature Biotechnology,* 15, 29-34.

GIUDICELLI, V., CHAUME, D. & LEFRANC, M. P. 2004. IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis. *Nucleic Acids Research,* 32, W435-W440.

GOHEEN, A. C., NYLAND, G. & LOWE, S. K. 1973. Association of a rickettsia-like organism with Pierces Disease of grapevines and alfalfa dwarf and heat therapy of disease in grapevines. *Phytopathology,* 63, 341-345.

GOTOH, N., WAKEBE, H., YOSHIHARA, E., NAKAE, T. & NISHINO, T. 1989. Role of protein-f in maintaining structural integrity of the *Pseudomonas-aeruginosa* outer-membrane. *Journal of Bacteriology,* 171, 983-990.

GUBLER, W. D., STAPLETON, J. J., LEAVITT, G. M., PURCELL, A. H., VARELA, L. G. & SMITH, R. J. 2006. UC IPM Pest Management Guidelines: Grape. *University of California Agriculture and Natural Resources, Publications.*

GUGLIELMI, L. & MARTINEAU, P. 2009. Expression of single-chain Fv fragments in *E. coli* cytoplasm. *Methods in molecular biology (Clifton, N.J.),* 562, 215-224.

GUILHABERT, M. R. & KIRKPATRICK, B. C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute to *X-fastidiosa* biofilm maturation and colonization and attenuate virulence. *Molecular Plant-Microbe Interactions,* 18, 856-868.

HANAHAN, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology,* 166, 557-580.

HANES, J. & PLUCKTHUN, A. 1997. *In vitro* selection and evolution of functional proteins by using ribosome display. *Proceedings of the National Academy of Sciences of the United States of America,* 94, 4937-4942.

HANNIG, G. & MAKRIDES, S. C. 1998. Strategies for optimizing heterologous protein expression in *Escherichia coli. Trends in Biotechnology,* 16, 54-60.

HE, M., COOLEY, N., JACKSON, A. & TAUSSIG, M. J. 2004. Production of human single-chain antibodies by ribosome display. *Methods in Molecular Biology,* 248, 177-189.

HE, M. & TAUSSIG, M. J. 1997. Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for *in vitro* display and evolution of antibody combining sites. *Nucleic Acids Research,* 25, 5132-5134.

HE, M. & TAUSSIG, M. J. 2002. Ribosome display: cell-free protein display technology. *Briefings in Functional Genomics and Proteomics,* 1, 204-212.

HE, M. & TAUSSIG, M. J. 2007. Eukaryotic ribosome display with in situ DNA recovery. *Nature Methods,* 4, 281-288.

HE, M. Y. & KHAN, F. 2005. Ribosome display: next-generation display technologies for production of antibodies *in vitro*. *Expert Review of Proteomics,* 2, 421-430.

HEARON, S. S., SHERALD, J. L. & KOSTKA, S. J. 1980. Association of xylem-limited bacteria with elm, sycamore, and oak leaf scorch. *Canadian Journal of Botany-Revue Canadienne De Botanique,* 58, 1986-1993.

HENNEBERGER, T. S. M., STEVENSON, K. L., BRITTON, K. O. & CHANG, C. J. 2004. Distribution of *Xylella fastidiosa* in sycamore associated with low temperature and host resistance. *Plant Disease,* 88, 951-958.

HEWITT, W. B., HOUSTON, B. R., FRAZIER, N. W. & FREITAG, J. H. 1946. Leafhopper transmission of the virus causing Pierces Disease of grape and dwarf of alfalfa. *Phytopathology,* 36, 117-128.

HILL, B. L. & PURCELL, A. H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and 4 other plants. *Phytopathology,* 85, 1368-1372.

HOPKINS, D. L. 1989. *Xylella fastidiosa* - xylem-limited bacterial pathogen of plants. *Annual Review of Phytopathology,* 27, 271-290.

HOPKINS, D. L. 2005. Biological control of Pierce's Disease in the vineyard with strains of *Xylella fastidiosa* benign to grapevine. *Plant Disease,* 89, 1348-1352.

HOPKINS, D. L. & ADLERZ, W. C. 1988. Natural hosts of *Xylella fastidiosa* in florida. *Plant Disease,* 72, 429-431.

HOPKINS, D. L. & MOLLENHA, HH. 1973. Rickettsia-like bacterium associated with Pierces Disease of grapes. *Science,* 179, 298-300.

HOPKINS, D. L. & MORTENSE, JA. 1971. Suppression of pierces disease symptoms by tetracycline antibiotics. *Plant Disease Reporter,* 55, 610-612.

HOPKINS, D. L. & PURCELL, A. H. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease,* 86, 1056-1066.

HUSTON, J. S., MUDGETTHUNTER, M., TAI, M. S., MCCARTNEY, J., WARREN, F., HABER, E. & OPPERMANN, H. 1991. Protein engineering of single-chain fv analogs and fusion proteins. *Methods in Enzymology,* 203, 46-88.

HUTCHINS, L. M., COCHRAN, L. C., TURNER, W. F. & WEINBERGER, J. H. 1953. Transmission of phony disease virus from tops of certain affected peach and plum trees. *Phytopathology,* 43, 691-696.

JACKSON, B. C., BLUA, M. J. & BEXTINE, B. 2008. Impact of duration versus frequency of probing by Homalodisca vitripennis (Hemiptera : Cicadellidae) on inoculation of *Xylella fastidiosa*. *Journal of Economic Entomology,* 101, 1122-1126.

KANE, J. F. 1995. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Current Opinion in Biotechnology,* 6, 494-500.

KANGA, L. H. B., JONES, W. A., HUMBER, R. A. & BOYD, D. W. JR. 2004. Fungal pathogens of the glassy-winged sharpshooter *Homalodisca coagulata* (Homoptera: Cicadellidae). *Florida Entomologist,* 87, 225-228.

KETTLEBOROUGH, C. A., SALDANHA, J., ANSELL, K. H. & BENDIG, M. M. 1993. Optimization of primers for cloning libraries of mouse immunoglobulin genes using the polymerase chain-reaction. *European Journal of Immunology,* 23, 206-211.

KHAN, N. A., SHIN, S., CHUNG, J. W., KIM, K. J., ELLIOTT, S., WANG, Y. & KIM, K. S. 2003. Outer membrane protein A and cytotoxic necrotizing factor-1 use diverse signaling mechanisms for *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. *Microbial Pathogenesis,* 35, 35-42.

KIPRIYANOV, S. M. 2002. High-level periplasmic expression and purification of scFvs. *Methods in molecular biology (Clifton, N.J.),* 178, 333-41.

KOEBNIK, R., LOCHER, K. P. & VAN GELDER, P. 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Molecular Microbiology,* 37, 239-253.

KOHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* 256, 495-497.

KOIDE, T., VENCIO, R. Z. N. & GOMES, S. L. 2006. Global gene expression analysis of the heat shock response in the phytopathogen *Xylella fastidiosa*. *Journal of Bacteriology,* 188, 5821-5830.

KOLAJ, O., SPADA, S., ROBIN, S. & WALL, J. G. 2009. Use of folding modulators to improve heterologous protein production in *Escherichia coli*. *Microbial Cell Factories,* 8, 9-25.

KOST, T. A., CONDREAY, J. P. & JARVIS, D. L. 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nature Biotechnology,* 23, 567-575.

KOSTAKIOTI, M., NEWMAN, C. L., THANASSI, D. G. & STATHOPOULOS, C. 2005. Mechanisms of protein export across the bacterial outer membrane. *Journal of Bacteriology,* 187, 4306-4314.

KOZAK, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Research,* 15, 8125-8148.

KREWER, G., DUTCHER, J. D. & CHANG, C. J. 2002. Imidacloprid insecticide slows development of Pierce's Disease in bunch grapes. *Journal of Entomological Science,* 37, 101-112.

KROGH, A. 1997. Two methods for improving performance of an HMM and their application for gene finding. *Proceedings / ... International Conference on Intelligent Systems for Molecular Biology ; ISMB. International Conference on Intelligent Systems for Molecular Biology,* 5, 179-186.

KRUGNER, R., JOHNSON, M. W., GROVES, R. L. & MORSE, J. G. 2008. Host specificity of Anagrus epos: a potential biological control agent of Homalodisca vitripennis. *Biocontrol,* 53, 439-449.

KURLAND, C. & GALLANT, J. 1996. Errors of heterologous protein expression. *Current Opinion in Biotechnology,* 7, 489-493.

KUZINA, L. V., MILLER, T. A. & COOKSEY, D. A. 2006. *In vitro* activities of antibiotics and antimicrobial peptides against the plant pathogenic bacterium *Xylella fastidiosa*. *Letters in Applied Microbiology,* 42, 514-520.

LACAVAL, P. T., AZEVEDO, J. L., MILLER, T. A. & S., H. J. 2009. Interactions of *Xylella fastidiosa* and Endophytic Bacteria in Citrus: A Review. *Tree and Forestry Science and Biotechnology, Global Science Books*, 40-46.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227**,** 680-5.

LAMPE, D. J., R., C., LAUZON, C. R. & MILLER, T. 2006. Development of symbiotic control of Pierce's Disease. http://*biopesticide.ucr.edu/abstracts/assets/Lampe_abstract.pdf*.

LEFRANC, M. P., GIUDICELLI, V., GINESTOUX, C., JABADO-MICHALOUD, J., FOLCH, G., BELLAHCENE, F., WU, Y., GEMROT, E., BROCHET, X., LANE, J., REGNIER, L., EHRENMANN, F., LEFRANC, G. & DUROUX, P. 2009. IMGT (R), the international ImMunoGeneTics information system (R). *Nucleic Acids Research,* 37, D1006-D1012.

LEI, S. P., LIN, H. C., WANG, S. S., CALLAWAY, J. & WILCOX, G. 1987. Characterization of the *Erwinia carotovora pelB* gene and its product pectate lyase. *Journal of Bacteriology,* 169, 4379-4383.

LI, Y. X., HAO, G. X., GALVANI, C. D., MENG, Y. Z., DE LA FUENTE, L., HOCH, H. C. & BURR, T. J. 2007. Type I and type IV pili of *Xylella*

fastidiosa affect twitching motility, biofilm formation and cell-cell aggregation. *Microbiology,* 153, 719-726.

LINDOW, S. E. & BRANDL, M. T. 2003. Microbiology of the phyllosphere. *Applied and Environmental Microbiology,* 69, 1875-1883.

LONCARIC, I., HEIGL, H., LICEK, E., MOOSBECKHOFER, R., BUSSE, H. J. & ROSENGARTEN, R. 2009. Typing of *Pantoea agglomerans* isolated from colonies of honey bees (Apis mellifera) and culturability of selected strains from honey. *Apidologie,* 40, 40-54.

MAKRIDES, S. C. 1996. Strategies for achieving high-level expression of genes in *Escherichia coli. Microbiological Reviews,* 60, 512-38.

MARKIV, A., ANANI, B., DURVASULA, R. V. & KANG, A. S. 2011. Module based antibody engineering: A novel synthetic REDantibody. *Journal of Immunological Methods,* 364, 40-49.

MARTIN, C. D., ROJAS, G., MITCHELL, J. N., VINCENT, K. J., WU, J., MCCAFFERTY, J. & SCHOFIELD, D. J. 2006. A simple vector system to improve performance and utilisation of recombinant antibodies. *BMC Biotechnology,* 6, 46-60.

MARTINEAU, P. & BETTON, J. M. 1999. *In vitro* folding and thermodynamic stability of an antibody fragment selected *in vivo* for high expression levels in *Escherichia coli* cytoplasm. *Journal of Molecular Biology,* 292, 921-929.

MATTHEAKIS, L. C., BHATT, R. R. & DOWER, W. J. 1994. An *in vitro* polysome display system for identifying ligands from very large peptide libraries. *Proceedings of the National Academy of Sciences of the United States of America,* 91, 9022-9026.

MATTHEWS, S., RAO, V. S. & DURVASULA, R. V. 2011. Modeling horizontal gene transfer (HGT) in the gut of the Chagas disease vector *Rhodnius prolixus*. *Parasites & Vectors,* 4, 77-85.

MENG, Y. Z., LI, Y. X., GALVANI, C. D., HAO, G. X., TURNER, J. N., BURR, T. J. & HOCH, H. C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *Journal of Bacteriology,* 187, 5560-5567.

MEYER, T. S. & LAMBERTS, B. L. 1965. Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips. *Biochim Biophys Acta,* 107, 144-145.

MILLER, T. 2007. Symbiotic control applications in agriculture and medicine. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 146, S218-S218.

MILLER, T. A. 2011. Paratransgenesis as a potential tool for pest control: review of applied arthropod symbiosis. *Journal of Applied Entomology,* 135, 474-478.

MINSAVAGE, G. V., THOMPSON, C. M., HOPKINS, D. L., LEITE, R. & STALL, R. E. 1994. Development of a polymerase chain-reaction protocol for detection of *Xylella fastidiosa* in plant-tissue. *Phytopathology,* 84, 456-461.

MIRCETICH, S. M., LOWE, S. K., MOLLER, W. J. & NYLAND, G. 1976. Etiology of almond leaf scorch disease and transmission of causal agent. *Phytopathology,* 66, 17-24.

MORANO, L. D., BEXTINE, B. R., GARCIA, D. A., MADDOX, S. V., GUNAWAN, S., VITOVSKY, N. J. & BLACK, M. C. 2008. Initial genetic analysis of *Xylella fastidiosa* in Texas. *Current Microbiology,* 56, 346-351.

MORTENSEN, J. A., STOVER, L. H. & BALERDI, C. F. 1977. Sources of resistance to pierces disease in vitis. *Journal of the American Society for Horticultural Science,* 102, 695-697.

MYERS, A. L., SUTTON, T. B., ABAD, J. A. & KENNEDY, G. G. 2007. Pierce's Disease of grapevines: Identification of the primary vectors in North Carolina. *Phytopathology,* 97, 1440-1450.

NADARASAH, G. & STAVRINIDES, J. 2011. Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiology Reviews,* 35, 555-575.

NOVY, R., DROTT, D., YAEGER, K. & MIERENDORF, R. 2001. Overcoming the codon bias of *E. coli* for enhanced protein expression. *InNovations (Novagen, Inc. newsletter),* 12, 1-3.

NYLAND, G., GOHEEN, A. C., LOWE, S. K. & KIRKPATR.HC 1973. Ultrastructure of a rickettsia-like organism from a peach tree affected with phony disease. *Phytopathology,* 63, 1275-1278.

O'TOOLE, G. A., PRATT, L. A., WATNICK, P. I., NEWMAN, D. K., WEAVER, V. B. & KOLTER, R. 1999. Genetic approaches to study of biofilms. *Methods in Enzymology,* 310, 91-109.

OLIVEIRA, A. C., VALLIM, M. A., SEMIGHINI, C. P., ARAUJO, W. L., GOLDMAN, G. H. & MACHADO, M. A. 2002. Quantification of *Xylella fastidiosa* from citrus trees by real-time polymerase chain reaction assay. *Phytopathology,* 92, 1048-1054.

PEREZ-DONOSO, A. G., GREVE, L. C., WALTON, J. H., SHACKEL, K. A. & LABAVITCH, J. M. 2007. *Xylella fastidiosa* infection and ethylene exposure result in xylem and water movement disruption in grapevine shoots. *Plant Physiology,* 143, 1024-1036.

PEREZ-DONOSO, A. G., SUN, Q., ROPER, M. C., GREVE, L. C., KIRKPATRICK, B. & LABAVITCH, J. M. 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa* infected grapevines. *Plant Physiology,* 152, 1748-1759.

PHILIBERT, P. & MARTINEAU, P. 2004. Directed evolution of single-chain Fv for cytoplasmic expression using the beta-galactosidase complementation assay results in proteins highly susceptible to protease degradation and aggregation. *Microbial Cell Factories,* 3, 16-32.

PILKINGTON, L. J., IRVIN, N. A., BOYD, E. A., HODDLE, M. S., TRIAPITSYN, S. V., CAREY, B. G., JONES, W. A. & MORGAN, D. J. W. 2005. Introduced parasitic wasps could control glassy-winged sharpshooter. *California Agriculture (REVIEW ARTICLE)*, 59, 223- 228.

PLUECKTHUN, A. 1991. Strategies for the expression of antibody fragments in *Escherichia coli*. *Methods (Orlando),* 2, 88-96.

PRASADARAO, N. V., WASS, C. A., WEISER, J. N., STINS, M. F., HUANG, S. H. & KIM, K. S. 1996. Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infection and Immunity,* 64, 146-153.

PRINZ, W. A., ASLUND, F., HOLMGREN, A. & BECKWITH, J. 1997. The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. *Journal of Biological Chemistry,* 272, 15661-15667.

PURCELL, A. H., FINLAY, A. H. & MCLEAN, D. L. 1979. Pierces Disease bacterium mechanism of transmission by leafhopper vectors. *Science,* 206, 839-841.

PURCELL, A. H. & HOPKINS, D. L. 1996. Fastidious xylem-limited bacterial plant pathogens. *Annual Review of Phytopathology,* 34, 131-151.

PURCELL, A. H., SAUNDERS, S. R., HENDSON, M., GREBUS, M. E. & HENRY, M. J. 1999. Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. *Phytopathology,* 89, 53-58.

PUSEY, P. L. 2002. Biological control agents for fire blight of apple compared under conditions limiting natural dispersal. *Plant Disease,* 86, 639-644.

RABINER, L. R. 1989. A tutorial on Hidden Markov Models and selected applications in speech recognition. *Proceedings of the Ieee,* 77, 257-286.

RAJU, B. C. & GOHEEN, A. C. 1981. Relative sensitivity of selected grapevine cultivars to Pierces Disease bacterial inoculations. *American Journal of Enology and Viticulture,* 32, 155-158.

RAJU, B. C., GOHEEN, A. C. & FRAZIER, N. W. 1983. Occurrence of Pierces Disease bacteria in plants and vectors in california. *Phytopathology,* 73, 1309-1313.

RAJU, B. C., WELLS, J. M., NYLAND, G., BRLANSKY, R. H. & LOWE, S. K. 1982. Plum leaf scald - isolation, culture, and pathogenicity of the causal agent. *Phytopathology,* 72, 1460-1466.

RAMIREZ, J. L., LACAVA, P. T. & MILLER, T. A. 2008. Detection of the bacterium, *Xylella fastidiosa*, in saliva of glassy-winged sharpshooter, Homalodisca vitripennis. *Journal of Insect Science,* 8, 34-40.

RAO, S., HU, S., MCHUGH, L., LUEDERS, K., HENRY, K., ZHAO, Q., FEKETE, R. A., KAR, S., ADHYA, S. & HAMER, D. H. 2005. Toward a live microbial microbicide for HIV: Commensal bacteria secreting an HIV fusion inhibitor peptide. *Proceedings of the National Academy of Sciences of the United States of America,* 102, 11993-11998.

REN, X. X., HOICZYK, E. & RASGON, J. L. 2008. Viral paratransgenesis in the malaria vector Anopheles gambiae. *Plos Pathogens,* 4, e1000135-e1000141.

RIO, R. V. M., HU, Y. J. & AKSOY, S. 2004. Strategies of the home-team: symbioses exploited for vector-borne disease control. *Trends in Microbiology,* 12, 325-336.

RITZ, D. & BECKWITH, J. 2001. Roles of thiol-redox pathways in bacteria. *Annual Review of Microbiology,* 55, 21-48.

ROBERTS, R. W. & SZOSTAK, J. W. 1997. RNA-peptide fusions for the *in vitro* selection of peptides and proteins. *Proceedings of the National Academy of Sciences of the United States of America,* 94, 12297-12302.

RODRIGUES, C. M., TAKITA, M. A., COLETTA-FILHO, H. D., OLIVATO, J. C., CASERTA, R., MACHADO, M. A. & DE SOUZA, A. A. 2008. Copper resistance of biofilm cells of the plant pathogen *Xylella fastidiosa*. *Applied Microbiology and Biotechnology,* 77, 1145-1157.

ROPER, M. C., GREVE, L. C., LABAVITCH, J. A. & KIRKPATRICK, B. C. 2007. Detection and visualization of an exopolysaccharide produced by *Xylella fastidiosa in vitro* and in planta. *Applied and Environmental Microbiology,* 73, 7252-7258.

ROSSER, M. P., XIA, W., HARTSELL, S., MCCAMAN, M., ZHU, Y., WANG, S. J., HARVEY, S., BRINGMANN, P. & COBB, R. R. 2005. Transient transfection of CHO-K1-S using serum-free medium in suspension: a rapid mammalian protein expression system. *Protein Expression and Purification,* 40, 237-243.

SANCHEZ, L., AYALA, M., FREYRE, F., PEDROSO, I., BELL, H., FALCON, V. & GAVILONDO, J. V. 1999. High cytoplasmic expression in *E coli,* purification, and *in vitro* refolding of a single chain Fv antibody fragment against the hepatitis B surface antigen. *Journal of Biotechnology,* 72, 13-20.

SANDKVIST, M. 2001. Biology of type II secretion. *Molecular Microbiology,* 40, 271-283.

SCHLAPSCHY, M., GRIMM, S. & SKERRA, A. 2006. A system for concomitant overexpression of four periplasmic folding catalysts to improve secretory

protein production in *Escherichia coli*. *Protein Engineering Design & Selection,* 19, 385-390.

SEIDEL, H. M., POMPLIANO, D. L. & KNOWLES, J. R. 1992. Phosphonate biosynthesis - molecular-cloning of the gene for phosphoenolpyruvate mutase from tetrahymena-pyriformis and overexpression of the gene-product in *Escherichia coli*. *Biochemistry,* 31, 2598-2608.

SHERALD, J., HEARON, S., KOSTKA, S. & MORGAN, D. 1982. Pathogenicity of a pierces disease (pd)-like bacterium cultured from leaf scorch affected sycamores. *Phytopathology,* 72, 710-710.

SHERALD, J. L. & LEI, J. D. 1991. Evaluation of a rapid elisa test kit for detection of *Xylella fastidiosa* in landscape trees. *Plant Disease,* 75, 200-203.

SHERALD, J. L., WELLS, J. M. & HURTT, S. S. 1987. Association of fastidious, xylem-inhabiting bacteria with leaf scorch in red maple. *Plant Disease,* 71, 930-933.

SHUSTA, E. V., VANANTWERP, J. & WITTRUP, K. D. 1999. Biosynthetic polypeptide libraries. *Current Opinion in Biotechnology,* 10, 117-122.

SIMPSON, A. J., REINACH, F. C., ARRUDA, P., ABREU, F. A., ACENCIO, M., ALVARENGA, R., ALVES, L. M., ARAYA, J. E., BAIA, G. S., BAPTISTA, C. S., BARROS, M. H., BONACCORSI, E. D., BORDIN, S., BOVE, J. M., BRIONES, M. R., BUENO, M. R., CAMARGO, A. A., CAMARGO, L. E., CARRARO, D. M., CARRER, H., COLAUTO, N. B., COLOMBO, C., COSTA, F. F., COSTA, M. C., COSTA-NETO, C. M., COUTINHO, L. L., CRISTOFANI, M., DIAS-NETO, E., DOCENA, C., EL-DORRY, H., FACINCANI, A. P., FERREIRA, A. J., FERREIRA, V. C., FERRO, J. A., FRAGA, J. S., FRANCA, S. C., FRANCO, M. C., FROHME, M., FURLAN, L. R., GARNIER, M., GOLDMAN, G. H., GOLDMAN, M. H., GOMES, S. L., GRUBER, A., HO, P. L., HOHEISEL, J. D., JUNQUEIRA, M. L., KEMPER, E. L., KITAJIMA, J. P., KRIEGER, J. E., KURAMAE, E. E., LAIGRET, F., LAMBAIS, M. R., LEITE, L. C., LEMOS, E. G., LEMOS, M. V., LOPES, S. A., LOPES, C. R., MACHADO, J. A., MACHADO, M. A., MADEIRA, A. M., MADEIRA, H. M., MARINO, C. L., MARQUES, M. V., MARTINS, E. A., MARTINS, E. M., MATSUKUMA, A. Y., MENCK, C. F., MIRACCA, E. C., MIYAKI, C. Y., MONTERIRO-VITORELLO, C. B., MOON, D. H., NAGAI, M. A., NASCIMENTO, A. L., NETTO, L. E., NHANI, A., JR., NOBREGA, F. G., NUNES, L. R., OLIVEIRA, M. A., DE OLIVEIRA, M. C., DE OLIVEIRA, R. C., PALMIERI, D. A., PARIS, A., PEIXOTO, B. R., PEREIRA, G. A., PEREIRA, H. A., JR., PESQUERO, J. B., QUAGGIO, R. B., ROBERTO, P. G., RODRIGUES, V., DE, M. R. A. J., DE ROSA, V. E., JR., DE SA, R. G., SANTELLI, R. V., SAWASAKI, H. E., DA SILVA, A. C., DA SILVA, A. M., DA SILVA, F. R., DA SILVA, W. A., JR., DA SILVEIRA, J. F., et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa.* The *Xylella fastidiosa* Consortium of the Organization for Nucleotide Sequencing and Analysis. *Nature,* 406, 151-159.

SORENSEN, H. P. & MORTENSEN, K. K. 2005. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *Journal of Biotechnology,* 115, 113-128.

SPYROPOULOS, I. C., LIAKOPOULOS, T. D., BAGOS, P. G. & HAMODRAKAS, S. J. 2004. TMRPres2D: high quality visual representation of transmembrane protein models. *Bioinformatics,* 20, 3258-3260.

STEIDLER, L. 2001. *Lactococcus lactis,* A Tool for the Delivery of Therapeutic Proteins Treatment of IBD. *Scientific World Journal,* 1, 216-217.

STEIDLER, L., HANS, W., SCHOTTE, L., NEIRYNCK, S., OBERMEIER, F., FALK, W., FIERS, W. & REMAUT, E. 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science,* 289, 1352-1355.

STEVENSON, J. F., MATTHEWS, M. A., GREVE, L. C., LABAVITCH, J. M. & ROST, T. L. 2004. Grapevine susceptibility to Pierce's Disease II: Progression of anatomical symptoms. *American Journal of Enology and Viticulture,* 55, 238-245.

STEVENSON, J. F., MATTHEWS, M. A. & ROST, T. L. 2005. The developmental anatomy of Pierce's Disease symptoms in grapevines: Green islands and matchsticks. *Plant Disease,* 89, 543-548.

TORCHIN, M. E., LAFFERTY, K. D., DOBSON, A. P., MCKENZIE, V. J. & KURIS, A. M. 2003. Introduced species and their missing parasites. *Nature,* 421, 628-630.

TOWBIN, H., STAEHELIN, T. & GORDON, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America,* 76, 4350-4354.

TRIAPITSYN, S. V., & PHILLIPS, P. A. 2000. First record of Gonatocerus triguttatus (Hymenoptera: Mymaridae) from eggs of Homalodisca coagulata (Homoptera: Cicadellidae) with notes on the distribution of the host. *Florida Entomologist*, 83, 200-203.

TSUMOTO, K., EJIMA, D., KUMAGAI, I. & ARAKAWA, T. 2003. Practical considerations in refolding proteins from inclusion bodies. *Protein Expression and Purification,* 28, 1-8.

VAN EMDEN, H. F. 1989. Pest control, *2nd Edition, Edward Arnold, London*, 117pp.

VAN SLUYS, M. A., DE OLIVEIRA, M. C., MONTEIRO-VITORELLO, C. B., MIYAKI, C. Y., FURLAN, L. R., CAMARGO, L. E. A., DA SILVA, A. C. R., MOON, D. H., TAKITA, M. A., LEMOS, E. G. M., MACHADO, M. A., FERRO, M. I. T., DA SILVA, F. R., GOLDMAN, M. H. S., GOLDMAN, G. H., LEMOS, M. V. F., EL-DORRY, H., TSAI, S. M., CARRER, H., CARRARO, D. M., DE OLIVEIRA, R. C., NUNES, L. R., SIQUEIRA, W.

J., COUTINHO, L. L., KIMURA, E. T., FERRO, E. S., HARAKAVA, R., KURAMAE, E. E., MARINO, C. L., GIGLIOTI, E., ABREU, I. L., ALVES, L. M. C., DO AMARAL, A. M., BAIA, G. S., BLANCO, S. R., BRITO, M. S., CANNAVAN, F. S., CELESTINO, A. V., DA CUNHA, A. F., FENILLE, R. C., FERRO, J. A., FORMIGHIERI, E. F., KISHI, L. T., LEONI, S. G., OLIVEIRA, A. R., ROSA, V. E., SASSAKI, F. T., SENA, J. A. D., DE SOUZA, A. A., TRUFFI, D., TSUKUMO, F., YANAI, G. M., ZAROS, L. G., CIVEROLO, E. L., SIMPSON, A. J. G., ALMEIDA, N. F., SETUBAL, J. C. & KITAJIMA, J. P. 2003. Comparative analyses of the complete genome sequences of Pierce's Disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *Journal of Bacteriology,* 185, 1018-1026.

VOEGEL, T. M., WARREN, J. G., MATSUMOTO, A., IGO, M. M. & KIRKPATRICK, B. C. 2010. Localization and characterization of *Xylella fastidiosa* haemagglutinin adhesins. *Microbiology-Sgm,* 156, 2172-2179.

WANG, Y. 2002. The function of OmpA in *Escherichia coli*. *Biochemical and Biophysical Research Communications,* 292, 396-401.

WAYADANDE, A., BRUTON, B., FLETCHER, J., PAIR, S. & MITCHELL, F. 2005. Retention of cucurbit yellow vine disease bacterium *Serratia marcescens* through transstadial molt of vector *Anasa tristis* (Hemiptera : Coreidae). *Annals of the Entomological Society of America,* 98, 770-774.

WELLS, J. M., RAJU, B. C., HUNG, H. Y., WEISBURG, W. G., MANDELCOPAUL, L. & BRENNER, D. J. 1987. *Xylella fastidiosa* gen-nov, sp-nov - gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas*-spp. *International Journal of Systematic Bacteriology,* 37, 136-143.

WELLS, J. M., RAJU, B. C. & NYLAND, G. 1983. Isolation, culture, and pathogenicity of the bacterium causing phony disease of peach. *Phytopathology,* 73, 859-862.

WELLS, J. M., RAJU, B. C., THOMPSON, J. M. & LOWE, S. K. 1981. Etiology of phony peach and plum leaf scald diseases. *Phytopathology,* 71, 1156-1161.

WINTER, G., GRIFFITHS, A. D., HAWKINS, R. E. & HOOGENBOOM, H. R. 1994. Making antibodies by phage display technology. *Annual Review of Immunology,* 12, 433-455.

WOODRUFF, W. A. & HANCOCK, R. E. W. 1989. *Pseudomonas aeruginosa* outer-membrane protein-f - structural role and relationship to the *Escherichia coli* ompa protein. *Journal of Bacteriology,* 171, 3304-3309.

YAN, X. H. & XU, Z. R. 2006. Ribosome-display technology: applications for directed evolution of functional proteins. *Drug Discovery Today,* 11, 911-916.

YONCE, C. E. & CHANG, C. J. 1987. Detection of xylem-limited bacteria from sharpshooter leafhoppers and their feeding hosts in peach environs monitored by culture isolations and elisa techniques. *Environmental Entomology,* 16, 68-71.

YOUSFI MONOD, M., GIUDICELLI, V., CHAUME, D. & LEFRANC, M.-P. 2004. IMGT/JunctionAnalysis: the first tool for the analysis of the immunoglobulin and T cell receptor complex V-J and V-D-J JUNCTIONs. *Bioinformatics (Oxford, England),* 20 Suppl 1, i379-i385.

ZHANG, H. K., TORKAMANI, A., JONES, T. M., RUIZ, D. I., PONS, J. & LERNER, R. A. 2011. Phenotype-information-phenotype cycle for deconvolution of combinatorial antibody libraries selected against complex systems. *Proceedings of the National Academy of Sciences of the United States of America,* 108, 13456-13461.

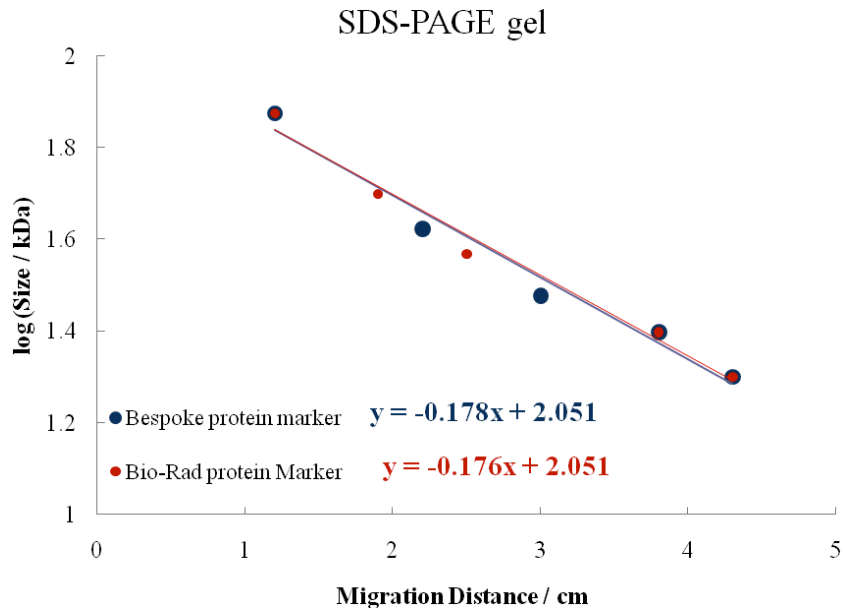
Appendix

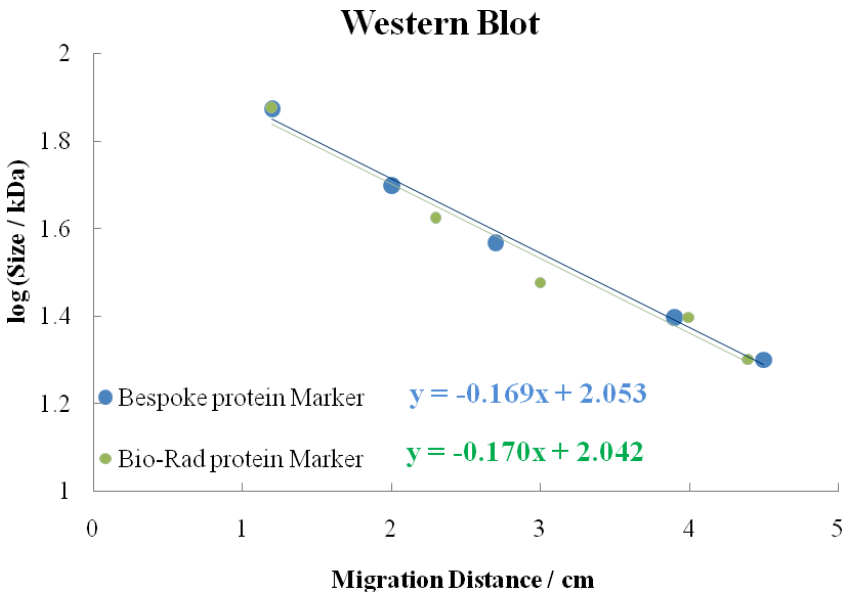
Appendix

Appendix 1

1.1 Bespoke and Bio-Rad protein markers size calibration graphs.

Size calibration graph was plotted for both SDS-PAGE gel and Western blot results to show the correlation between two sets of protein marker.





1.2 Full length mature and truncated mopB sequence in pSANG10-3F vector.

Showing: the *Nde*I, *Not*I and *Hind*IIIsites, 6xHis-tag and Tri-Flag.

a) Full length mature *mopB* in pSANG (1084 bp)

```
atacatatggcccaggaattcgatgaccggtggtatctcgctggttccaccgggtttaac
 I  H  M  A  Q  E  F  D  D  R  W  Y  L  A  G  S  T  G  F  N
ttccaagataagcgtcgtcttactaacgatgctccttttttcgctcttggaattggtaag
 F  Q  D  K  R  R  L  T  N  D  A  P  F  F  A  L  G  I  G  K
ttcatcaatccagtttggtcgatcgacggcacactgaactatcagaacccgcagttcaaa
 F  I  N  P  V  W  S  I  D  G  T  L  N  Y  Q  N  P  Q  F  K
agaaacaaagatctaaactggagccaatatggcttctctgtcgattttcgtcgccatttc
 R  N  K  D  L  N  W  S  Q  Y  G  F  S  V  D  F  R  R  H  F
atccaggataaccgcggctggaatccttacttattgttaggagcggggtaccaacgttca
 I  Q  D  N  R  G  W  N  P  Y  L  L  L  G  A  G  Y  Q  R  S
gaggaggaatttgataacaccccaaacgtaaactcccctggcgaacgtaagaaaggcggc
 E  E  E  F  D  N  T  P  N  V  N  S  P  G  E  R  K  K  G  G
tttgctgctaaggtaggcgctggcttgcagacaacattcagaagccgtgtcgccgtccgt
 F  A  A  K  V  G  A  G  L  Q  T  T  F  R  S  R  V  A  V  R
gctgaagtagcttatcgtggtgaccatgataaggatagcgtgcgtcagccggtggctggt
 A  E  V  A  Y  R  G  D  H  D  K  D  S  V  R  Q  P  V  A  G
gttgcggcaccttccagcaagaaatggtttggtgataccttggcttcgattggtgtcgtg
 V  A  A  P  S  S  K  K  W  F  G  D  T  L  A  S  I  G  V  V
atcccatttggtccagccgcttcctcaccaacgccaccacctgctccgcctgctccaaca
 I  P  F  G  P  A  A  S  S  P  T  P  P  P  A  P  P  A  P  T
ccgccgccgccaccgcctgctccaagctgtgctgaattggatagcgatggagatggagtc
 P  P  P  P  P  P  A  P  S  C  A  E  L  D  S  D  G  D  G  V
aacgactgtgacgacaagtgcccgaactcacaaccaggccagacgatcggaccagatgga
 N  D  C  D  D  K  C  P  N  S  Q  P  G  Q  T  I  G  P  D  G
tgtccggtgccagtgtcgattgacttaaaaggcgtaaacttcgattttgacaagtctaag
 C  P  V  P  V  S  I  D  L  K  G  V  N  F  D  F  D  K  S  K
cttcgtcctgacgccgttgcggttcttaaggaggctaccgaaatcttgagacgctaccct
 L  R  P  D  A  V  A  V  L  K  E  A  T  E  I  L  R  R  Y  P
gacttgcacgtagaggtggctggccataccgattcgacaggtccggctgcttataacaaa
 D  L  H  V  E  V  A  G  H  T  D  S  T  G  P  A  A  Y  N  K
aagctatcggaacgtcgcgctaaagtcgtgtatgactacctgcgtaacaatggtatcgac
 K  L  S  E  R  R  A  K  V  V  Y  D  Y  L  R  N  N  G  I  D
gcatctcgcctgattgggccagtcggatatggggagacccatccaattgatacaaacaaa
 A  S  R  L  I  G  P  V  G  Y  G  E  T  H  P  I  D  T  N  K
accccagcaggtcgtgcgaagaaccgccgtactgagttgaacgttcagaatgcggccgca
 T  P  A  G  R  A  K  N  R  R  T  E  L  N  V  Q  N  A  A  A
ttatcacatcatcatcaccatcacaagctggactacaaagaccatgacggtgattataaa
 L  S  H  H  H  H  H  H  K  L  D  Y  K  D  H  D  G  D  Y  K
gatcatgacatcgattacaaggatgacgatgacaagtaataaaagcttttaataagtcgag
 D  H  D  I  D  Y  K  D  D  D  D  K  -  -  K  L  -  -  V  E
caccac
 H  H
```

b) Truncated *mopB* in pSANG (564 bp)

```
atacatatggcccaggaattcgatgaccggtggtatctcgctggttccaccgggtttaac
 I   H   M   A   Q   E   F   D   D   R   W   Y   L   A   G   S   T   G   F   N
ttccaagataagcgtcgtcttactaacgatgctccttttttcgctcttggaattggtaag
 F   Q   D   K   R   R   L   T   N   D   A   P   F   F   A   L   G   I   G   K
ttcatcaatccagtttggtcgatcgacggcacactgaactatcagaacccgcagttcaaa
 F   I   N   P   V   W   S   I   D   G   T   L   N   Y   Q   N   P   Q   F   K
agaaacaaagatctaaactggagccaatatggcttctctgtcgattttcgtcgccatttc
 R   N   K   D   L   N   W   S   Q   Y   G   F   S   V   D   F   R   R   H   F
atccaggataaccgcggctggaatccttacttattgttaggagcggggtaccaacgttca
 I   Q   D   N   R   G   W   N   P   Y   L   L   L   G   A   G   Y   Q   R   S
gaggaggaatttgataacaccccaaacgtaaactcccctggcgaacgtaagaaaggcggc
 E   E   E   F   D   N   T   P   N   V   N   S   P   G   E   R   K   K   G   G
tttgctgctaaggtaggcgctggcttgcagacaacattcagaagccgtgtcgccgtccgt
 F   A   A   K   V   G   A   G   L   Q   T   T   F   R   S   R   V   A   V   R
gctgaagtagcttatcgtggtgaccatgataaggatagcgtgcgtcagccggtggctggt
 A   E   V   A   Y   R   G   D   H   D   K   D   S   V   R   Q   P   V   A   G
gttgcggcaccttccagcaagaaatggtttggtgataccttggcttcgattggtgtcgtg
 V   A   A   P   S   S   K   K   W   F   G   D   T   L   A   S   I   G   V   V
atcccatttggtgcggccgcatgctcacatcatcatcaccatcacaagctggactacaaa
 I   P   F   G   A   A   A   C   S   H   H   H   H   H   H   K   L   D   Y   K
gaccatgacggtgattataaagatcatgacatcgattacaaggatgacgatgacaagtaa
 D   H   D   G   D   Y   K   D   H   D   I   D   Y   K   D   D   D   D   K   -
Taaaagcttaataagtcgagcaccac
 -   K   L   -   -   V   E   H   H
```

1.3 Recovered anti-MopB scFv's (4XfMopB1-4) antibodies DNA sequences.

Showing: the *Nco*I and *Not*I sites, variable light chain, linker and variable heavy chain.

a) 4XfMopB1

The GenBank accession number for the 4XfMopB1 scFv is JQ606804.

```
                                                    accatggcagaaatt
                                                     T  M  A  E  I
gtgctgacccagtttccaacatccctgtccgtggctacaggagaaaaagtcactatcaga
 V  L  T  Q  F  P  T  S  L  S  V  A  T  G  E  K  V  T  I  R
tgcataaccagcactgatattgatgatgatatgaactggtaccagcagaagccaggggaa
 C  I  T  S  T  D  I  D  D  D  M  N  W  Y  Q  Q  K  P  G  E
ccccctaagctccttatttcagaagccaatactcttcgtcctggagtcccatcccgattc
 P  P  K  L  L  I  S  E  A  N  T  L  R  P  G  V  P  S  R  F
tccagcagtggctatggtacggattttgtttttacaattgaaaacatgctctcagaagat
 S  S  S  G  Y  G  T  D  F  V  F  T  I  E  N  M  L  S  E  D
gttgcagattactactgtttgcaaagtgataacatgccttacacgctcggaggggggacc
 V  A  D  Y  Y  C  L  Q  S  D  N  M  P  Y  T  L  G  G  G  T
aaactggaggtgctacgtggtggtggtggttctggtggtggtggttctggcggcggcggc
 K  L  E  V  L  R  G  G  G  G  S  G  G  G  G  S  G  G  G  G
tccggtggtggtggttctcagctgaaggactcaggacctggcctggtggcgccctcacag
 S  G  G  G  G  S  Q  L  K  D  S  G  P  G  L  V  A  P  S  Q
agcctgtccatcacttgcactgtctctgggttttcattaaccagctatggtgtacactgg
 S  L  S  I  T  C  T  V  S  G  F  S  L  T  S  Y  G  V  H  W
gttcgccagcctccaggaaagggtctggagtggctgggagtaatatgggctggtggaagc
 V  R  Q  P  P  G  K  G  L  E  W  L  G  V  I  W  A  G  G  S
acaaattataattcggctctcatgtccagactgagcatcaacaaagacaactccaagagc
 T  N  Y  N  S  A  L  M  S  R  L  S  I  N  K  D  N  S  K  S
caagttttcttaaaaatgaacagtctgcaaagtgatgacacagccatgtactactgtgcc
 Q  V  F  L  K  M  N  S  L  Q  S  D  D  T  A  M  Y  Y  C  A
agagatcaggtctacggtagtagctactatgctatggactactggggtcaaggaacccct
 R  D  Q  V  Y  G  S  S  Y  Y  A  M  D  Y  W  G  Q  G  T  P
ctcaccgtttcctcggcggccgca
 L  T  V  S  S  A  A  A
```

*Nco*I CCATGG

*Not*I GCGGCCGC

Linker ggtggtggtggttctggtggtggtggttctggcggcggcggctccggtggtggtggttct

b) 4XfMopB2

```
                                        accatggcagacgtccagataatccag
                                         T  M  A  D  V  Q  I  I  Q
actccagcctccctatctgtatctgtgggagagactgtcaccatcacatgtcgagcaagt
 T  P  A  S  L  S  V  S  V  G  E  T  V  T  I  T  C  R  A  S
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 E  N  I  Y  S  N  L  A  W  Y  Q  Q  K  Q  G  K  S  P  Q  L
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 L  V  Y  G  A  T  N  L  A  D  G  V  P  S  R  F  S  G  S  G
tctgggcaagattattctctaaccatcagcagcctggagtctgacgatacagcaacttat
 S  G  Q  D  Y  S  L  T  I  S  S  L  E  S  D  D  T  A  T  Y
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 Y  C  L  Q  H  G  E  S  P  L  T  F  G  A  G  T  K  L  T  V
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 L  R  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S  G  G  G
ggttctcaggggtggagtctgggggaggcttagtgaagcctggagggtccctgaaactc
 G  S  Q  G  V  E  S  G  G  G  L  V  K  P  G  G  S  L  K  L
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 P  E  K  R  L  E  W  V  A  S  I  S  S  G  I  T  Y  Y  P  D
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 S  V  K  G  R  F  T  I  S  R  D  N  A  R  N  I  L  N  L  Q
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 M  S  S  L  R  S  E  D  T  A  M  Y  Y  C  A  R  Y  Y  Y  G
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 T  S  F  Y  W  Y  F  D  V  W  G  A  G  T  P  L  T  V  S  A
gcggccgca
 A  A  A
```

*Nco*I CCATGG

*Not*I GCGGCCGC

Linker ggtggtggtggttctggtggtggtggttctggcggcggcggctccggtggtggtggttct

c) 4XfMopB3

The GenBank accession number for the 4XfMopB3 scFv is JQ606805.

```
                                                      accatggcagac
                                                        T  M  A  D
gtccagatgatccagactacagcctccctatctgcatctgtgggacaaactgtcaccacc
 V  Q  M  I  Q  T  T  A  S  L  S  A  S  V  G  Q  T  V  T  T
acatgtcgagcaagtgagaatattaacagttgtttagcatggtatcagcagaaacaggga
 T  C  R  A  S  E  N  I  N  S  C  L  A  W  Y  Q  Q  K  Q  G
aaatctcctcagctcctggtctataatgcaaaaaccttagcagaagatgtgccatcaagg
 K  S  P  Q  L  L  V  Y  N  A  K  T  L  A  E  D  V  P  S  R
ttcagtggcagtggatcaggcacacagttttctctgaagatcaacagcctgcagcctgaa
 F  S  G  S  G  S  G  T  Q  F  S  L  K  I  N  S  L  Q  P  E
gattttgggagttattactgtcaacatcattatggtactccgtggacgttcggtggaggc
 D  F  G  S  Y  Y  C  Q  H  H  Y  G  T  P  W  T  F  G  G  G
accaagctgaatctccaacgtggtggtggtggttctggtggtggtggttctggcggcggc
 T  K  L  N  L  Q  R  G  G  G  G  S  G  G  G  G  S  G  G  G
ggctccggtggtggtggttctcagatgcagcagcctggggcagaggttatgaagcctggg
 G  S  G  G  G  G  S  Q  M  Q  Q  P  G  A  E  V  M  K  P  G
gcctcagtcaagttgtcctgcacagcttctgacttcaacattaaagacacctttatgcac
 A  S  V  K  L  S  C  T  A  S  D  F  N  I  K  D  T  F  M  H
tgggtgaagcagaggcctgaacagggcctggagtggattggcaggattgatcctgcgatt
 W  V  K  Q  R  P  E  Q  G  L  E  W  I  G  R  I  D  P  A  I
ggtgaaactaaatatgacccgaagttccagggcaaggccactatagaagcagacacatcc
 G  E  T  K  Y  D  P  K  F  Q  G  K  A  T  I  E  A  D  T  S
tccaacacagcctacctgcagctcaccagcctgacatctgaggacactgccgtctattac
 S  N  T  A  Y  L  Q  L  T  S  L  T  S  E  D  T  A  V  Y  Y
tgtgcttttccccactactggggccaaggcacctcactcaccgtctcttcggcggccgca
 C  A  F  P  H  Y  W  G  Q  G  T  S  L  T  V  S  S  A  A  A
```

*Nco*I CCATGG

*Not*I GCGGCCGC

Linker ggtggtggtggttctggtggtggtggttctggcggcggcggctccggtggtggtggttct

d) 4XfMopB4

```
                                                accatggtacaaaatctt
                                                 T  M  V  Q  N  L
ctcacacagtctccagcaatcatgtctgcatctccaggggagaaggtcaccatgacctgc
 L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T  M  T  C
agtgccagctcaagtgtaagttacatgcactggtaccagcagaagtcaggcacctccccc
 S  A  S  S  S  V  S  Y  M  H  W  Y  Q  Q  K  S  G  T  S  P
aaaagatggatttatgacacatccaaactggcttctggagtccccgctcgcttcagtggc
 K  R  W  I  Y  D  T  S  K  L  A  S  G  V  P  A  R  F  S  G
agtgggtctgggacctcttactctctcacaatcagccgaatggaggctgaagatgctgcc
 S  G  S  G  T  S  Y  S  L  T  I  S  R  M  E  A  E  D  A  A
acttattactgtcagcaaaggagtagttacccactcacgttcggtgctgggacaaagctg
 T  Y  Y  C  Q  Q  R  S  S  Y  P  L  T  F  G  A  G  T  K  L
gctatgaaacgtggtggtggtggttctggtggtggtggttctggcggcggcggctccggt
 A  M  K  R  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S  G
ggtggtggttctcagcttcaggagtcgggacctgagctggtgtagcctggggcctcagtg
 G  G  G  S  Q  L  Q  E  S  G  P  E  L  V  -  P  G  A  S  V
aagatttcctgcaaagattctggctacacattcagtagtccttggataaactgggtgaag
 K  I  S  C  K  D  S  G  Y  T  F  S  S  P  W  I  N  W  V  K
cagaggcctggacagggtcttgagtggattggacggatttatcctggagatggaagaact
 Q  R  P  G  Q  G  L  E  W  I  G  R  I  Y  P  G  D  G  R  T
gactacaatggggacttcagggacaaggccacactgactgcagacaagtcctccagcaca
 D  Y  N  G  D  F  R  D  K  A  T  L  T  A  D  K  S  S  S  T
gcctacatgcagctcagtagcctgacatctgaggactctgcggtctattactgtgcaaga
 A  Y  M  Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R
tcaagggatggtaactacggatattactatgctatggactactggggtcaaggaacccca
 S  R  D  G  N  Y  G  Y  Y  Y  A  M  D  Y  W  G  Q  G  T  P
ctcaccgtctcctcggcggccgca
 L  T  V  S  S  A  A  A
```

*Nco*I CCATGG

*Not*I GCGGCCGC

Linker ggtggtggtggttctggtggtggtggttctggcggcggcggctccggtggtggtggttct

Stop codon tag

1.4 4XfMopB3 scFv antibody DNA sequences in pAHAHis expression vector.

Showing: the *Nco*I and *Not*I sites, variable light chain, linker, variable heavy chain, HA-tag and 6xHis-tag.

```
            accatggcagacgtccagatgatccagactacagcctccctatctgca
             T  M  A  D  V  Q  M  I  Q  T  T  A  S  L  S  A
tctgtgggacaaactgtcaccaccacatgtcgagcaagtgagaatattaacagttgttta
 S  V  G  Q  T  V  T  T  T  C  R  A  S  E  N  I  N  S  C  L
gcatggtatcagcagaaacagggaaaatctcctcagctcctggtctataatgcaaaaacc
 A  W  Y  Q  Q  K  Q  G  K  S  P  Q  L  L  V  Y  N  A  K  T
ttagcagaagatgtgccatcaaggttcagtggcagtggatcaggcacacagttttctctg
 L  A  E  D  V  P  S  R  F  S  G  S  G  S  G  T  Q  F  S  L
aagatcaacagcctgcagcctgaagattttgggagttattactgtcaacatcattatggt
 K  I  N  S  L  Q  P  E  D  F  G  S  Y  Y  C  Q  H  H  Y  G
actccgtggacgttcggtggaggcaccaagctgaatctccaacgtggtggtggtggttct
 T  P  W  T  F  G  G  G  T  K  L  N  L  Q  R  G  G  G  G  S
ggtggtggtggttctggcggcggcggctccggtggtggtggttctcagatgcagcagcct
 G  G  G  G  S  G  G  G  G  S  G  G  G  G  S  Q  M  Q  Q  P
ggggcagaggttatgaagcctggggcctcagtcaagttgtcctgcacagcttctgacttc
 G  A  E  V  M  K  P  G  A  S  V  K  L  S  C  T  A  S  D  F
aacattaaagacacctttatgcactgggtgaagcagaggcctgaacagggcctggagtgg
 N  I  K  D  T  F  M  H  W  V  K  Q  R  P  E  Q  G  L  E  W
attggcaggattgatcctgcgattggtgaaactaaatatgacccgaagttccagggcaag
 I  G  R  I  D  P  A  I  G  E  T  K  Y  D  P  K  F  Q  G  K
gccactatagaagcagacacatcctccaacacagcctacctgcagctcaccagcctgaca
 A  T  I  E  A  D  T  S  S  N  T  A  Y  L  Q  L  T  S  L  T
tctgaggacactgccgtctattactgtgcttttccccactactggggccaaggcacctca
 S  E  D  T  A  V  Y  Y  C  A  F  P  H  Y  W  G  Q  G  T  S
ctcaccgtctcttcggcggccgcagatgtgccggattatgcgagcctcgagcaccaccac
 L  T  V  S  S  A  A  A  D  V  P  D  Y  A  S  L  E  H  H  H
caccaccactgagatccggctgctaacaaagcccgaaagagttatccttt
 H  H  H  -  D  P  A  A  N  K  A  R  K  S  Y  P
```

1.5 4XfMopB3 scFv antibody DNA sequences in pARM4 plasmid

Showing: the constitutive promoters, *pelB* sequence, *Nde*I, *Nco*I, *Not*I and *Hind*III sites, variable light chain, linker, variable heavy chain and 6xHis-tag.

```
                    catatgaaatacctgctgccgaccgctgctgctggtctgctg
                     H  M  K  Y  L  L  P  T  A  A  A  G  L  L
ctcctcgctgcccagccggccatggcagacgtccagatgatccagactacagcctcccta
 L  L  A  A  Q  P  A  M  A  D  V  Q  M  I  Q  T  T  A  S  L
tctgcatctgtgggacaaactgtcaccaccacatgtcgagcaagtgagaatattaacagt
 S  A  S  V  G  Q  T  V  T  T  T  C  R  A  S  E  N  I  N  S
tgtttagcatggtatcagcagaaacagggaaaatctcctcagctcctggtctataatgca
 C  L  A  W  Y  Q  Q  K  Q  G  K  S  P  Q  L  L  V  Y  N  A
aaaaccttagcagaagatgtgccatcaaggttcagtggcagtggatcaggcacacagttt
 K  T  L  A  E  D  V  P  S  R  F  S  G  S  G  S  G  T  Q  F
tctctgaagatcaacagcctgcagcctgaagattttgggagttattactgtcaacatcat
 S  L  K  I  N  S  L  Q  P  E  D  F  G  S  Y  Y  C  Q  H  H
tatggtactccgtggacgttcggtggaggcaccaagctgaatctccaacgtggtggtggt
 Y  G  T  P  W  T  F  G  G  G  T  K  L  N  L  Q  R  G  G  G
ggttctggtggtggtggttctggcggcggcggctccggtggtggtggttctcagatgcag
 G  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S  Q  M  Q
cagcctggggcagaggttatgaagcctggggcctcagtcaagttgtcctgcacagcttct
 Q  P  G  A  E  V  M  K  P  G  A  S  V  K  L  S  C  T  A  S
gacttcaacattaaagacacctttatgcactgggtgaagcagaggcctgaacagggcctg
 D  F  N  I  K  D  T  F  M  H  W  V  K  Q  R  P  E  Q  G  L
gagtggattggcaggattgatcctgcgattggtgaaactaaatatgacccgaagttccag
 E  W  I  G  R  I  D  P  A  I  G  E  T  K  Y  D  P  K  F  Q
ggcaaggccactatagaagcagacacatcctccaacacagcctacctgcagctcaccagc
 G  K  A  T  I  E  A  D  T  S  S  N  T  A  Y  L  Q  L  T  S
ctgacatctgaggacactgccgtctattactgtgcttttccccactactggggccaaggc
 L  T  S  E  D  T  A  V  Y  Y  C  A  F  P  H  Y  W  G  Q  G
acctcactcaccgtctcttcggcggccgcatccgcacatcatcatcaccatcaccaccat
 T  S  L  T  V  S  S  A  A  A  S  A  H  H  H  H  H  H  H  H
cactaataaaagcttttaggggaggttccctttagtgagggttaattgcgcgcttggcgt
 H  -  -  K  L  L  G  E  V  P  F  S  E  G  -  L  R  A  W  R
```

Appendix 2

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