FOOD SAFETY IMPROVEMENTS BY RECOMBINANT PROTEIN

TECHNONOLGIES

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FOOD SAFETY IMPROVEMENTS BY RECOMBINANT PROTEIN TECHNOLOGIES

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FOOD SAFTY IMPROVEMENTS BY RECOMBINANT PROTEIN

TECHNOLOGIES

Ywh-Min Tzou

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Ywh-Min Tzou, son of Te-Tsen and So (Tsai) Tzou, was born on March 20, 1969, in Chia-Yi, Taiwan. He graduated from National Chung-Hsing University, Taichung, Taiwan, in 1992, and after 2 years of military service, he worked in the Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, as a technician. With a specialization of immuno-electron microscopic research with rice (*Oryza sp*) storage proteins for 2 years, he decided to study abroad. He entered the Department of Botany & Microbiology at Auburn University and started to investigate tobacco (*Nicotiana tabacum*) salinity tolerant genes with transgenic *Arabidopsis thaliana* in August 1996 and earned his M.S. degree in December, 2002. He was accepted as a doctorate student by the department of Nutritional Science at Oklahoma State University, Stillwater, Oklahoma in 2002. In the August, 2005 he was accepted by the Department of Nutrition and Food Science at Auburn University.

DISSERTATION ABSTRACT

FOOD SAFTY IMPROVEMENTS BY RECOMBINANT PROTEIN TECHNOLOGIES

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Food safety is a scientific term to describe proper handling, preparation and storage of foods to prevent the spread of potential hazards. Health hazards associated with foods are usually categorized as biological, chemical, allergic and physical hazards. Of those, biological hazards, foodborne pathogens, cause the most foodborne illnesses. Foodborne illness is a serious public health problem worldwide. The Centers for Disease Control and Prevention (CDC) estimates that each year in the US, 76 million persons contract foodborne illnesses, of those 325,000 are hospitalized and 5,000 die. The true incidence of foodborne illness cases could be much higher, with estimates of economic costs ranging from $1.3 to $4.0 billion each year. Moreover, a recent concern about bioterrorism elevates the possibility that foodborne pathogens

could be used as weapons against the public. Therefore, improvements in food safety will be beneficial to the food industry and public health, and most importantly, bring safe food to the consumers.

To achieve this goal, it is widely believed that rapid and accurate detecting methods along with good foodborne pathogens control procedures to eliminate the hazards from production to the dining table. In this study, several food safety issues were researched. These include: 1. a engineered recombinant protein to improve PCR detections; 2. a cost effective method based on magnetic nano-particles to separate food particles from the foodborne pathogens; 3. alternative sources for antibodies to improve the detection of foodborne pathogens; 4. a possible candidate as a vaccine to control *Listeria monocytogenes* infections among animals; 5. a recombinant fungicidal protein from *Nicotiana tabacum* to control fungal growth during the processes.

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I. INTRODUCTION

Food safety is a scientific term to describe proper handling, preparation and storage of foods and the prevention of transmission of foodborne diseases. Health hazards associated with foods are usually categorized as biological, chemical, and physical hazards. Of those, biological hazards (foodborne pathogens) result in the most foodborne illnesses. Foodborne illness is a serious worldwide public health problem. The Centers for Disease Control and Prevention (CDC) estimates that 76 million persons contract foodborne illnesses, of those 325,000 are hospitalized and 5,000 die each year due to foodborne illnesses in the United States (FoodNet, 2004). Estimates of the true incidence of foodborne cases are much higher, with estimates of economic costs ranging from $1.3 to $4.0 billion each year (El-Gazzar and Marth, 1992; Todd, 1989). Moreover, recent concerns about bioterrorism elevates the possibility that foodborne pathogens can be used as weapons against the public (Pappas and others 2006). Therefore, improvements in food safety detection techniques will be beneficial to the food industry, public health, and most importantly, the consumer.

1. Improvements of pathogen detection

1a. Improvements of Immuno-Polymerase Chain Reaction.

Development of a cost effective magnetic beads. According to The Bad Bug Book by USFDA (United State Food and Drug Administration), detection and identification of foodborne pathogens could be achieved by microbiological, biochemical, immunological, and sequence homology identification methods (FDA, 2004). These methods have advantages and disadvantages over each other. Generally microbiological and biochemical methods require days for colony development or the enzymes accumulation enough for differences to be distinguished. Immunological methods provide rapid results, but the epitopes might be absent due to the environment or food where the pathogens grow. Detection methods based on nucleotide sequencing, such as PCR, are the fastest. However, false positives or negatives could happen due to genetic material been exchanged between species (United States. Food and Drug Administration., 1992; Feng, 1997). To overcome this situation, many research methods of combined Immunological and PCR, called Immuno-PCR, (Lundeberg and others 1996; Warshawsky and Miller, 1994; Widjojoatmodjo and others 1991) have been developed to significantly increase accuracies. Generally, magnetic beads conjugated with the antibodies against species of interest are used to separate pathogens from the substances in foods that may have influence on PCR reactions.

Microorganisms eluted from magnetic beads are then further confirmed with PCR. However, while helping some small food companies to monitor their sanitation conditions, we sensed that they would rather take the food safety risks than pay for more accurate pathogen analysis, using magnetic beads. Small food companies are associated with significant portions of foodborne outbreaks due to less experience and facilities in monitoring and controlling the pathogens (FoodNet, 2004). Therefore, we believe that the development of a cost effective form of magnetic beads for improving pathogen detection could be beneficial.

Polymerase Chain Reaction (PCR) is a technique that uses thermally stable DNA polymerases to amplify specific fragments of DNA *in vitro* (Sambrook and Russell, 2001). Over the past three decades, this technique has been improved dramatically and is widely used in molecular cloning, medical diagnoses, species identification, and other DNA related procedures (Arezi and others 2003; Weissensteiner and Lanchbury, 1996). Due to its sensitivity, time effectiveness, low cost, and reliability, PCR has been used in detecting foodborne pathogens in food microbiology laboratories around the world (Elizaquivel and Aznar, 2008; Hagren and others 2008; Saroj and others 2008; Bailey and Cosby, 2003). In a foodborne illness outbreak, food microbiologists are responsible for providing medical professionals rapid information about species of pathogens and their capability to resist medical treatments, such as the use of antibiotics. Unlike traditional microbiological methods, PCR takes only several hours to get the information medical professionals need.

Although, current PCR techniques show promise in giving this type of information, scientists are not satisfied and have not stopped seeking improvements. A more rapid detection system would result in less suffering and more lives saved. To shorten the overall test time, food microbiologists and molecular biologists focus on increasing the effectiveness in pre-enrichment/enrichment, lowering the number of PCR cycles, and shortening the extension duration in PCR reactions.

Recent studies of enzymatic mechanisms of thermally stable DNA polymerases indicate that fusing a thermally stable single strand of DNA binding protein from *Sulfolobus solfataricus* (sso7d) increases processivity and salt tolerance of both group A (Taq) and group B (Pfu) DNA polymerases (Wang and others 2004). Although these astonishing findings have been quickly grasped by molecular biologists to develop higher fidelity DNA polymerases, the benefit of the higher performances of fused enzymes have not been adopted and tested by food microbiologists. Studies have also shown that thermal stable DNA polymerases from the genus *Thermococcus* showed better thermal stability and faster catalytic activities than Taq. However, due to their miss-paring repairing (3' exonuclease) activities, DNA polymerases from *Thermococcus* usually yield less DNA and have less specificity when used for detection or screening purposes. Since the purpose of the 3' exonuclease activities is for higher fidelity in cloning, the deactivation of this site of DNA polymerase should help yield and specificity in pathogen detections.

Another PCR enzymatic study found that the activities of group B thermally stable DNA polymerases could be inhibited by the accumulation of 2'-deoxyuridine

5’-triphosphate (dUTP), which is converted from 2’-deoxyadenosine 5’-triphosphate (dATP) during the thermal cycles. A lot of research has contributed to the information for “detoxification” of this dUTP inactivation to thermally stable polymerase. Among these, dUTPases isolated from Achaea sources are most effective for PCR (Dabrowski and Kiaer Ahring, 2003; Hogrefe and others 2002). The potential application of dUTPases in PCR for pathogen detection is that these enzymes could increase the stability of polymerase and allow PCR to stay active for more cycles. As a consequence, the duration of the pre-enrichment/enrichment step in pathogen detection could be shortened, since the time for PCR reaction for DNA to be doubled is usually in three minutes, and bacteria need at least 20 minutes to double their population. Therefore, the overall test times could be shortened up to several hours.

1b. Sources of Antigen Recognition Molecules.

Development of monoclonal antibody for detecting methicillin-resistant *Staphylococcus aureus* (MRSA). Among all the foodborne pathogens, *S. aureus* obtained significant attention because of its relatively thermal stable enterotoxins (Dack, 1963; O'Hehir and others 1991). Therefore, unlike other heat liable pathogens, regular cooking cannot completely prevent the ingestion of the pathogenic agents released from *S. aureus* (O'Hehir and others, 1991; Dack, 1963). Among all the *S. aureus*, MRSA is lethal to humans and very difficult to eliminated once someone has

contacted the organism (Livorsi and others 2008; Burkhardt and others 2007; French, 2006b; French, 2006a).

Several methods of using PCR (Rossney and others 2008; van Hal and others 2008; Borgmann and others 2008), ELISA (Daeschlein and others 2006), and latex agglutination (Chomvarin and others 2004; Lee and others 2004; Chediac-Tannoury and Araj, 2003; Cavassini and others 1999) to detect MRSA have been developed. However, many of these tested methods that are either not reliable or rapid enough (Vodyanoy, personal communication). To improve the detection of MRSA, it is believed that having a better monoclonal antibody is feasible. Yet, many of the antibodies that are commercially available were tested and determined to be not sensitive enough (Vodyanoy, personal communication).

Alternative source of IgG. Antibodies have been widely used in the detection of foodborne pathogens (Roitt and others 2001; Playfair and Chain, 2005; Roitt and Delves, 2006). A reliable source of immunoglobulin is the key to detect the hazardous substances in foods. However, in many situations, antibodies did not produce as expected. These situations include instabilities of the hybridoma (Coco-Martin and others 1992; El-Abdallah and Ford, 2005; Harris and others 1990), difficulty in purification of the antigen (Huang, personal communications), and toxicity of antigens (Kato and others 1990). A universal approach is to obtain mRNAs isolated before the death of host animals, or the stop of immunoglobulin production, from either spleen or

hybridoma cells, encoding both heavy chain and light chain of immunoglobulin (Sepulveda and Shoemaker, 2008). With reverse-transcriptase PCR (rt-PCR), and a linker encoding a random coil, both heavy chain and light chain at Fv regions are linked as a single open reading frame (ORF). This open reading frame is then inserted in appropriate vectors and transformed and expressed in mammalian cells (Pokorny and others 2008; Cardinale and others 1998; Brocks and others 1997), yeast (Orr and others 2003; Siegel and others 2004; Wang and Shusta, 2005), *E. coli* (Ye and others 2008; Kandilogiannaki and others 2001; Rippmann and others 1998) or bacteriophage (Baek and others 2002; Zacher and others 1980; Zakharova and others 2005).

2. Control of Foodborne Pathogens

2a. Recombinant internalin A (INLA) for potential oral

Listeriosis is a deadly invasive infection caused by a Gram positive bacterium, *Listeria monocytogenes.* Epidemiological studies on the prevalence of this disease have indicated that meat and poultry are of the most common vectors of *L. monocytogenes*, and the major contamination pathways are from animal feces during slaughter (Fenlon and others 1996; Lovern, 2001). Therefore, the reduction of the bacteria in feces could lower *L. monocytogenes* contamination in foods. Traditional

control of listeriosis relies mainly on antibiotics. Although it is rarely reported that the use of antibiotics produces a "super bug" strain of *Listeria*, improper use of antibiotics remains risky to select antibiotics resistant pathogens that co-exist with *L. monocytogenes* in the gastrointestinal (GI) tracks (Threlfall, 1992). These observations indicate that an alternative method to control listeriosis is necessary. Among these alternatives, very few documents were found to employe immunization to control listeriosis.

Although *L. monocytogenes* is widely used as an immunization vector to carry antigens for intracellular responses (Bruhn and others 2007; Shen and others 1995), few research reports are available relative to vaccination against listeriosis (CDC, 2001; Linde and others 1995; Potel and Schulze-Lammers, 1985). The possible reasons for the limited cases are that the antigenic agents displayed on *L. monocytogenes* surfaces are diverse, depends on the strains (Potel and Schulze-Lammers, 1985) and the environment in which the cultures were grown (Jaradat and Bhunia, 2003; Potel and Schulze-Lammers, 1985). This diversity of antigens in *L. monocytogenes* makes it necessary to combine several strains at 10^8 live cells (each) for efficient protection in mice (Potel and Schulze-Lammers, 1985). However, this dosage exceeds the LD_{50} for mice (10^7) reported in other research (Willers and others 1982) implying that vaccination with live *L. monocytogenes* at this dosage might not be safe.

2b. Expression and fungicidal activity assay of osmotin.

Pathogenesis-related proteins (PR) are produced by plants in response to environmental stress, such as extreme salinity, and pathogen infections (Kononowicz and others 1992). Because of the nature and purpose of host plants, it makes sense that PR proteins are good candidates to control foodborne pathogens. Among PR proteins, osmotin was the most studied, shown to kill fungi by triggering the signal transduction cascade leading to apoptosis (Narasimhan and others 2005). Since the mechanisms of osmotin are somewhat hormonal, it is theoretically much more potent than enzymatic and enzyme-inhibitory fungicides. Moreover, the consumption of osmotin is safer because this protein is present in many plant tissues, which means that we consume osmotin in our daily dietary vegetables, fruit or nuts.

Despite the early discovery and characterization (Singh and others 1987) and potential medical applications in treating yeast infections, diabetes, and obesity (Min and others 2004; Narasimhan and others, 2005), osmotin has never been widely applied because of its availability. There are three difficulties that make it impossible to obtain at significant amount of osmotin: First, osmotin is a PR protein that only expresses during certain types of stress, and the only way to obtain this protein is from a salt-adapted tobacco cell line, Wisconsin 38; Next, it is difficult to purify because of its tendency to form up to 20-mer making this protein impossible to be eluted (Singh,

personal communication); third, osmotin is somewhat toxic to bacteria, giving very little yield of recombinant proteins.

II. REVIEW OF LITERATURE

There is no doubt that foodborne illnesses cause economic and public health distress worldwide. The Centers for Disease Control and Prevention (CDC) estimates that 76 million persons contract foodborne illnesses of those 325,000 are hospitalized and 5,000 die each year due to foodborne illnesses in the United States (FoodNet, 2004). These estimates are assumed to be much higher as the CDC bases their data on confirmed foodborne cases. Estimates of the true incidence of foodborne cases are much higher, with estimates of economic costs ranging from $1.3 to $4.0 billion each year (El-Gazzar and Marth, 1992; Todd, 1989). Moreover, since the September 11, 2001 Attack on the World Trade Center, New York, bioterrorism becomes a worldwide threat (Brandon, 2001; Shahi and others 2001) and brings the possibility that terrorists could use foodborne pathogens as weapons to threaten public (Pappas and others, 2006). Therefore, having a better detection and control system for foodborne pathogens is more important than ever.

1. Improvements of pathogen detection

1a. Development of Immuno-Polymerase Chain Reaction.

Development of cost effective magnetic beads. Because food matrices are usually made of living organisms and share many characteristics with pathogens, these impurities influence detection significantly (Taylor and others 2005). Traditional methods to remove food particles include filtration (Rafii and Lunsford, 1997) and differential centrifugation (Fujikawa and Shimojima, 2008; Wolffs and others 2007; Johnston and others 2005). However, the conditions given by methods are food sample specific, and hence, further optimizations are required for different foods. Moreover, significant amounts of food matrices remain in the samples that interfere with detection (Taylor and others, 2005). Unlike filtration and differential centrifugation, magnetic beads are attracted only to magnetic fields. Therefore, those with similar sizes, and Svedberg units will be removed efficiently (Olsvik and others 1994). This method was widely accepted and used in many foods microbiological detections since its introduction (Himathongkham and others 2007; Gehring and others 2006; Gehring and others 2004; Bennett and others 1996).

Magnetic particles are rapid and powerful tools to isolate, purify, or concentrate substances of interest from a mixture (Halling and Dunnill, 1980). The most critical step for obtaining a suitable magnetic matrix to carry the affinity agent is

to obtain nano-sized particles. The compound, magnetic Fe_3O_4, was found to be capable of forming nano-particles at sizes that can be controlled with proper reacting conditions. In 1980, Sugimato (Sugimoto and Matijevic, 1980) developed a method to synthesize Fe_3O_4 magnetite from iron (II) sulfate. He oxidized $FeSO_4$ with nitrate in an alkaline condition. Although Sugimato demonstrated an efficient way to produce nano-magnetite, these particles are actually quite unstable, especially with acid. Therefore, a further coating with some inert substances such as polystyrene (Qiu and others 2007; Huang and Tang, 2004) or silica (Kim and others 2008a) is needed to help stabilize the particles. Although polystyrene has better physical and chemical properties over silica, coating with polystyrene involves complicated processes (Huang and Tang, 2004; Vestal and Zhang, 2002). Therefore, for cost efficiency, a silica coating might be better than polystyrene. Silica coated magnetite was first synthesized for purification of plasmid DNA (Taylor and others 2000; Butterworth and others 1996). To coat the beads with silica, Butterworth employed sodium silicate to deposit silica on the Fe_3O_4 crystals in tetramethylammonium hydroxide (TMA) buffer pH 9.5. This protected the nano-particles so they could tolerate the acidity of hydrochloric acid up 2.0M for hours. In 2000, Taylor modified the silica coating with boric acid and obtained a better acid resistat magnetite (Taylor and others, 2000).

There are a number of methods to conjugate the magnetic beads with proteins. Traditionally, protein molecules were linked to cyanogen bromide activated agarose with amino groups on proteins (David and others 1974). Although cyanogen bromide activation is a reliable method for conjugation, it is not suitable because the coating

does not have polysaccharide and will not be activated. Therefore, an alternative conjugation via amino groups (Guisán and others 1993) or aldehyde groups (McMaster and Carmichael, 1977; Guisan and others 1991; Guisan and Blanco, 1987) is required. Taylor and others (2000) provided an alternative method using 3-Aminopropyl-trimethoxysilane (APTMOS) to deposit the silica on the crystal. In addition to the deposition of silica, APTOMS introduces amino groups on the surface of the beads. These amino groups are then suitable to react with the carboxyl groups of the proteins. Further modifications on the APTMOS-treated beads with glutaraldehyde, converted the H_3N^+- group on the nano-particles to aldehyde (Liao and others 2007).

Cloning and expression of a recombinant staphylococcal protein A (SPA)

This magnetic bead has been conjugated with anti-*Salmonella* outer membrane protein rabbit IgG, and has been shown to efficiently remove the food matrix (Carter, 2008). However, the total bacteria counts following elution and washing of the beads did not match the innocua, implying that significant amounts of bacteria were not eluted effectively and stayed on the beads (Huang, personal communications). One of the possibilities to explain this observation is that acid might not be able to break all the antibody-antigen interaction (Kaiser and Micheel, 1991). Kaiser and Micheel were able to effectively elute human alpha-fetoprotein only by alkaline conditions. Because this acidic-stable Fab-antigen interaction is unpredictable, using a sandwich method with Staphylococcal protein A (SPA) to capture the IgG bound bacteria is a possible way to solve this problem. Although, there were reports that SPA-Fc interactions

could be acidic-resistant and required alkaline conditions such as ethanolamine to break the binding (Shelver and others 2000; Kim and others 1998; Inoue and others 1996), the difficulties shall be insignificant if IgG for conjugation was SPA purified. However, native SPA contains a C-terminal peptidylglycan domain to anchor itself to the bacterial cellwall (Pfam database, http://www.ncbi.nlm.nih.gov/). This motif of SPA will be problematic because it will nonspecifically bind to any bacteria, especially to Gram (+) bacteria.

SPA is an extracellular protein that is produced by *Staphylococcus aureus*. Composed of a signal peptide at the N-terminus, followed by five immunoglobulin binding domains (domain B), and ending with a peptidylglycan binding domain at N-terminus, SPA has been widely used to purify immunoglobulin for decades (Kronvall and others 1970a; Kronvall and others 1970b; Kronvall and others 1970c; Dossett and others 1969; Kronvall, 1967). The first recombinant SPA was cloned and expressed in *E. coli* in 1979 (Coffin, 1979) by inserting a restriction-digested genomic fragment of *S. aureus* in a plamid. Since the expression of this recombinant SPA is controlled by its native topological promoter, relax-supercoil forms of DNA (Gao and Stewart, 2004), the yield is relatively low in comparison to the overexpressed format.

Improvement of polymerase chain reaction. Since the introduction of the Polymerase Chain Reaction (PCR) by Kary Mullis in 1987(Mullis and Faloona, 1987), this technique has been widely applied in cloning, detection, or whenever a specific

fragment nucleic acid amplifications is required. In foodborne pathogen detection, PCR provides species (Agarwal and others 2002), serotype (Carvalho and others 2007; Zhao and others 2006; Suh and Song, 2006), and antibiotic resistance determination (Kim and others 2005) more rapidly and precisely than most methods.

Over the past 30 years, many improvements have been made in the area of PCR. These include new enzymes (Arezi and others, 2003), a modified thermal cycling programming (Condorelli and others 1998; Tarleton and Schwartz, 1991; Simard and others 1991), and the incorporation of new additives (Kang and others 2005; Rasmussen and others 1994; Sun and others 1993). Current progresses in technologies allowed scientists to reach volcanos in deeper sea and collect new hyperthermophilic Achaea species for isolations of new enzymes (Canals and others 2006; Niemann and others 2006). These evolutions of new enzymes seem to be very helpful and have attracted attention in the biotechnology industry as well as in academic research. However, in pathogen detection, many scientists do not appreciate the enzymatic improvements and tend to stay focused on the original enzyme, (Taq), for their basic PCR experiments. With Taq being more robust in results, it is not perfect in several aspects, such as being less thermally stable and less rapid, than type B polymerases (Arezi and others, 2003).

Although the 3'-5' exonuclease activities of type B polymerases significantly increases the fidelity of DNA amplification, the degradation of primes at 3' end generated unspecific annealing and resulted nonspecific amplification. The degradation of the primers also lowered the annealing temperatures and got very low

yields, and hence, having an exonuclease deficient polymerase B was observed to increase the yield (Angers and others 2001) and the length of amplicon (Nishioka and others 2001). Investigating the 3-dimentional structure of type B polymerase (Kim and others 2008b; Hashimoto and others 2001; Hopfner and others 1999) helped us to understand the consensus sequence and critical region of the exonuclease (Figure 1). Exonuclease deficient DNA polymerase from *Pyrococcus furiosus* was the first achievement (Cline and others 1996). However, the method for the mutagenesis remained a secret. The first documented mutation method was done with *Thermococcus kodakaraensis*, KOD polymerase (Nishioka and others, 2001). In this report, double substitutions of Asp 141 → Ala, and Glu 143 → Ala deactivated the exonuclease completely.

Another dramatic improvement was inspired by N-terminal exonuclease deletion of Taq polymerase (Kim and others 1997; Park and others 1997). Researchers found that in these deletions, the activity of Taq DNA polymerase was significantly lower. The comparison of wild type and mutated proteins with the 3-dimentional structures, concluded that the deleted domains include a helix-turn-helix (HTH) structure, implying that DNA binding activity near the active site of DNA polymerase is critical for DNA synthesis. Further studies on the structures of polymerase-DNA complex showed that HTH specific to single strain DNA would help the most because the active site of DNA synthesis is closer to the 3' end, and that is a single strand. Therefore, fusing a thermally stable single strand of DNA binding protein from *Sulfolobus solfataricus* (sso7d) increases processivity and salt tolerance of both group

A (Taq) and group B (Pfu) DNA polymerases (Wang and others, 2004). Although these findings have been quickly grasped by molecular biologists to develop higher fidelity DNA polymerases, the benefit of the higher performances of fused enzymes have not yet been adopted and tested by food microbiologists.

Although the ingredients for PCR all proved to be thermally stable, thermal cycling is an extreme condition for certain biochemicals. During this repeated heating and cooling condition, a small portion of dATP was converted into dUTP. The converted dUTP has been proven to inhibit the activities of group B thermally stable DNA polymerases (Arezi and others, 2003; Dabrowski and Kiaer Ahring, 2003; Hogrefe and others, 2002) and create unwanted miss-pairing in the DNAs synthesized by both type A and B polymerases (Dabrowski and Kiaer Ahring, 2003). Much research has contributed a lot of information for "detoxification" of this dUTP inactivation to thermally stable polymerase. Among these, dUTPases isolated from Achaea sources are most effective for PCR (Dabrowski and Kiaer Ahring, 2003; Hogrefe and others, 2002). Therefore, if these enzymes stabilize the polymerase, it is possible that reactions can be performed for more cycles. As a consequence, the duration of pre-enrichment/enrichment procedures for detecting bacteria could be shortened. Since the doubling time of PCR reaction is usually three minutes, and bacteria need at least 20 minutes to double their population, the overall test times could be shortened up to several hours.

1b. Sources of Antigen Recognition Molecules

Development of monoclonal antibody for detecting methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is a *Staphylococcus aureus* that carries *mecA* in its chromosome encoding penicillin binding protein 2a (pbp2a). *MecA* does not generate any phenotype unless there is β-lactam in the growth medium. Under this antibiotic environment, pbp2a can complement the deactivation of penicillin binding protein 2 (pbp2) and help to synthesize the peptidylglycan (Lu and others 1999). Although several effective cures were found, (Livorsi and others, 2008; Burkhardt and others, 2007; French, 2006b; French, 2006a), the capability of this "super bug" to transfer *mecA* gene to other species of bacterial pathogens has created a terrible scenario that "super bug" could appear soon (Brody and others 2008). Therefore, the prevention of MRSA from further spread in our environments should proceed immediately, and accurate detection of MRSA is the first effective act.

The detection methods using PCR (Rossney and others, 2008; van Hal and others, 2008; Borgmann and others, 2008) could be effective at least for screening. However, due to the possibilities of false positives and false negatives, PCR method has not been certificated by official methods such as Bacteriological Analytical Manual (United States. Food and Drug Administration., 1992). Latex agglutination (Chomvarin and others, 2004; Lee and others, 2004; Chediac-Tannoury and Araj, 2003; Cavassini and others, 1999) to detect MRSA has been developed, and is perhaps, the most popular method. The inconvenience of this method is that it might require

professionals to interpret the results, and the agglutination depends largely on the proper amount of pbp2a. Immunological method such as ELISA (Daeschlein and others, 2006) is theoretically the best method, because the difference between regular *S aureus* and MRSA is only pbp2a. However, many of these methods tested were either not reliable or rapid enough (Vodyanoy, personal communication). Moreover, many of the antibodies that are commercially available were also tested and shown to be not specific enough (Vodyanoy, personal communication).

The Pbp2a protein is composed of a transmembrane domain at N-terminus, followed by a penicillin binding domain, and ending with a transpeptidase at C-terminus (NCBI Pfam database). Comparison the amino sequences of pbp2a using BLAST (Basic Local Alignment Search Tool) against database hosted at NCBI (National Center for Biotechnology Information) showed that there is some diversity in the penicillin binding domain among all the staphylococcal penicillin binding proteins. Therefore, the use of this domain of pbp2a as an antigen might be the best opportunity to obtain a better antibody.

Alternative source of IgG. Antibody, especially IgG, is the primary source of molecules that is widely used as recognition agents to detect and track very small amount of substances of interested in various types of samples (Roitt and others, 2001; Playfair and Chain, 2005; Roitt and Delves, 2006). Having a reliable source of immunoglobulin is the key ingredient for ELISA, western blotting and many

commonly used methods to detect and identify the hazardous substances in foods. However, in many situations, antibodies would be not produced as expected due to the instabilities of hybridoma (Coco-Martin and others, 1992; El-Abdallah and Ford, 2005; Harris and others, 1990), or the death of host animal because of the toxicity of antigens (Kato and others, 1990). In some cases, antigens could be difficult or impossible to purify and result in development of nonspecific antibodies (Huang, personal communications). Studies have demonstrated that some antigens tend to have poor immunization responses (Bell and others 1999; Silk and others 1990), or generate immunoglobulin other than the preferred IgG (Huang, personal communication). To solve these problems, mRNAs, from either spleen cells or hybridoma cells, encoding both heavy chain and light chain of immunoglobulin must be isolated before the host animals die or hybridoma stopp producing antibodies. With reverse-transcriptase PCR (rt-PCR), and a linker encoding a random coil, both heavy chain and light chain at Fv regions (scFv) were linked as a single open reading frame (Sepulveda and Shoemaker, 2008). This open reading frame was then inserted in appropriate vectors and transformed and expressed in mammalian cells (Pokorny and others, 2008; Cardinale and others, 1998; Brocks and others, 1997), yeast (Orr and others, 2003; Siegel and others, 2004; Wang and Shusta, 2005), *E. coli* (Ye and others, 2008; Kandilogiannaki and others, 2001; Rippmann and others, 1998) or bacteriophages (Baek and others, 2002; Zacher and others, 1980; Zakharova and others, 2005).

Because the posttranslational modification, ScFv expressed in mammalian cells is theoretically gives a better quality antibody. However, it is also expected to

have very low yields, and is very expensive to produce. Overexpression of scFv in *E. coli* provides economical advantage in maintenance and the highest yield among all the alternative methods (Kandilogiannaki and others, 2001; Ye and others, 2008), but were reported to have folding problems (Jurado and others 2006; Pantoliano and others 1991), and the quality of antibodies were the worst among all the alternatives.

Although, scFv displayed on phages provided similar folding quality as those expressed in *E. coli,* scFv-phages have many advantages over others. These advantages, included superior signal amplification and excellent properties to conjugate with gold nano-particle, (Moll and Guo, 2007; Kretova and others 1988), and thus, made this platform very attractive. Although, there are many other bacteriophages that have been reported to produce scFv successfully (Kalnina and others 2008), filamentous phages, such as M13 and fd, have been the most popular because of their easy handling and simple genome (Weiner and others 1991; Zacher and others, 1980; Zakharova and others, 2005).

2. Control of Foodborne Pathogens

2a. Recombinant internalin A (INLA) for potential oral vaccine

Listeriosis is a deadly invasive infection caused by Gram positive *Listeria monocytogenes.* Epidemiological studies on the prevalence of this disease have indicated that meat and poultry are of the most common vectors of *L. monocytogenes*, and the major contamination pathways are from animal feces during slaughter (Fenlon and others, 1996; Lovern, 2001). Therefore, the reduction of the bacteria in feces could lower *L. monocytogenes* contamination in foods. Traditional control of listeriosis relies mainly on antibiotics. Although it is rarely reported that the use of antibiotics produces "super *Listeria*", improper use of antibiotics remains risky to select antibiotics resistant pathogens that co-exist with *L. monocytogenes* in GI tracts (Threlfall, 1992). These observations indicated that an alternative method to control listeriosis is necessary. Among these alternatives, very little research indicates that these methods was able to control listeriosis that employed immunization.

Although *L. monocytogenes* is widely used as an immunization vector to carry antigens for intracellular responses (Bruhn and others, 2007; Shen and others, 1995), very little research about vaccination to protect host animals from listeriosis can be found (CDC, 2001; Linde and others, 1995; Potel and Schulze-Lammers, 1985). The possible reasons are that the antigenic agents displayed on bacteria surface are diverse,

depending on the genetics of strains (Potel and Schulze-Lammers, 1985) and the environments of cultures (Jaradat and Bhunia, 2003; Potel and Schulze-Lammers, 1985). This characteristic of *L. monocytogenes* makes it necessary to combine several strains at 10^8 live cells (each) for efficient protection in mice (Potel and Schulze-Lammers, 1985). However, this dosage exceeds the LD_{50} for mice (10^7) reported in other research (Willers and others, 1982) implying that vaccination with live *L. monocytogenes* at this dosage might not be safe.

2b. Expression and fungicidal activity assay of osmotin

Pathogenesis-related proteins (PR) are produced by plants in response to environmental stress, such as extremes of salinity, and pathogen infections (Kononowicz and others, 1992). Because of the nature and purpose of host plants, it makes sense that PR proteins are good candidates to control foodborne pathogens. Among PR proteins, osmotin is one of most well studied, shown to kill fungi by triggering the signal transduction cascades leading to apoptosis (Narasimhan and others, 2005). Since the mechanisms of osmotin are somewhat hormonal, it is theoretically much more potent than enzymatic and enzyme-inhibitory fungicides. Moreover, the consumption of osmotin is theoretically safe because this protein is present in many plant tissues, which means that we are already consuming osmotin in our daily dietary vegetables, fruit or nuts.

Despite the early discovery and characterization (Singh and others, 1987) and potential medical applications in treating yeast infections, diabetes, obesity (Min and others, 2004; Narasimhan and others, 2005), and cancer (Singh, personal communication), osmotin (OSM) has never been widely applied because of availability. At present, the only source to purified OSM is from salt-adapted tobacco cell culture of Wisconsin 38 with a purification method as described (Singh and others, 1987). Having a transgenic plant carries OSM driven by a strong promoter, such as CAMV from Cauliflower mosaic virus, is theoretically the best source for this protein. However, no documentation can be found to purify from transgenic plant, even in the laboratory who successfully obtained OSM overexpression potato by *Agrobacterium*-mediated transformation (Liu and others 1994). The reason why plant biologist and pathologist strictly followed the classical prototocol developed by Singh is that OSM tends to form up to 20mer (Singh, personal communication). This phenomenon of OSM makes this protein impossible to be eluted, and certain plant tissues, such as vascular fibers, make the situation even worse. Therefore, scientists have been seeking some easier expression vehicles, such as *E. coli*. However, because OSM is somewhat toxic to bacteria, giving very little recombinant proteins are produced.

III. MATERIALS AND METHODS

1. Statements about the materials and methods

Chemicals, supplies and instruments. We are confident for any scientist to duplicate the experiment and receive similar results with the conditions/parameters given in this dissertation using common research grades of chemicals, supplies, and instruments. However, if any specifications were indicated, such as vendors, grades, purity, or model numbers, the specifications are theoretical or tested to be critical, and those specifications must be met for duplications to be expected.

Laboratory safety, handling biohazard and recombinant DNAs, and animal uses. All the biohazard related experiments in this dissertation were done in Room 137, in the Poultry Science Building at Auburn University, which is a certified biohazard level II laboratory, and has sufficient safety protections to meet the regulations of EPA, USDA, CDC, and Auburn University. Individuals that participated in any of the projects were properly trained by Auburn University Office of Safety and Environmental Health. We also strictly enforced the regulations listed in

the Laboratory Safety Manual and Chemical Hygiene Plan published by Auburn University Office of Safety and Environmental Health. All the biohazard and recombinant DNAs were inactivated with appropriate oxidants/disinfections and autoclaved before disposal. Animals used in this dissertation were approved by Auburn University before we conducted any experiment (Appendix D.) The lab animals were cared for by Auburn University Animal facility, and the experimental treatments were given by an individual with proper training (Appendix E.) Any animal experiments associated with monoclonal antibodies and hybridoma were executed in the Auburn University Hybridoma Lab, and the applications for use of these animals were filed and approved separately.

2. Routine molecular biology methods

Bioinformatics and computational biology. Genomic information of human, mouse, *E. coli, L. monocytogenes*, and other necessary species were obtained from several databases, including the Entrez server of the National Center for Biotechnology Information (NCBI Entrez, http://www.ncbi.nlm.nih.gov/entrez/), Workbench 3.2 hosted at San Diego Supercomputer Center (SDSC, http://workbench.sdsc.edu/), and The Institute for Genomic Research (TIGR, http://www.tigr.org/). Three dimensional structures of proteins were obtained from protein databases provided by the Research Collaboratory for Structural

Bioinformatics (RCSB, http://www.rcsb.org/pdb/), and images were processed locally with RasMol version 2.7.2.1.1 for Windows (http://www.bernstein-plus-sons.com/software/RasMol_2.7.2.1.1/) and Vector NTI version 10.1 for Windows (Invitrogen, Carlsbad, CA). Both DNA and protein sequencing data were analyzed with Basic Local Alignment Search Tool (BLAST) services provided by NCBI (http://www.ncbi.nlm.nih.gov/blast/) and ClustalW provided by SDSC. Sequencing results obtained from Auburn University Genetic Analysis Lab were visualized with Chromas version 1.61 (Technelysium Pty Ltd, Austria) or Vector NTI version 10.1 for Windows (Invitrogen, Carlsbad, CA). Promoter regions and transcription start sites were predicted with geWorkbench developed by Columbia University. DNA restriction cutting cites were analyzed with NEB Cutter provided by New England Lab (NEB, Ipswich, MA, http://tools.neb.com/NEBcutter2/index.php). PCR primers were designed with the help of OligoAnalyzer 3.0 provided by Integrated DNA Technologies (IDT, Coralville, IA) hosted at https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/. PCR reactions were simulated in the web-based UCSC *In-Silico* PCR (http://genome.ucsc.edu/cgi-bin/hgPcr), or *in silico* amplification hosted at http://insilico.ehu.es/PCR/, depending on the virtual DNA templates provided.

Targeting of surface-expressed proteins was predicted by PSORTI, PSORTb, Proteome Analyst, SubLoc, CELLO, PSLpred, LOCtree and P-Classifier as described by (Gardy and Brinkman, 2006). To prevent recombinant proteins from forming inclusion bodies, protein sequences were analyzed at http://www.biotech.ou.edu/to

predict the solubility and optimize conditions for induction (Wilkinson and Harrison, 1991). Recombinant proteins were virtually analyzed with web-based Protein Calculator developed by The Scripps Research Institute (http://www.scripps.edu/~cdputnam/protcalc.ht ml) for the protein properties that were needed for protein purification and identification.

Polymerase Chain Reaction (PCR) and PCR cloning. Primers in this project were designed manually, and all the oligo-nucleotide sequences are listed in Appendix C. Sequences of interest from several species or strains were downloaded from databases chosen, and further analyzed by SDSC ClustalW. Suitably identical regions of DNA were picked for primers annealing. PCR reactions were simulated as mentioned to confirm the design of primers, and annealing temperatures were predicted with IDT OligoAnalyzer 3.0. Primers were mixed with distilled water, dNTPs, buffer, and templates in PCR tubes (USA Scientific, Ocala, FL) and placed in Px2 thermal cycler (Thermo, Waltham, MA) for programmed thermal cycles. High fidelity thermal stable DNA polymerase, STgo produced and purified in lab, Tgo (Roche, Basel, Switzerland), iProof (Bio-Rad, Hercules, CA), or Phusion (NEB, Ipswich, MA) were added into the reagents when the temperature reached a programmed 90°C. Quality of PCR products were examined by 1.0% agarose gel electrophoresis with TAE buffer at the electric field of 5 volt/cm. PCR products were purified by PCR Clean-Up columns (Novagen, Madison, WI) or eluted from gel with

PerfectPrep Gel Clean-Up columns (Eppendorf, Westbury, NY) before they were ligated with PCRscript PCR Cloning kit (Stratagene, La Jolla, CA).

Plasmid isolation and restriction digestion. The method for plasmid isolation in this project followed the instruction of the Cold Spring Harbor Laboratory's manual 2nd Edition (Sambrook and others 1989) with some minor modifications. A Single colony of bacteria that carried plasmid was picked, inoculated in LB broth (Miller formulate, 1% tryptone, 1% sodium chloride, 0.5% yeast extract) with appropriate antibiotics, and grown at 37°C with shaking at 250 rpm. Bacteria from overnight cultures were collected by centrifugation, suspended with solution I (50 mM glucose, 50 mM Tris pH 7.9, 10 mM EDTA), and lysed with solution II (0.2N sodium hydroxide, 1% SDS). After proteins and chromosomal DNA were removed by Solution III (3M sodium acetate, pH 4.8) and centrifugation, plasmid DNA was precipitated with 1 volume of isopropanol. Plasmid DNA was further purified by a FastPrep plasmid preparation kit (Eppendorf, Westbury, NY) for restriction digestion or transformation. DNA quantities were measured with spectrophotometry. Every 200ng of DNA was reacted with 5 units of appropriate restriction enzymes in 20 µl under manufacturer's instructions. Digestions were monitored with agarose gel electrophoresis with suitable agarose percentage. Gel electrophoresis images were visualized by ethidium bromide illuminated with UV 265nm and captured with UVP MultiDoc-It and Doc-It software (UVP, Upland, CA). DNA fragments of interest were obtained by digesting of 3 µg of plasmid in suitable restriction enzymes. Calf intestine

alkaline phosphatase (Fermentas, Hanover, MD) was also added to the restriction reaction if dephosphorylation was required. DNA in the agarose gel was located with UV 365nm with ethidium bromide. Solidified agarose with the DNA of interest was cut with a razor blade and melted with 1M guanidine buffer at 50°C, and DNA was purified with PerefectPrep Gel Cleanup kit (Eppendorf, Westbury, NY).

Ligation and Transformation. Linear DNA fragments were mixed at the ratio of 1: 6 (linearized plasmid: insert) if the ends of the plasmids were dephosphorylated or 1:50 to 1:100 if not dephosphorylated. T4 DNA Ligase (NEB, Ipswich, MA), ligation buffer, and 20% of PEG 6000 were added to the mixture. The reactions were performed at room temperature for 1hr or over night at 16°C, depending on the activities of the enzymes. Followed by 60°C inactivation of ligase, 100 µg of beef glycogen were added into every 20 µl of reactions for precipitation of DNA with 50% isopropanol. DNA pellets were collected by centrifugation and suspended in ddH_2O for transformation.

Electroporation and heat-shock methods were used to deliver DNA into bacteria. For electroporation, the competent cells were prepared by collecting 3 L of LB culture at log phase (OD 0.6-0.8). Bacteria collected were washed twice with 3 L of ice-cold chilled ddH_2O, and once with 3L of cold 10% sterilized glycerol. Pellets of washed bacteria were suspended with 10% glycerol at the final volume of 5 ml per OD per litter. Bacteria were then aliquot, frozen in liquid nitrogen and placed in -80°C for

long-term storage. Forty microlitters of this competent cell were mixed with 1ng of purified plasmid or 1-4 µl of ligation products in chilled electroporation cuvettes. Water condensation on the surface of cuvettes were wiped off with Kimwipes, and cuvettes were placed in the chamber in the BTX Electroporator ECM399 (BTX, San Diego, CA). An electric field of 18 Kvolts/cm was applied with 36 µF of capacitance and 150 Ω of resistance. Discharge duration was measured by the built-in timer in electroporator to predict the efficiency of transformation. Usually, 5ms (millisecond) yielded the highest transformation. A duration shorter than 2ms implied very bad transformation, and better quality of DNAs and re-transformations were required. Bacteria were suspended with 960 µl of 42°C pre-warmed SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) and were transferred to test tubes for incubation at 37°C with 250 rpm shaking for a least 1hr. Appropriate amount of this medium were plating on LB agar with suitable antibiotics or differentiation markers, and were incubated at 37°C overnight.

For the heat shock method, competent cells were prepared by the method developed by Inoue (Inoue and others 1990) with modifications. A single colony of bacteria strains were grown in 100 ml of SOB medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$) with appropriate antibiotics at 37°C overnight. While bacteria were growing overnight, transformation buffer (TB, 10 mM PIPES pH 6.7, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$) was precisely made as described below. To make the working solution of TB, 20 ml of 500 mM PIPES pH 6.7 was added to 800 ml of deionized water and the rest of the components

were added as crystals or pH -unadjusted stock solutions to meet the concentration desired. After bringing the volume to 1L, the solution was sterilized by filtration. This TB was then chilled in a refrigerator overnight before use, and stored in a refrigerator for months. The overnight culture was diluted 1:100 with SOB and grown at 18°C with 250 rpm shaking. The use of antibiotics was avoided in the medium unless they were required for the plasmids to be maintained. Bacteria density was constantly monitored by OD_{600nm}. Bacteria were harvested when OD reach 0.55 with 4000 G centrifugation for 10 minutes. For each 250 ml of OD=0.55 culture, 80 ml of chilled TB were used to wash and very gently suspend the pellets on ice. After centrifugation at 2500G for 10 minutes 20 ml of chilled TB for every 250 ml original culture were used to gently suspend the washed pellets. When bacteria were all suspended, Dimethyl sulfoxide (DMSO) was added to the suspension at the final concentration of 7%. The chemically competent cells were quickly aliquoted to 200-1000 µl in 2.0 ml SEAL-RITE microcentrifuge tubes (USA Scientific) and sharp frozen with liquid nitrogen or -70°C methanol bath. The transformation efficiencies of the competent cells were then tested with a control plasmid, and the efficiencies were calculated as CFU/µg of control plasmid. The efficiencies of those competent cells are expected to be at least 10^8 or 10^6 CFUs if antibiotics were required in the preparation of the competent cells.

The heat shock transformations followed the method described in the Molecular Cloning, 3rd Edition (Sambrook and Russell, 2001) with modifications. Up to 20 µl transformation mixture or 1 µg of plasmid DNA was pipetted into 2.0 ml

SEAL-RITE microcentrifuge tubes and placed on ice. Once the tubes were chilled, 200 µl of competent cells were pipetted and mixed with DNAs with gently swirling. The tubes were placed on ice for 30 minutes; meanwhile, a 42°C water bath and an ice-water bath were prepared. The tubes with competent cells were then heat shock at 42°C for 45 seconds and quickly incubated in ice-water bath for at least one minute. It is critical to not shake of the tubes while transferring them between water bathes and during the one minute incubation in ice-water bath. After the incubation in ice-water bath, 800 µl of SOC were added to the tubes and incubate at 37°C without shaking for 10 minutes, and then shaking at 250 rpm for another 50 minutes. The transformation mixtures were then plated for colony development. If containers other than 2.0 ml SEAL-RITE microcentrifuge tubes were used, the duration of heat shock must be optimized otherwise the transformation efficiencies could be low or no colonies developed.

Overexpression of recombinant proteins in *E. coli*. Once the ORFs with correct sequences were inserted in the T7 driven expression vectors, plasmids were transformed into DE3 strains of *E. coli* and plated on LB agar with 10 mM $MgSO_4$ and appropriate antibiotics for single colony. To ensure consistency of expression, colonies were picked with wooden toothpicks and transferred to LB MG medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 10 mM $MgSO_4$, and 10 mM glucose), once they were visible. These LB MG cultures were carefully monitored and harvested once turbidity was noticed. Small scales of recombinants were induced by growing 100 µl

of LBGM culture in 3 ml of auto-induction medium, ZYM5052 (1% tryptone, 0.5% yeast extract, 0.5% glycerol, 50 mM NH_4Cl, 25 mM KH_2PO_4, 25 mM Na_2HPO_4, 5 mM Na_2SO_4, 0.05% glucose, 0.2% lactose, 10 μM $FeCl_3$, 4 μM $CaCl_2$, 2.5 μM each of $MnCl_2$ and $ZnSO_4$, and 0.4 μM each of $CoCl_2$, $CuSO_4$, $NiCl_2$, Na_2MoO_4, Na_2SeO_3 and H_3BO_3), and grown at 37°C overnight with 300 rpm shaking (Studier, 2005). The cultures were diluted 10 times with ZYM5052 medium or water for OD_{600nm} measurement and equal amount of bacteria were lysed with SDS sample buffer and analyzed with SDS PAGE. Final concentration of 8% glycerol was added to the cultures with expressible plasmids and frozen in -80°C or -20°C for storage (Novagen, 2006). For large scale expression, 10-100 μl of glycerol stocks were added to 100 ml of LB MG in 250 ml flasks and incubated at 37°C overnight with 300 rpm shaking. These overnight cultures were diluted 1:100 with ZYM5052 and proteins were induced overnight. Bacteria with overexpressed recombinant were collected by centrifugation and suspended with 1/100 of original culture volume of 10 mM Tris pH 8.0. If no lysis was observed, EDTA was added at the final concentration of 1 mM to wash away excess divalent metallic ions. EDTA-washed bacteria were then centrifuged and resuspended in the buffers that compatible with downstream purifications or assays. To lyse the cells, solid lysozyme was added to the suspension at the final concentration of 1 mg/ml. After incubation at 37°C for 30 min, compatible detergents (Triton X-100, Tween-20, NP-40 or BRJI-35) were added to the lysates to increase the solubility recombinant proteins. Once viscosity was observed, the lysates were chilled on ice for 10min and sonicated to break down the DNAs. After centrifugation at 20,000G for 20 minutes, supernatant and pellets were collected

separately, and analyzed with SDS PAGE for existence of recombinant proteins. These lysates and pellets were used for further purification or assay.

3. Development of cost effect Immuno-PCR

Synthesis of magnetic beads. Fe_3O_4 magnetite was prepared by the method of Sugimato (Sugimoto and Matijevic, 1980) with modification. Briefly, 4.5 L of 0.42 M of iron (II) sulfate was degassed in a 6 L Erlenmeyer flask. Once degassed, a three-hole # 9 ½ stopper which fit a thermometer, a plastic propeller stick attached to an overhead stirrer, and a nitrogen inlet, was used to seal the flask. The flask was heated on hot plate, and nitrogen gas was passed through to the flask from the inlet and exhausted from the hole for propeller stick. Once the iron(II) solution was heated to 95˚C, 1.5 L of 0.8 M potassium nitrate and 3.4 M potassium hydroxide (degassed at 65˚C) were added into the flask. The mixture was then kept at 92-96˚C for an hour with stirring and constant purging of nitrogen gas for one hour. After the solution was cooled to room temperature, the flask was placed on a 4 × 4 × ¼ (inches) of N42 strength of neodymium magnetic plate for 10 minutes, and the supernatant was removed by suction. The black magnetite was repeatedly washed by suspended with deionized water, pulled by the magnetic plate, and suction, until the pH of supernatant was lower than 7.0. The yield of the magnetic beads was roughly 1.5 L in bed volume, and these beads were store in deionized water. About 50 ml bed volume of this

magnetic beads were washed with 95% ethanol, and the washed beads were placed on a pre-weighted weighting boat and dried in 90˚C oven to measure the dried weigh of the beads. Roughly, there were 450 mg per ml bed-sized of the beads. The magnetic beads were examined by transmitting electron microscope (TEM).

To coat the beads with silica, sodium silicate was used to deposit silica on the particles (Taylor and others, 2000; Butterworth and others, 1996). 100 g of sodium silicate solution (40-42˚ Bé), 5.3 g of boric acid, 3.3 g NaOH were dissolved in 1 L of deionized water. Fifty grams of dry weight of Dowex-50 or Amberlite H-120 were washed with deionized water, regenerated with 1.0 M of hydrochloric acid, and deionized water. This regenerated resin was mixed with 800 ml of sodium silicate solution with slowly stirred for one minute and the resin was removed by vacuum filtration. The filtrated solution was adjusted to pH 9.5 with unfiltered sodium silicate solution and mixed with 200 ml bed volume of uncoated magnetic beads. While the slurry was stirred, 100 ml of 1.0 M of tetramethylammonium hydroxide (TMA) was added, and the pH of the mixture were slowly adjusted to 10.0 with 0.5 M hydrochloric acid over a period of about one hour, and reacted for another two hour with stirring. The beads were then washed with deionized water until the pH of the supernatant reach nearly neutral. Fifty microlitters bed volume of coated beads were examined by reaction with 1 ml of 1.0, 2.0, 3.0, and 4.0 M of hydrochloric acid in microcentrifuge tubes for two hours. One hundred millilitter bed volume of these coated beads were suspended in 1000 ml of 95% ethanol, in which, 10 ml of 3-Aminopropyl-trimethoxysilane (APTMOS) were added to deposit the silicate to add

the H_3N^+- group onto the nano-particles (Liao and others, 2007). The mixture was stirred at room temperature for two hours then transfer to a 90˚C water bath with stirring until the temperature reach 70˚C for 10 minutes. The beads were then washed twice with ethanol, and twice with deionized water, one time with 10 mM pyridine-NaOH buffer pH 9.0, and suspended in 800 ml of the same buffer. Two hundred millilitter of 25% glutaraldehyde pH 9.0 were added to the suspension to introduce aldehyde groups to the ammonium group on the beads (Liao and others, 2007). After two hours of reaction with stirring at room temperature, the beads were washed with deionized water until the pH reached neutral and suspended in 1 L of 95% ethanol. Ten percent of acetate anhydride was added to the beads and stirred at room temperature for 30 minutes to block the free ammonium groups on the beads. The glyoxal beads were then washed with deionized water and stored in refrigerator. To measure the availability of the free aldehyde groups on the beads, 0.1 ml bead volume of beads were suspended in 1.5 ml 0.2M K_2HPO_4 in a 2- ml tube. 15 mg of nitrilotriacetic acid (NTA) were added to the suspension. After reacting at room temperature with agitation for 2 hours, several grains of $NaBH_4$ at average crystals size were added to the suspension to stabilize the crosslinkages for another one hour. After three washes with water, these NTA coupled beads were incubated with 1.5 ml of 200 mM $CoCl_2$ for 15 minutes with agitation. The cobalt beads were then washed three times with water and chelated cobalt ions were eluted twice with 100 µl of 100 mM EDTA pH 8.0. The fractions were pooled, and the concentration of the cobalt was measured with spectrophotometry. Briefly, 10 µl of 2M imidazole were added to 100 µl of the elution, and a serial dilution from 0-20 mM of Co^{2+} in 100 mM EDTA was

made. The OD 518nm of the elution was plotted on the standard curve, and the amount of the cobalt was calculated. Theoretically, one cobalt ion occupied one aldehyde group on beads. A scheme with a simplified procedure from the synthesis of beads for the conjugation of antibodies was represented in Figure 5.

Expression, purification, and activity assay of Staphylococcal Protein A (SPA). DNA encoding only domain-Bs (immunoglobulin binding domains) was obtained by PCR with primers spa6538_3_eco and spa6538_5_nde and with *S. aureus* ATCC6538 as templates. A DNA fragment with correct sequences was inserted in expression vector pET20b and transformed into *E. coli* BLR (DE3) (Novagen) and expressed as mentioned. Lactose induced bacteria were collected with centrifugation and lysed with 8 M urea and 10 mM Tris pH 7.8. Supernatant obtained after 20,000 G centrifugation was mixed with 10 mM pH 7.8 Tris buffer-equilibrated DEAE-cellulose. After washing with 10 times bed volume of 10 mM Tris pH 7.8, 10 times bed volumes of 10 mM Tris pH 6.5, 5 times bed volume of 10 mM Tris pH 7.2, recombinant proteins were eluted with 500 mM NaCl in 10 mM Tris pH 7.2. Fractions were examined with SDS PAGE, and the ones with high protein content of interested were pooled, and imidazole was added at the final concentration of 5 mM. SPA was then purified with cobalt-chelated agarose (BD Biosciences). The immunoglobulin binding activity was assayed by conjugating SPA with glyoxal-agarose (Agarose Bead Technologies, Tampa, FL), in 100 mM K_2HPO_4 and 20 mM Sodium cyanoborohydride for 8 hrs (Guisan and others, 1991; Ito and others 1985). After

blocking excesses aldehyde groups on beads with (1 mg/ml of beads) ethanolamine, SPA-agarose beads were washed 3 times with 5 bed volume of 100 mM K_2HPO_4 (Guisan and others, 1991; Ito and others, 1985) and were ready to be packed in a column for immunoglobulin binding. Ten to 50 percent of saturated ammonium sulfate precipitation of rabbit IgGs were dialyzed in 100 mM K_2HPO_4 pH 9.5 and applied to the SPA-agarose column. After washing with 10 times bed volumes of 100 mM K_2HPO_4 pH 9.5, the immunoglobulin was eluted with 100 mM citrate buffer pH 3.0. Fractions of elutants with dense immunoglobulin bands (55kd and 18kd) in SDS PAGE were pooled and dialyzed in phosphate buffered saline (PBS). The content of immunoglobulin was measured by Bradford protein assay (Bio-Rad) with the reference of bovine γ-globulin.

Expression, purification, and activity assay of thermal stable enzymes for PCR. Coding sequence of *Sulfolobus solfataricus* single strand DNA binding protein (sso7d) was assembled with PCR and synthetic oligonucleotides as described (Wang and others, 2004) with modification. Briefly, T4 polynucleotide kinase (Fermentas) phosphorylated oligonucleotides (sec1, sec2, sec3, and sec4) were precisely assembled by pairing with primers (B1, B2, B3) at the junctions of each fragments, and covalently linked with T4 ligase (New England Bio Lab). This ligation mixture was further amplified with primers (SSO5_EcorI, SSO3XHO), and yielded a 230 base pairs DNA fragment. DNA encoding Tgo polymerase was obtained by PCR with primers (TGO5_nde, TGO3_eco) and the template of genomic DNA isolated from

Thermococcus gorgonarius (ATCC700654). After inserting Tgo coding sequence in the cloning vector, pCRscript, the codons encoding ASP141, Ile142, and Glu143 (DIE) in confirmed clone were substituted with Asn, Ile, and Gln (NIQ) by site-directed mutagenesis with primers (tgo_die_5, tgo_die_3). This mutated DNA was named Niq, and confirmed sso7d was added at its 3' end of Niq and wild type Tgo and yielded SNiq and STgo. Lyophilized *Pyrococcus furiosus* (ATCC 43587) cells were used as templates to obtain the dUTPase gene with primers ((P45_3, P45_5). Confirmed expression vectors were then transformed into codon supplemented (Baca and Hol, 2000) DE3 *E. coli* strain Rosetta2 (Novagen). After the expressed bacteria were suspended in 0.5% Brij-35, 0.5% NP-40, 10 mM Tris pH 8.5 and 50 mM KCl and lysed as mentioned above, lysates were incubated in a 70°C water bath for 30 min with shaking before they were centrifuged at 20,000 G for 20 min. Supernatants collected were then purified with cobalt-agarose, and the purified proteins were dialyzed with buffers that are compatible with downstream assays. The purity of recombinant was determinate by SDS PAGE and the protein content was measured with Bradford protein assay with Bovine serum albumin (BSA) as the reference.

Polymerase activities were measured using a plasmid pJET 1.2 (Fermentas) inserted with the coding sequence of GFP and primer PJET F and PJET R. With a serial dilution of enzymes, relative enzyme activity was measured and the optimal concentration of enzyme for PCR reaction was obtained. Exonuclease activity of STgo, SNiq, Niq, and Taq were measured by incubating enzymes at their optimal concentration for PCR at 72°C, with their own buffer systems, and 60 base pairs

synthesized oligonucleotide but without DNA templates and dNTPs. Reactions were stopped by adding 1 mM EDTA at 0, 10, 30, 60, 120 and 180 mins after incubation and stored in -20°C for later analysis. Reagents were mixed with ¼ volumes of 20% Ficoll-400, and these were analyzed with TAE polyacrylamide gel electrophoresis. The degradation of DNAs was visualized with ethidiumbromide and UV 320nm. To reduce the effects of variation of annealing temperatures different enzymes required, gradient PCR programming from 58°C to 70°C were applied to compare the velocities of DNA synthesis catalyzed by Taq, STgo and SNiq. With genomic DNA of *L. monocytogenes* and 2 pairs of primers (intl_485-510_forward, intl_800-780_rev, LLO_242_forward, LLO_714_reverse) enzymes were added into their own reagents with optimized buffer systems.

4. Sources of antigen recognition molecules

Preparation of antigen for MRSA detection. PCR amplification aimed at the coding sequence of penicillin binding motif was used (primer MCA3 Sal and MCA5Nbg). The sequenced confirmed PCR product was then legated with plasmid pET20B and transformed into *E. coli* strain BL21 (DE3. The recombinant pbp2a (rpbp2a) was induced by 0.2% lactose in ZYM5052 broth incubated at 37°C overnight. Bacteria were lysed with 6 M guanidine and rpbp2a was affinity purified with immobilized cobalt matrix. To ensure correct translation, this recombinant protein

was tested with PBP latex aggregation test and penicillin inactivation assays. The purity of the recombinant was determinate by SDS PAGE and the protein content was measured with the Bradford protein assay. Confirmed protein was sent to Hybridoma Facility at Auburn University for immunization. Native pbp2a was also prepared for screening the specificity and sensitivity of monoclonal antibody as followed. MRSA and regular *S. aureus*, and other Gram (+) bacteria were grown in TSB (Trypticase Soy Broth, EMD Bioscience) supplemented with 0.5% yeast extract and 0.1% K_2HPO_4 for overnight at 37°C with 250 rpm shaking. Bacteria were harvested by centrifugation and suspended with 1/40 of the original culture volume of water. The final concentration of 100 mM NaOH was added, and heated in a boiling water bath for three minutes. Final concentrations of 130 mM Tris-Base and 100 mM HCl were added to the lysate, and supernatants collected after 3000 G centrifugation for 15min were saved as native antigens. The sensitivity and specificity were tested with ELISA.

Constructions of PIII deleted helper M13 (M13dp3A), cloning pGSV9, and expression (pKscFv) vectors. With M13KE (NEB Biolabs) and primers p3d+nhe65 and p3d-hxo63, coding sequence of PIII was removed (Rakonjac and others 1997; Baek and others, 2002), and another PCR product encoding β-lactamase was ligated to compensate the expression level PVI that downstream of PIII with appropriated restriction enzymes. The ligation mixture was transformed into a non-F' *E. coli* strain, MachOne (Invitrogen), and the transformants grew in a serial dilution of ampicillin in LB medium. Single strand DNA (ssM13dp3, Figure 2.) was isolated

from the culture with 4 μg/ml of ampicillin with the methods as described (Sambrook and others, 1989). Polynucleotide linker for expression and cloning was assembled by PCR as described for ssod7, with primers SCFV_P1, SCFV_P2, pelb5, scfv3, and scfv_B1. Leading with a pelb signal peptide, targeting periplasmic space, followed by SalI, NcoI, a coding sequence for ggggsgipgggsggggs, XhoI, HindIII, and a poly-his tag, this fragment of DNA was cloned into the backbone of pUC18 (pGSV9) for cloning, and the pBR322 ori, and kanamycin resistance operon backbone of pET28 (pKscFv, Figure 3.) for expression. Other components in pKscFv including *rrnB* terminator (pTRC, Invitrogen), phage shock promoter *ppsp* and its regulator *pspa* from *E. coli* strain NEB Turbo, and PIII from M13KO7 (NEB Biolabs) were amplified by PCR and assembled by ligation.

Cloning of scFv for expression on phages and yeast. Total RNAs from anti-*Bacillus anthracis* spore hybridoma clones 1B1 (simplified as B), 1C2 (C), 3F8 (F), were purified by mini RNA purification kitII (Zymo Research). Five microgram of RNA were used for a 40 μl of reverse transcription reaction with 10 pmole of oligo-adp-$(dt)_{22}$ (ADP2 22) (Sambrook and Russell, 2006) and catalyzed with SuperscriptIII (Invitrogen) at 50°C for 30 min and at 60°C for another 30 min. After 70°C inactivation of SuperscriptIII for 15 min, RNAse A, RNAse iF, RNA H and 1 mM EDTA were added to the reagents and incubated at 37°C for 10 minutes to digest RNA. With twice phenol extraction, this first strand DNA was separated from the primer, enzymes, salts, and dNTPs by spin dialysis with Montage PCR Cleaning

(Millipore). The purified first strand DNAs were then reacted with 1 μM dATP and terminal transferase (NEB Biolabs) for addition of 3' poly-A tails. After another spin dialysis, another adapter primer with $(dt)_{17}$ (ADP17), dNTPs, and buffer were added for second strand synthesis (Sambrook and Russell, 2001). After denature at 98°C for one minute, STgo polymerase was added, followed by annealing at 60°C for 30sec, second strand DNA was synthesized at 68°C for 10min. Excess primers was then removed by additional spin dialysis, and purified double strand DNAs were added to PCR reactions with primer ADP and adp2 (58.3) for 10 cycles. These three PCR cDNA libraries were called, pclB, pclC and pclF, and stored at -20°C for downstream cloning. 3' primers Igγ1, Igγ2A and Igγ2B were used to combine with primer ADP for amplify the variable regions of heavy chains from cDNA libraries and yield three DNA (fhB, fhC and fhF), whereas, only primer ADP and LC_3 were required to obtain light chains' variables (flB, flC and fF). The sequence data of fhB, fhC, fhF, flB, flC and flF were analyzed with Vector NTI (Invitrogen) and amino acid sequences were predicted with web-based software SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) for signal peptidease cutting sites. The signalP and sequence data of fhB, fhC, fhF, flB, flC and flF were used to design primers (h_b5Sal66, H_bf_3nco67.5, h_c_5sal65, h_c3nco, h_f_sal66, lb5sal, lcb5xho66, lclfj5xho65, lC_C5xho66 and l_c_all_3Hind66, for precise translation of scFv, and yielded six pieces of DNA, hB, hC, hF, lB, lC, and lF, with PCR. After hB, hC, hF were inserted at the restriction sites of SalI and NcoI at pGSV9, lB, lC, and lF were cloned after the linker, ggggsgipgggsggggs, in every clones of pGSV9 of hB, hC, hF and yielded 9 clones of pGSVBB, pGSVBC, pGSVBF, pGSVCB, pGSVCC,

pGSVCF, pGSVFB, pGSVFC, and pGSVFF. The insertions in these pGSVs were digested with SalI and HinDIII and cloned into pKscFv and transformed into F' strain of *E. coli* OmniMax and yielded of pKscFvBB, pKscFvBC, pKscFvBF, pKscFvCB, pKscFvCC, pKscFvCF, pKscFvFB, pKscFvFC, and pKscFvFF. For easier presentation, we called these 9 pKscFv clones as pKBa. pKscFv clones confirmed with correct restriction fragments were ready to infect with helper phage, and produce antibody phages. The insertions in the pGSVs were further modified with primers METHB5MFE, METhC5mfe, METHF5MFE, METLB3Bg2 and METLC3Bg2 to introduce restriction recognition sites for secretory expression of scFv in *Pichia methanolica*.

Producing of helper and scFv phages. The helper phages were first produced by electro-transformation of ssM13dp3 into a clone of OmniMax with empty pKscFv. The transformants grew in serial dilution of ampicillin from 1-10 μg/ml in LB (Miller formula, USB Corp. Cat# 75854, Lot#122245) with 5 mM MgSO4, and 6.25 μg/ml of kanamycin for overnight. The helper phages were purified from the cultures with 1 μg/ml of ampicillin with the methods as described (New England Biolabs, 2006). Helper phage M13dp3A obtained from this culture was further purified on top-LB-agarose for single plagues as described (New England Biolabs, 2006). Tips of wooden toothpick were saturated with the liquid in the plague area by contact the plaque for several seconds and put in 5 ml of 100x diluted overnight culture of OmniMax with pKscFv in LB with 5 mM MgSO4, and 6.25 μg/ml of kanamycin. After growing at

37°C for 5-6hrs, the helper phages were purified and titered. For large scale proliferation of helper phages, 10^{10} PFU of M13dp3A, were used to infect every 10 ml of OD_{600nm} 0.6 culture of OmniMax with pKscFv; for large scale proliferation of antibody phages, 10^{10} PFU of helper phages, were used to infect every 10 ml of OD_{600nm} 0.6 culture of OmniMax with pKBa. After growing at 37°C for 5-6 hrs with 250 rpm shaking, phages were harvested and titered. Due to possibilities loses of infection activities, the PFU of antibody phage were estimated with spectrophotometry using the equation of $PFU/ml = OD269nm \times 6.5 \times 10^{12}$. About 10^8 of phages were used to analyze the specificity and sensitivity with ELISA. To test the possibility of miss packing of pKscFv, 10^{12} of M13dp3A were used to infect 4 ml of TOP10F' (Str^R) in 4 ml of LB with 5 mM MgSO4 at OD_{600nm} 0.6. After recovery for 30min, the bacteria were centrifuged and plated on two LB agar with 25 μg/ml kanamycin and 15 μg/ml streptomycin.

Transformation and expression of scFv in *Pichia methanolica*. Confirmed yeast expression vectors were linearized with compatible restriction endonucleases to separate the expression cassette from plasmids. Linearized plasmids were precipitated and purified by silica spin columns, and DNA concentrations were measured with OD_{260nm}. There microgram of these purified DNAs were incubated with *P. methanolica* competent cells prepared as recommendations of manufacturer. After incubation on ice for three minutes, competent cells were transferred to 2 mM gaped electroporation cuvettes, and a single pulse was applied with 750v, 25μF and a

resistance setting of ∞Ω on a GenePluser (Bio-Rad), and 1 ml of YPAD (1% yeast extract, 2% peptone, 2% glucose, 0.01% adenine) was added into each cuvatte and transferred to a 2 ml microcentrifuge tube. After incubation at 30°C for 1hr without shaking, transformants were spun at 1,500 G for a minute and washed with 1 ml of 2x strength of yeast nitrogen base (YNB, obtained from Invitrogen) to remove the excess adenine. The washed transformants were then collected with centrifugation and suspended with 100 µl of 2x YNB, and spread on 2 minimal dextrose agar plates (MDA, 2x YNB, 2% glucose, 40 ppm biotin, 1.5% agar) for ADE2 selection (Hiep and others 1993). Single colonies were expected to be visible after 4 days of incubation at 30°C, and were further purified by streaking on another MDA plate. To reduce the bias caused by somatic variations, two single colonies were picked from each MDA plate for small scale expression. Colonies were picked with wooden toothpicks and grown in 12 ml of Buffered Dextrose-complex Medium (BMDY, 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 2X YNB, 40 ppm, and 2% glucose) for 16-18 hrs at 30°C with shaking. One ml of these overnight cultures was glycerol-stocked and the rest of the culture were used for induction by replacing the BMDY media with 4 ml of Buffered Methanol-complex Medium (BMDY, 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 2X YNB, 40 ppm biotin, and 0.5% methanol) (Invitrogen, 2002). For every 24 hrs, additional 0.5% of methanol was fed to the culture, as well as, 1% soytone (Zhao and others 2008) at the first methanol feed, and 1% casmino (Slininger and others 2006; Hu and others 2006; Rahbarizadeh and others 2006) at third feed to minimize the protease activities. Twenty four hours after the fourth methanol feed, the medium was

harvested by centrifugation (Invitrogen, 2002). Proteins in the medium were precipitated with 85% ammonium sulfate, and purified by cobalt chelated agarose for ELISA assays.

5. Potential oral-vaccine for the control of listeriosis.

Antigen Preparation: *L. monocytogenes* Scott A overnight culture was used in PCR as template with primers inlA-5, inlA-3 for amplifying the sequence encoding INLA from 34th to 495th AAs, and introducing *Nde*I or *Eco*RI at 5' ends and *Xho*I or *Bam* HI at 3' ends. The PCR products were inserted into plasmid pCRscript for confirming DNA sequences. Confirmed fragments of DNA were then ligated with expression vectors, pET20B+ to yield pETinla or pTX101 (Francisco and others 1992) to yield pTXinla. Plasmid pTXinla was transformed into *E. coli* TOP10 (Δ*LacI*) for constitutionally expressing rINLA on the surface of bacteria (Figure 4). Recombinant INLA was induced by IPTG with *E. coli* strain BL21 Gold (DE3) carrying pETinla. rINLA from expressed bacteria was purified with 70% saturated ammonium sulfate, followed by IMAC and DEAE columns (Figure 20) and dialyzed against proper buffer to meet the requirements for downstream experiments

Immunization, IgG purification, and titering. Four hundred microgram of rINLA were emulsified in 1 ml of adjuvant to immunize a female New Zealand rabbit. Every four weeks, the rabbit was boosted with 400 μg of rINLA, and the blood was collected one week after the boost. The rabbit IgGs were purified with protein A affinity column. Purified anti-INLA IgGs were then titered by ELISA against BSA, *E. coli, L. monocytogenes*, recombinant rINLA, and native INLA. Titers were defined as the highest dilutions (equivalent to those of blood) of those absorbance >1.0 after 30 min incubation.

Invasion Study. Monolayers of human CACO2 cells were cultured, in 24-well plate, in Eagle's Minimum Essential Medium supplemented with 20% FBS at humidified 5% CO_2, 37°C for overnight. *E. coli, Listeria innocua* and *L monocytogenes* G7757 cells were collected at log phase and washed through centrifugation with PBS. The populations of bacteria were estimated by spectrophotometry through a pre-constructed equation and adjusted to desired multiple of infection (MOI) with CACO2's growth medium. Anti-INLA IgG or rINLA was added to the medium immediately before bacteria were pipetted onto the cells. After incubated at 37°C for one hour, medium and bacteria were removed and cells were washed once with PBS containing 1% BSA. The non-invaded bacteria were killed by incubating the cells with 10 μg/ml of gentamycin in growth medium for 30 min. Cells were then washed three times with PBS contained 1% BSA to remove the antibiotic,

and were lysed with chilled water. The lysates were serial diluted and plated on TSA to determine the population of invaded bacteria.

6. Expression and biological activity assay of osmotin

Cloning, expression and purification of osmotin (OSM) from *Nicotiana tabacum*. Primers OSM5ndeB and OSM3_Bam were used to remove the vacuole targeting signal peptide and introducing NdeI and BamHI sites for precisely expression of osmotin in *E. coli.* After restricting cuts, the PCR product was gel-purified and inserted in pET20b, and transformed into TOP10 *E coli*. Confirmed clones pETOSM14, pETOSM15 and pETOSM16, were infected with λCE6 to induce osmotin at OD_{600nm} 1.0 or 1.5 (Novagen, 2008). SDS PAGE was used to examine the induction, and plasmids capable of translation were picked for sequencing. Sequence confirmed plasmids were transformed into DE3 strains of *E. coli,* Rosetta2pLYSS (Novagen) and the optimal overexpression pH was determined by growing the transformants in ZYM5052 at the pH of 6.9. 7.0 and 7.1. Confirmed culture was 100X diluted in ZYM505 (1% tryptone, 0.5% yeast extract, 0.5% glycerol, 50 mM NH_4Cl, 25 mM KH_2PO_4, 25 mM Na_2HPO_4, 5 mM Na_2SO_4, 0.05% glucose, 10 μM $FeCl_3$, 4 μM $CaCl_2$, 2.5 μM each of $MnCl_2$ and $ZnSO_4$, and 0.4 μM each of $CoCl_2$, $CuSO_4$, $NiCl_2$, Na_2MoO_4, Na_2SeO_3 and H_3BO_3) with 50 μg/ml of ampicillin and 25 μg/ml of chloramphenicol, and induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside

(IPTG) at OD_{600nm} 4.0 for eight hours. The pH of the medium was monitored with 5 ml of the induced culture, and 10M NaOH or 15M NH_4OH were added to adjust the pH of medium to between 6.9 and 7.0 at 0, 2, 4 and 6hr after induction. The expressed bacteria collected from centrifugation were lysed with sonication in 1 mM EDTA and 50 mM Tris pH 8.0. Fraction of inclusion bodies were washed twice with 50 mM Tris pH 8.0, and dissolved in 8 M urea, 10 mM Tris pH 6.0. Low capacity (0.66meq/g) CM-cellulose (Sigma C-2883) was used to absorb the denatured OSM in a flask and packed in a column. After washing with one bed volume of 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 10 mM Tris pH 6.0 and 8 M urea, 5 bed volume of 1 mM EDTA, 10 mM Tris pH 6.0 and 8 M urea, 10 bed volumes of 10 mM Tris pH 7.5 and 8 M urea, the CM-cellulose was transferred into 50- ml conical tubes and OSM were eluted twice with one bed volume of 500 mM NaCl, 50 mM Tris pH 8.5 and 8M urea for 12hr/each. Imidazole was added to this elution at the final concentration of 5 mM and denatured OSM was bound on cobalt-agarose beads in a conical tube. After washing with 5 bed volumes of wash buffer (50 mM Tris pH 7.0 and 8 M urea), beads were packed in a column and washed for an additional 5 bed volumes of wash buffer. After drained completely, agarose beads were transferred into a conical tube, and OSM was eluted twice with 100 mM imidazole, 50 mM Tris pH 7.0 and 8M urea for 12hrs/each. The purified OSM was dialyzed in 20 mM sodium acetate pH 5.2. The purity of recombinant was determinated by SDS PAGE and the protein content was measured with Bradford protein assay.

Biological activity of osmotin. One hundred microlitter of YPAD (1% yeast extract, 2% peptone, 2% glucose, 0.01% adenine), 0.02 OD_{650nm} of yeast, *Candida albicans* (ATCC11651), *Cryptococcus neoformans* (ATCC13690), *Saccharomyces cerevisiae* (BWG7a), *Saccharomyces cerevisiae* (GRF167), *Pichia methanolica* (PMAD11), *Pichia methanolica* (PMAD16) and *Pichia methanolica* (PMAD16/pMETαB/HAS) diluted from fresh overnight cultures were grown in the wells of microplates with a serial dilution of OSM from 0-450 μg/ml. Plates were incubated at 30°C for 48 hr, and the OD_{650nm} of cultures were determinate with a microplate reader. Each reading was blanked with the culture without inoculums at the same OSM concentration, and dived by the highest OD reading in its own strain, as relative growths. The relative growths were further analyzed with SAS Version 9.2 for ANOVA and Turkey grouping. *Vibrio cholera* and yeast strains mentioned were streaked on 0.8% low-melting agarose YPAD plates with 0, 12.5 and 25 μg/ml of OSM, and grown the microorganisms at 30°C for 48 hr. To measure the ferric (III) binding capacity, recombinant OSM was placed in dialysis tube (molecular weight cut off 6-8 kd) and human heatshock protein 60 (HSD1) was used to blank the possible binding of poly-histidine tag. Tubes were placed in 2l of 10 mM or 1 mM $FeCl_3$ buffer with sodium or ammonium at pH 5.2, and allowed protein to absorb the ions for 24hrs. Followed by three changes with deionized water and total dialysis time of 4 days, proteins in tubes were quantitated by SDS with references of serial dilution of BSA at 1-40 μg/well. After 4 hr baking at 450°C, iron was dissolved in 25 ml of 1% hydrochloric acid for atomic absorption analysis.

IV. RESULTS

1. Improvements of pathogen detection

1a. Development of Immuno-Polymerase Chain Reaction.

Synthesis of magnetic beads and expression of SPA. We were able to produce about 450 g of magnetite from 6 L of reagents. The particle size was determined under a transmitting electron microscope and observed to be 10-90nm (Figure 7). The synthesized beads resisted the erosion with up to 4M hydrochloric acid (Figure 6). The capacity of magnetic beads for aldehyde groups were about 20 μM/ml of beads. The recombinant SPA was able to be expressed at the level of at least 1 g at the molecular weight of 33 kd as expected, and several hundred milligrams of them could be purified (Figure 8). Agarose beads that conjugated with recombinant SPA were able to separate the IgG from rabbit serum (Figure 9) and the binding capacity was about 2 mg IgG per ml of agarose beads.

Enzymes for polymerase chain reaction. Enzymes were successfully purified (Figure 10). The polymerases were titered for optimal concentration of enzymes in PCR (Figure 11). Both STgo and SNiq have similar optimal enzyme concentration at 3 ng/50 µl of reaction. As expected, SNiq catalyzed much more target DNA fragments. Moreover, higher concentrations of STgo showed inhibition to amplicon amplification but not SNiq. Exonuclease activities were not detected when Niq and SNiq were incubated at 72 °C for up to 3 hrs (Figure 12). However, noticeable degradation of DNA by STgo was observed as early as 1hr. Reactions catalyzed by STgo and SNiq were much faster than Taq (Figure 13). Bands at sizes up to 3.5 kb, and reaction rate in SNiq was calculated to be 4 kb/minute, while Taq has a reaction rate of was about 0.75kb/minutes. PCR products in SNiq showed more specificity than that of STgo. STgo and SNiq also showed better salt tolerance over the pH of 8.0, 8.5, and 8.8 (Figure 14). For the dUTPase activity, recombinant protein showed at least a two fold increase of PCR products at the enzyme ranges of 0.4-0.006 µg/50 µl of reaction (Figure 15). When enzyme concentrations were greater than 0.8 µg/50 µl, the detoxification was inhibited; however, the PCR yield is not less than the control.

1b. Sources of Immunological recognition molecule

MRSA monoclonal antibodies. Greater than 95% purity of the rpbp2a protein was achieved (Figure 16). A slight reduction in the toxicity of penicillin G was

observed when greater than 280 μg of this recombinant protein was dropped on the plate (Figure 17). An agglutination test demonstrated that purified rpbp2a created the largest amount of precipitation (Figure 18). The biological activity assay indicated that the protein was properly translated and folded. The monoclonal antibodies obtained were all specific against the lysates of MRSA.

Phage antibodies. Because detectable amounts of plasmid pKscFv and significant amounts of genomic of host *E. coli* were co purified (Figure 19), we designed an experiment to evaluated the leak by miss-packing plamid DNA. From 10^{12} PFU of M13dp3A, 20 (±3.2) streptomycin resistant colonies were found, indicating that there was leakage at 2.0 in 10^{11} PFU of phages. At present, 12 clones of antibody phages were successfully constructed. The antibody showed highest reactivity with *B. anthracis* (Table 1)

Yeast scFv. Although there was an instrument failure in the Bio-Rad GenePulser, 16 stable clones were obtained after adenine (ADE2) selection. Although, expression level of scFv was not able to be detected by SDS PAGE or slot blot, some distinguishable readings were obtained from ELISA (Table2)

2. Control of foodborne pathogens.

Evaluation of the efficacy of the anti-listeriosis vaccine. A female New Zealand rabbit was immunized with a 50kd fragment of internalin A (INLA, Figure 20) from *L. monocytogenes* including the domains that bind to E-cadherin (CDH1). The immune response was very strong. The titer of the serum was greater than 500 in one week after the first immunization. Since then, the titer remained consistently high between 4,000 and 8,000 after one week of the boost. Invasion studies using protein-A purified from this polyclonal antiserum to attenuate listerial INLA in COCA2 cell culture showed at least 92% of the invasions were blocked. Our results indicated that if properly delivered, INLA is a good candidate for use as an oral vaccine preventing listeriosis. In our safety evaluation of rINLA surface expressed *E. coli,* the transformed bacteria (clone 33) seems to have similar invasion as its host TOP 10 (Figure 21). Anti-INLA IgG blocked the invasion up to 99% at a multiple of infection (MOI) of 100 (Figure 22) and 95% at MOI of 10 (Figure 23). The *Listeria innocua* invasions were significantly increased when the amount of purified rINLA were increased (Figure 24)

Expression and fungicidal activity assay of osmotin. OSM inducible clones were able to be screened by the infection of bacteriophage lambda carrying T7 RNA polymerase gene (λCE6) at both OD_{600nm} of 1.0 and 1.5. However, the induction level

by λCE6 was too low to be confirmed with SDS PAGE, and hence, western blotting with anti-OSM antibody were used to detect the expressions (Figure 25). Because higher bacteria density yielded better OSM, the OSM was induced at a very late log phase. The largest amount of proteins were induced at pH 7.0, therefore this pH was used for large scaled induction. The recombinant proteins remained in the inclusion body when we tried to wash some of the soluble protein away (Figure 26). OSM-expressed bacteria were observed to be very fragile, and could be lysed by only deionized water. OSM was purified by CM-cellulose and cobalt-agarose (Figure 27) in urea denatured form at a very high purity. It was estimated by the density of the bands in SDS gel that at least 80% of osmotin remained on CM-cellulose and Co-Agarose (Figure 28) Fortunately, at least 60% of OSM remained could be recovered with the modified protocols mentioned in the Materials and Methods Section.

Although, osmotin growth inhibition ($p<0.002$) in every strain tested, only *Cryptococcus neoformans* was completely inhibited (Figure 29). Similar results were also observed from solid state inhibition (Figure 30). In addition to the total abolition of *Cryptococcus neoformans*, the growth of the *Saccharomyces cerevisiae* strain BWG7a was slightly inhibited. We also proved that OSM would bind to ferric (III) ions (Figure 31.) Every Fe^{3+} bound to 5 molecules of dissolved proteins or 4 precipitated OSM. This binding decreased as the concentration of the iron increased, and was totally blocked by ammonium (Table 4).

V. CONCLUSIONS

1. Improvements of pathogen detection

1a. Development of Immuno-Polymerase Chain Reaction

Synthesis of magnetic beads and expression of staphylococcal protein A.

We were able to produce magnetite at a particle size of 10-90 nm under a transmitting electron microscope (Figure 7). The synthesized beads resisted the acid erosion with up to 4 M of hydrochloric and had aldehyde group capacity of 20 μM/ml of beads. The magnetic beads were conjugated with anti-*Salmonella* IgG and determined to work efficiently in chicken skin samples (Carter, 2008). However, we also noticed that the bacteria cannot be eluted thoroughly, thus we designed a sandwich method based on SPA-Fc interaction to capture the IgG bound bacteria. Although we have not tested this method yet, there are several reasons why we believe that SPA magnetite would perform better than that directly conjugated with IgG. First, bacteria can be thoroughly eluted. This is extremely important because it is impossible to be quantitated without complete elution. Second, IgG could be inactive when the Fab domains are reacted

with the aldehydes in the direct conjugation. To compensate for this adversary effect we used excess amounts of precious antibodies, usually tens of milligrams. The inactivation by aldehydes will be very insignificant because every SPA has five binding domains and any of them can bind to IgG independently. Third, the SPA conjugation is flexible. A single batch of SPA conjugated beads can universally react with any IgG purified by SPA. Last, and the most attractive one is that the SPA conjugated beads allow us to adjust the antibody concentrations for better specificity and sensitivity. In the case when antibodies were conjugated directly to the beads, the amounts of IgG needed to be compromised to the binding capacity of the beads. For most antibodies, no matter how specific they are, nonspecific captures will be present in excess of IgG. On the other hand, bacteria might not be captured if there are not enough antibodies.

We encountered several difficulties when we tried to clone and express the SPA. The *spa* gene is composed of several repeated domains. Inside each domain, it is composed of several repeats, too. These continual repeats make not only PCR very difficult but also instability of the gene in the recombinase A ($recA^+$) *E. coli* strains such as NEB Turbo. Since *spa* has repeat sequences, it makes sense that recA might recognize it as damages and remove some fragments of DNA (Puopolo and others 2001; Hsieh and others 1992). It is worth to mention that *spa* is probably not stable in *S. aures*, neither. It was discovered that *spa* is one of the most vaiant ORF in *S. aures*. The instability of *spa* in *S. aureus* made this gene vary in the sequences and lengths. This phenomenon of *spa* was then quickly grasped by microbiologist to develop *spa*

typing to distinguish the strains in *S. aureus*. However, once we switched the host to recA$^-$ strains, such as MachOne or Top10, the stability of *spa* seemed to be acceptable. Very low yield was obtained when SPA was induced in regular BL21 (DE3) *E. coli.* Induction of SPA using transformation mixture slightly increased the SPA production, but the yield is only 1/10 of that in *E. coli* strain BLR (DE3, recA$^-$).

The binding capacity of SPA-agarose beads was observed to be 2 mg/ml beads. This is far below our expectation of 10 mg/ml. Further modification of the conjugation method by substitution of sodium cyanoborohydride with sodium borohydride increased the capacity significantly, implying that some critical amino acid residue can be modified by cyanogens and inactive the IgG binding activities.

Enzymes for polymerase chain reaction. In addition to better thermal stability, thermal stable DNA polymerases from the genus *Thermococcus* showed better and faster catalytic activities than Taq (Arezi and others, 2003). Therefore, DNA polymerases from *Thermococcus* are perfect candidates for PCR pathogen detection. However, because many research studies have achieved poor PCR yields due to the exonuclease activities in type B polymerases, we modified a thermal stable DNA polymerase encoded by *Thermococcus gorgonarius* (Tgo) at its exonuclease domain I to improve the productivity. The exonuclease activity of the Niq was not detected after incubation with oligonucleotide at 72˚C for 3 hours. Niq shows similar amplification patterns as native Tgo but tolerates a wider range of polymerase

concentrations, yields more DNA, and reduces in minor bands in agarose electrophoresis gel. Further modifications on Niq with a single strand DNA binding domain (sso7d) to make this enzyme, SNiq, catalyzes the DNA synthesis with a reaction rate at 1kb/15sec. SNiq was also shown to tolerate a very wide range of ionic strength and pH . PCR products catalyzed by SNiq showed little differences between the conditions of 10-120 mM Tris and pH 8.0-8.8. This salt and pH insensitive characteristic of SNiq indicated that this enzyme is very likely to tolerate more salts in microbiological media and tolerate more acids produced by microorganisms, and is more robust and reliable for pathogen detection.

The rapid reaction rate of STgo and SNiq make longer DNA synthesis while annealing, and thus, they require at least 6°C higher annealing. Therefore, not only by reducing the extension time but also reducing the time required for temperature in the thermal cyclers to ramp down. In addition to rapid reaction rates, the higher thermal stability of group B DNA polymerase also allowed the denature times to be reduced to as low as 10 sec at 98°C whereas, Taq required at least 30sec at 95°C. For 1 kb amplification in 35-cycle PCR, STgo and SNiq could be completed in 1.5 hrs in a peltier-cooling thermal cycler, whereas, Taq might require 2.5 hrs.

2b. Source of Immunological recognition molecules

Phage antibodies. Like *spa* cloning, the *gIII* for the construction of the pKscFv plasmids was found to be slightly unstable in the recA^{+} *E. coli* strains such as NEB Turbo. Several researchers have indicated the low fidelity of *gIII* during the display of scFv (Krumpe and others 2006). Since *gIII* contains several repeat sequences, it would appear that recA might recognize these defects and remove some fragments of DNA (Puopolo and others, 2001; Hsieh and others, 1992). However, once the host was switched to recA^{-} strains, such as OmniMax or Top10 F', the stability of *gIII* seemed to be improved.

Following the detection of plasmid pKscFv and after co-purification of a significant amount of *E. coli* genomic DNA (Figure 19), we designed an experiment to evaluate the leak by miss-packing of pKscFv plamid. From 10^{12} PFU of M13dp3A, 20 (±3.2) streptomycin resistant colonies were found, indicating that the leakage was 2.0 in 10^{11} PFU of phages. At the beginning, we designed this binary expression system to prevent M13 from packing the scFv coding sequences, thus, the manufacturers will be guaranteed of their customers' continuous business. At this time, we do not know if this level of leakage is acceptable to prevent the customer's propagations of phage antibodies. However, we believe further optimization in the time of phage harvest will help. Although, only 12 clones of antibody phages were successfully constructed, the

antibodies showed highest reactivity with *B. anthracis* (Table 1) Of course, further optimization is still necessary.

One potential problem regarding this binary expression system needs to be mentioned. This expression system largely depends on the trace amount of lactose to produce enough phage shock promoter regulator protein (PSPA) to suppress the *ppsp* promoter activity, so helper phages can infect efficiently (Rakonjac and others, 1997). On the other hand, too much lactose in the medium would theoretically produce phages without PIII-scFv fusions. Lactose contents in Tryptone were found to have significant effect on the *lac* promoter, and vary by manufacturers, lot, grade, preparation and even the age of storage (Grossman and others 1998; Studier, 2005). Lennox formula (Cat#75852, Lot#118557) and Miller formula (Cat#75854, Lot#122245) of LB media from USB Corporation, Cleveland, OH, were tested for PFU and the sizes of the plaques. Slightly more PFU but significantly lager plaques were found in Miller formula. It is not clear that if NaCl, batch or freshness made this difference. However, any one would like to repeat our experiments with different basis of tryptone should try several different sources to find a better media.

Yeast scFv. Because yeasts are eukaryotes and have similar posttranslational modification as mammalian, they are good platforms for scFv production (Chang and others 2008; Cunha and others 2004). Among all the yeast expression systems, *Pichia* has been proved to produce much more cell mass (Fan and others 2008; Chang and

others, 2008; Cunha and others, 2004; Shi and others 2003). Because anti-*B. anthracis* scFv showed toxicity to *E. coli,* and no colonies were found when expression vectors were transformed, we picked the system, *Pichia methanolica*, of which the expression level can be controlled. Although, the expression level of *Pichia methanolica* induced by methanol is much less than that in *Pichia pastoris* driven by AOX promoter, several expressions have been successfully induced by methanol in *Pichia pastoris* (Cunha and others, 2004).

Because of instrument failure in the Bio-Rad GenePulser, only 16 stable clones were obtained after ADE2 selection. This is far lower than Invitrogen's suggestion for selection of high expression level clones. Although the expression level of scFv was not able to be detected by SDS PAGE or slot blot, some distinguishable readings were obtained from ELISA (Table 2), indicating that this project is feasible.

Because of its ability to survive long periods of time high mobility, *Bacillus anthracis* is recognized as a bioterrorism threat. Although *Bacillus anthracis* is not a foodborne but bioterrorism threat, this model of phage antibody can be easily adapted for food microbiological detection. For bioterrorism threats, many of these situations occur in battle fields, and soldiers are not very likely to bring an ELISA reader, and refrigerator. Therefore, the format of detection and thermal stabilities of the antibody become very important. For the thermal stability, Fc domains of native immunoglobulin will be irreversibly inactivated at the temperature above 55°C in minutes (Vermeer and Norde, 2000), whereas M13 phage can tolerate 65°C for days (Auburn University Detection and Food Safety, personal communications) On the

other hand, scFv varies in amino acid sequences and the thermal stability could be different. A research project using a yeast expression system to screen scFvs that tolerate up to 61°C (Orr and others, 2003) inspired us that fusing the scFv with PIII in M13 has a chance to obtain antibodies that tolerate a temperature beyond 55°C.

2. Control of foodborne pathogens.

Evaluation of the efficacy of the anti-listeriosis vaccine. Effective vaccinations rely largely on how well vaccines represent the pathogens. Using INLA as an antigen for vaccination provides several advantages over live *L. monocytogenes* cells. First, all the pathogenic *L. monocytogenes* expresses INLA, thus immunity to INLA will provide protection against all pathogenic *L. monocytogenes.* Second, once immunoglobulin encounters pathogenic *L. monocytogenes*, it instantly neutralizes INLA which is the key pathogenic molecule in animals. Third, immunizing animals with INLA is a much safer procedure than immunizing them with live *L monocytogenes*.

Unlike many reports (Lecuit and others 2001; Willers and others, 1982), our invasion studies with *L. innocua* indicate that even un-anchored INLA shows activity to facilitate the invasions (Figure 24). This unexpected result further emphasizes the importance of INLA for *Listeria* pathogenesis and implies that the neutralization of INLA by antibodies might also reduce the chance of other less invasive pathogens,

such as *Salmonella spp* infection. Further examination of this hypothesis with anti-INLA IgG indicated that the neutralization of INLA blocks most invasions. The results indicate that oral vaccination with INLA-surface-expressed *E. coli* to protect animals from listeriosis is feasible. Currently, only two questions remain to be addressed: first, will the INLA surface-expressed *E coli* stimulate significant IgA production in the gastrointestinal tract. Second, will IgA be produced in the gastrointestinal tract after oral immunization providing the same anti-*Listeria* activity as in our *in vitro* study?

Expression and fungicidal activity assay of osmotin. Some difficulties were experienced when cloning the OSM gene for expression. In the first attempt, the blunt-ended PCR product was cloned in the pJET1.2 (Fermentas), in which the multiple cloning sites are located in an ORF encoding restriction enzyme (AvaII) for positive selection of the insert. The OSM insertion was toxic to *E. coli*. Very few clones were obtained, and all the restriction confirmed clones were sequenced to have frame shift at the primer recognition sites. Therefore, it was determined that to clone the PCR products in pETs and screen the plasmid in non-DE3 strain (TOP10) with λCE6. OSM inducible clones were able to be screened by the infection of λCE6 at both OD_{600nm} of 1.0 and 1.5. However, the induction level by λCE6 is too low to be confirmed with SDS PAGE, and hence, western blotting with anti-OSM antibodies were used to detected the expressions (Figure 25)

The largest amount of proteins were induced at pH 7.0, therefore this pH was used for large scale induction. Interestingly, the pH in the induced culture was 5.5 if the induction pH is 7.1, whereas 6.0 for the pH 6.9 and 7.0 induction, and roughly 6.3 for non-OSM induction. The reason for this pH decrease is not known. However, because OSM-expressed bacteria were determined to be very fragile, and can be lysed by only deionized water, it is very possible that the pH drop might be the cytosol leak. However, when we analyzed the coding sequences of OSM in the WorkBench software hosted in San Diego Supercomputer Center, we did not find any transmembrane possibility (Table 5) Therefore, the lysis of bacteria might be caused by other mechanisms.

Although, OSM can be purified by CM-cellulose and followed by cobalt-agarose in a urea denatured form at very high purity with a standard protocol. It was estimated by the density of the bands in SDS gel, which at least 80% of osmotin remained on CM-cellulose and Co-Agarose (Figure 28). Fortunately, by a modification mentioned above at least 60% of OSM remained could be recovered.

In a study by a research team in Purdue University using a functional complementary method to investigate the mechanism how osmotin kills yeast cells. Yeasts were incubated with osmotin in medium and spread on plates to determine the survive (Yun and others 1997). Since osmotin trends to aggregate, there are chances that osmotin did not kill but rather than bound and aggregated the yeast cells, and thus, less yeast colonies formed. Moreover the experiment designed did not simulate the conditions associated with foods. Therefore, we designed a liquid and solid state

inhibition for evaluation of the fungicidal activity of OMS. Only *Cryptococcus neoformans* was completely inhibited by OSM in liquid inhibition assay. The inhibition could only be determined statistically ($p<0.002$) in other strains (Figure 29.) In addition to *Cryptococcus neoformans*, the growth of *Saccharomyces cerevisiae* strain BWG7a (OSM sensitive) was slightly inhibited in a solid state inhibition test (Figure 30). This inhibition of BWG7a gave similar results as described (Yun and others, 1997), indicating that posttranslational modifications in *E. coli* did not affect the activity of OSM. The data were further analyzed for the liquid state with Turkey Post Hoc tests; It was determined that higher concentrations of OSM slightly inhibited the fungicidal activity (Table 3). This U-shaped curve implied that there might be self-regulation mechanisms associated with this protein. The *in silico* analysis in WorkBench lists several regulation possibilities by phosphorylation, glycosylation and amidation (Table 5).

At our very first try of purification, our "purified" protein was slightly red. However, OSM was purified to be colorless (Singh and others, 1987). By the communications, we know that several cancer research teams are using OSM as a human adiponectin analogue in preventing cancer formation. Their unpublished results indicated that not only the phenotypes the adiponectin induced, but cancer cells treated with OSM also showed the syndromes of iron deficiency, meanwhile, the iron deficiency was not present to be significant in the normal cells. Therefore, we believe that the redness was caused by iron, and we tried to remove it from the proteins. Although we did not try any ferric chelators as described (Fernandez and Winkelmann,

2005; Hermes-Lima and others 2000), the removal of iron could be accomplished by reducing the Fe (III) to Fe (II) and absorbs it on EDTA, as mentioned in the Materials and Methods Section. Although every OSM only bound 1/4 -1/5 ferric (III) ions (Figure 31), the binding was very tight. At least three changes of buffer would not remove Fe^{3+} (Table 4). Therefore, not only for food safety control, we believe that OSM has great potential in cancer preventions.

3. Overall conclusions

There is no doubt that better food safety conditions reduce foodborne illnesses. In this dissertation, we contributed several examples how to improve food safety using recombinant protein technologies. Relative to foodborne pathogen detections, we developed cost effective SPA magnetic beads. We also improved reaction rates, specificity, yields and tolerances to pH and ionic strength of thermal stable DNA polymerase for PCR. By combining with SPA magnetic beads, the assay of PCR and immunological methods could be confirmed in a single run. Therefore, possibilities of false negatives or positives could be significantly reduced.

We also used recombinant protein to remove the regions of pbp2a that is homologous to other penicillin binding proteins to prepare an antigen for antibody immunization. Because the antigen prepared is less complex, and many other *S. aureus* proteins are excluded, we can have a very good antibody for MRSA detection.

Although further optimization is required, our phage and yeast scFv showed promising results. Moreover, we might be able to obtain a thermal stable antibody that meets the criteria of the military.

For the control of pathogens, our anti-*Listeria* oral vaccine was effective in our *in vitro* studies. We are the first team in the world to expressthe tobacco osmotin at the level of tens of milligrams. Although there are still many unanswered questions, we believe that we made this protein practically available. This big step would provide us enough opportunities to discover the full potencies of osmotin.

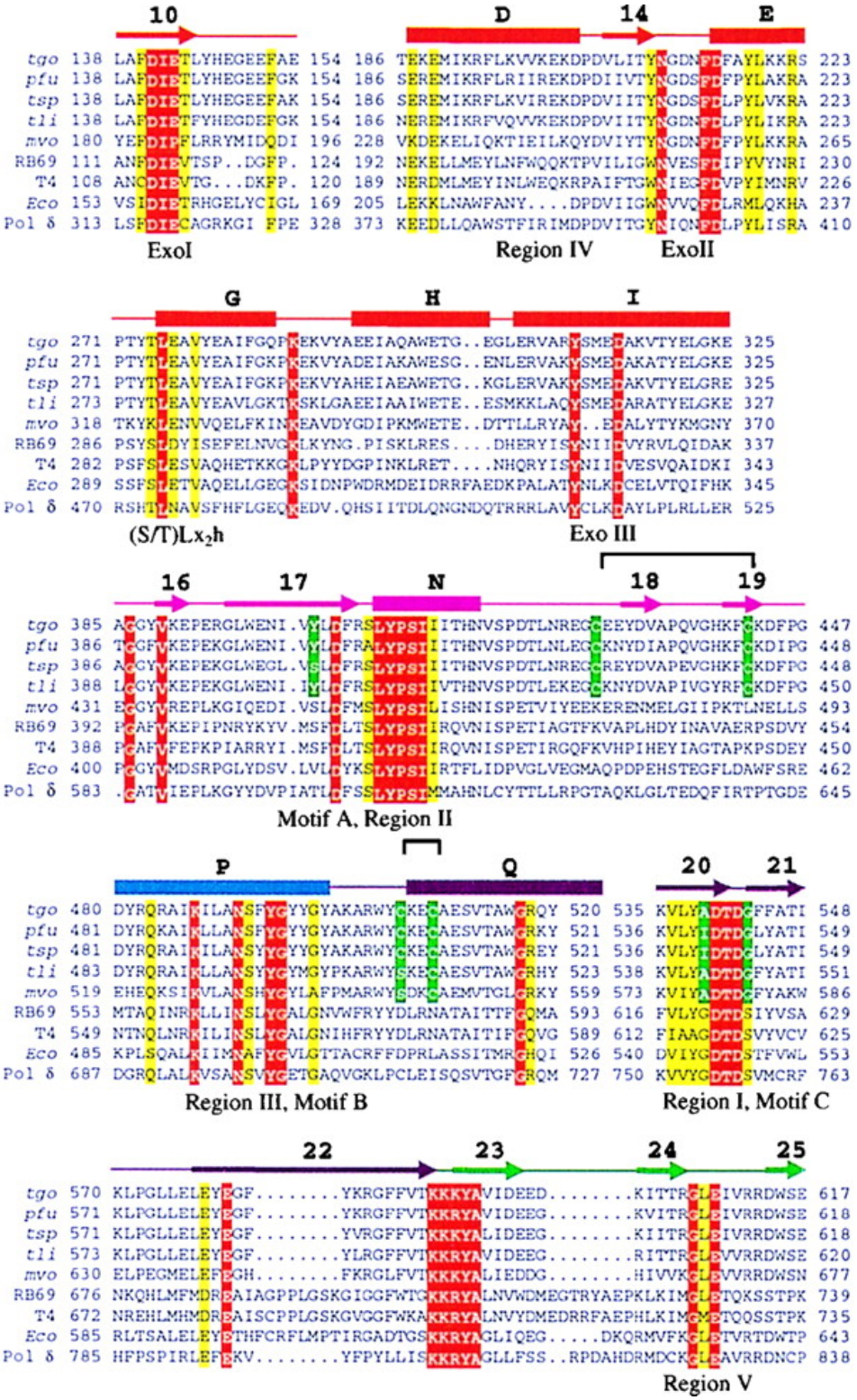

Figure 1. Amino acid sequence alignment of group B DNA polymerases (Hopfner, 1999).

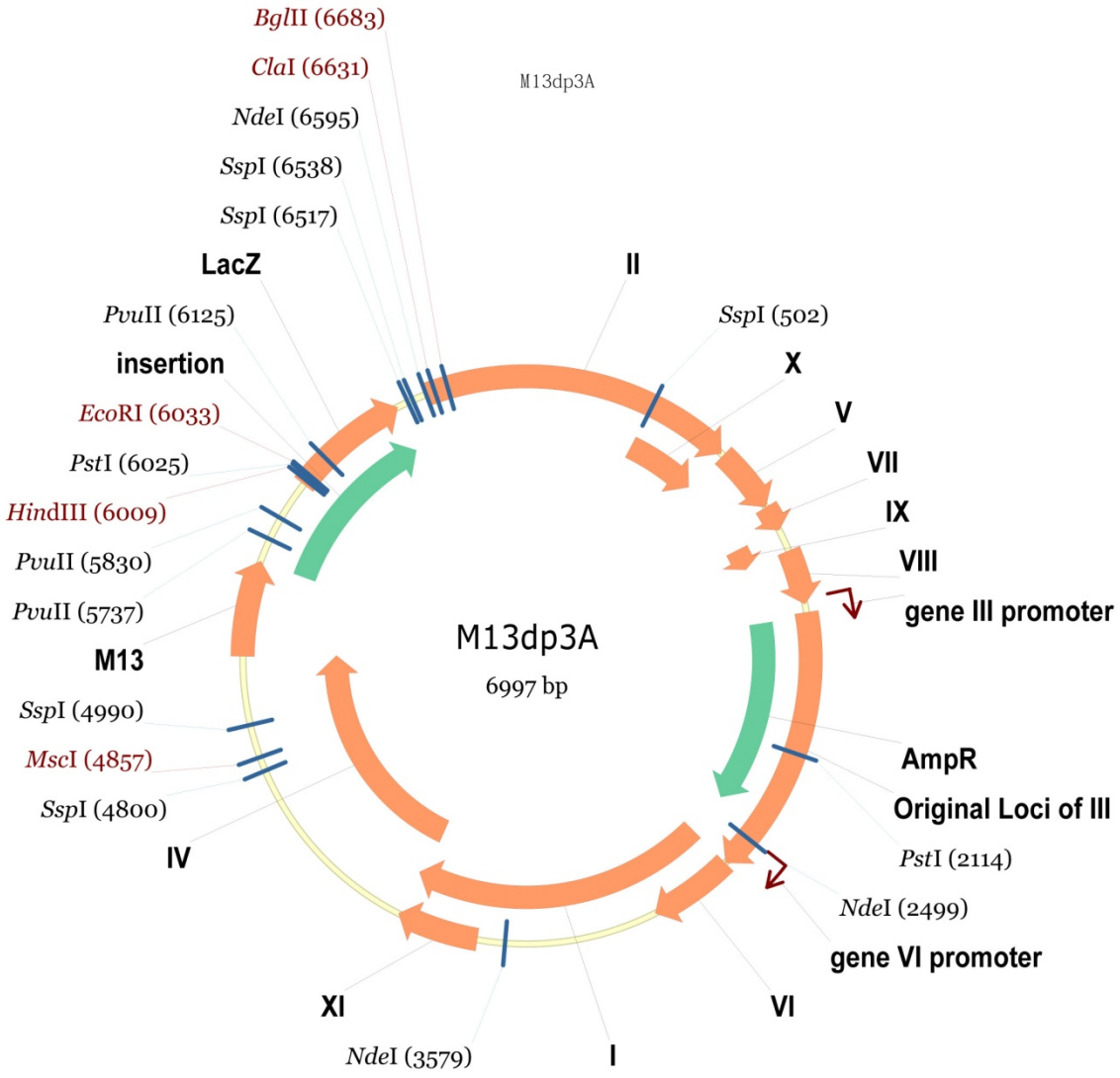

Figure 2. Vector map of M13dp3A.

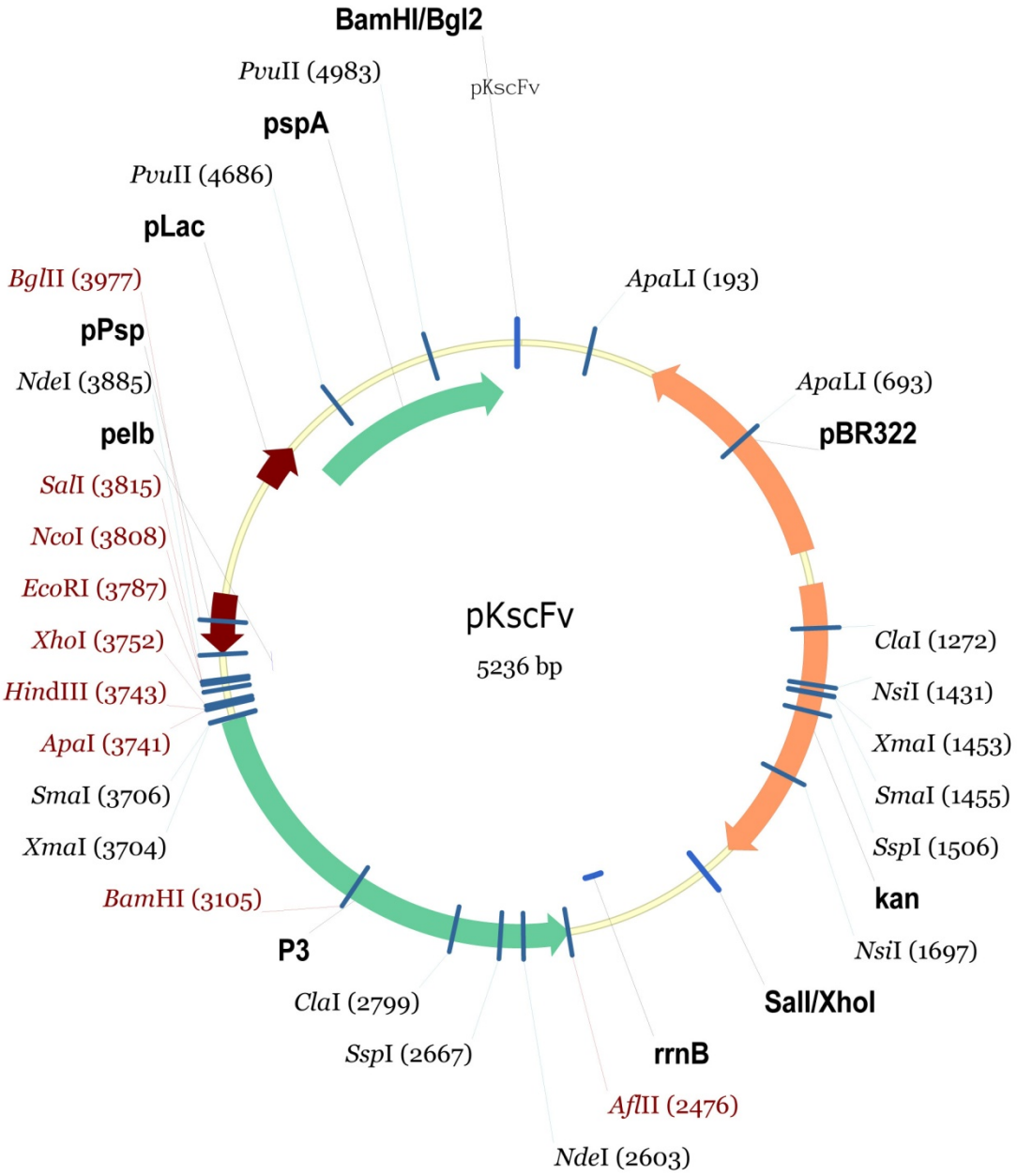

Figure 3. Plasmid map of pKscFv.

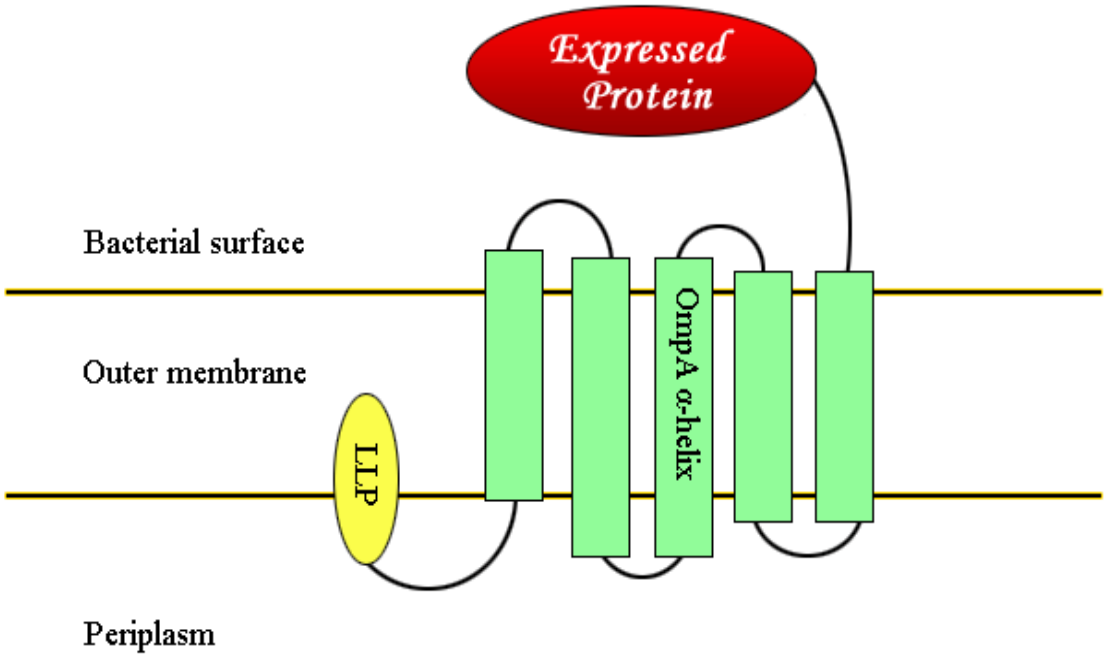

Figure 4. Protein targeting and possible structure of gene product encoding in the surface expression vector pTX101. (Francisco, 1992)

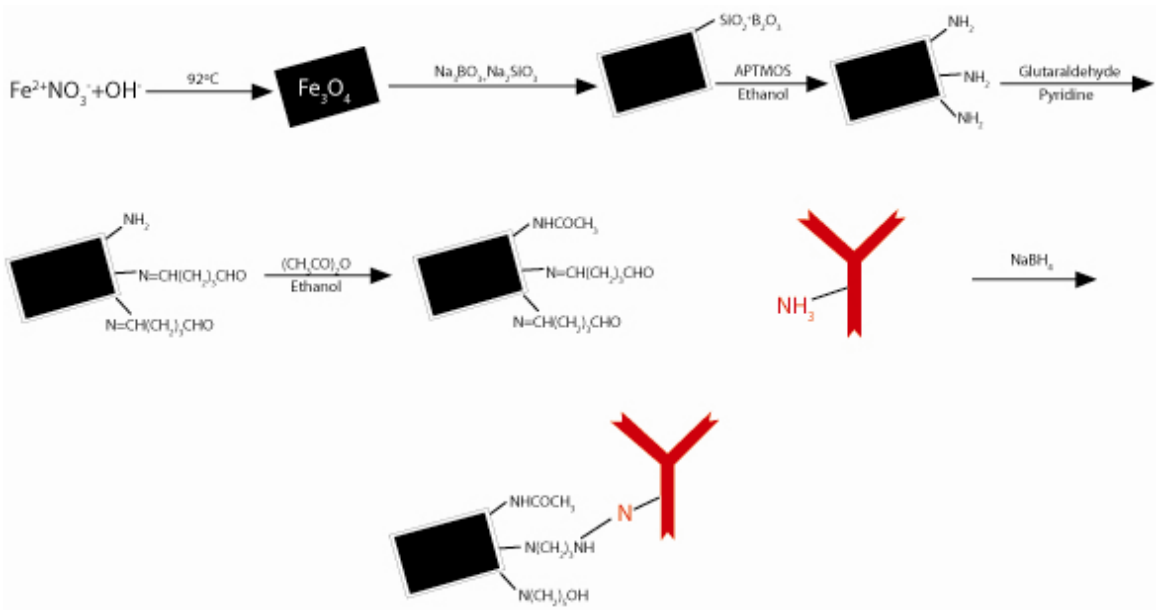

Figure 5. Scheme of procedure for synthesis of antibody conjugated magnetic beads.

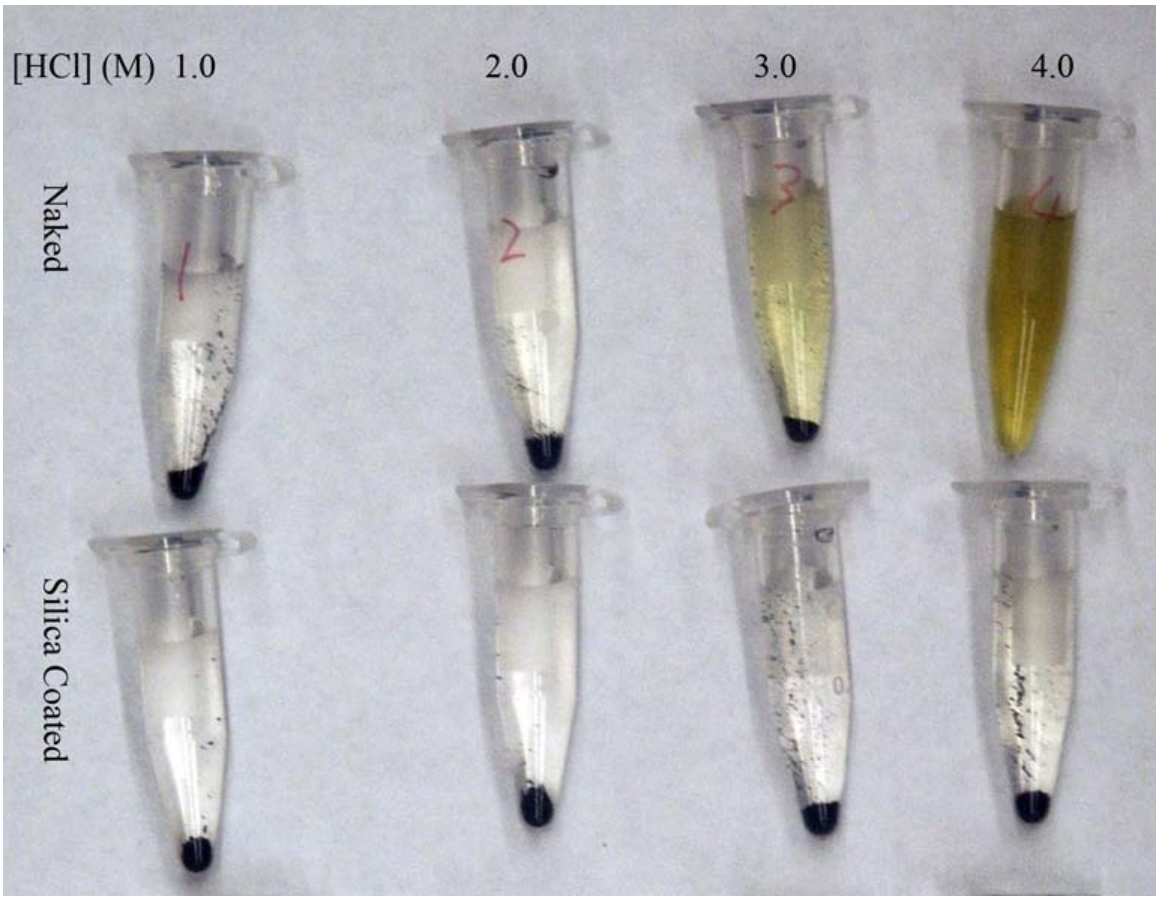

Figure 6. Efficiency of silica coating of magnetic beads.

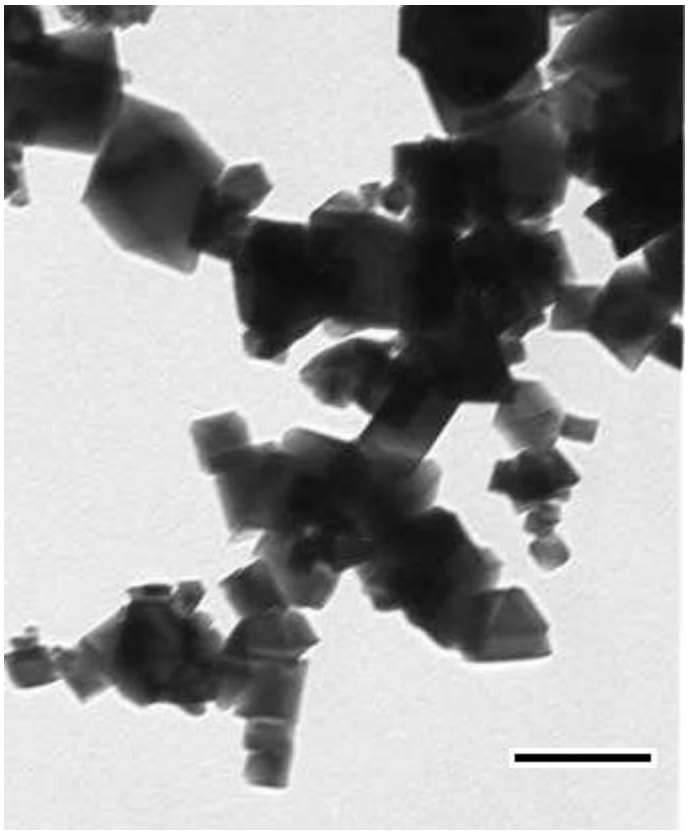

Figure 7. Transmitting electron microscopic photography of magnetic beads synthesized. The bar at the right-bottom corner represented 100nM

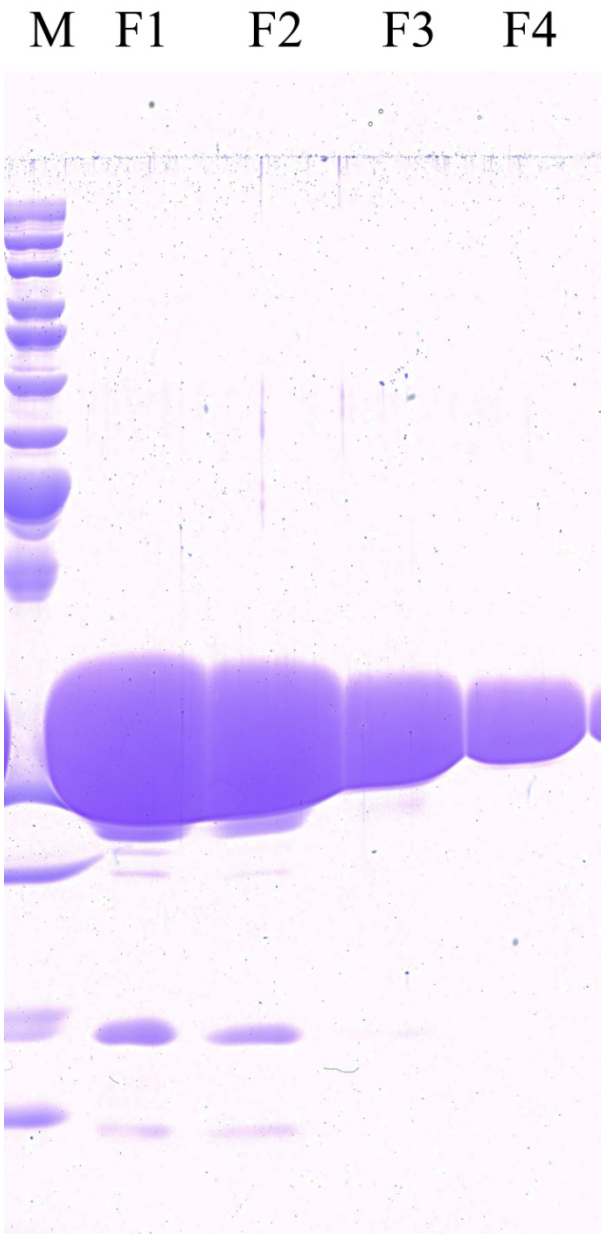

Figure 8. Cobalt chelated agarose elution of SPA. Lane F1 – F4 are fraction collected from the elution, M is molecular weight marker (Fermentas Page Ruler) representing the migration of 200, 150, 120, 100, 52, 70, 60, **50**, 40, 30, 25, 20kd proteins in a 12% SDS PAGE.

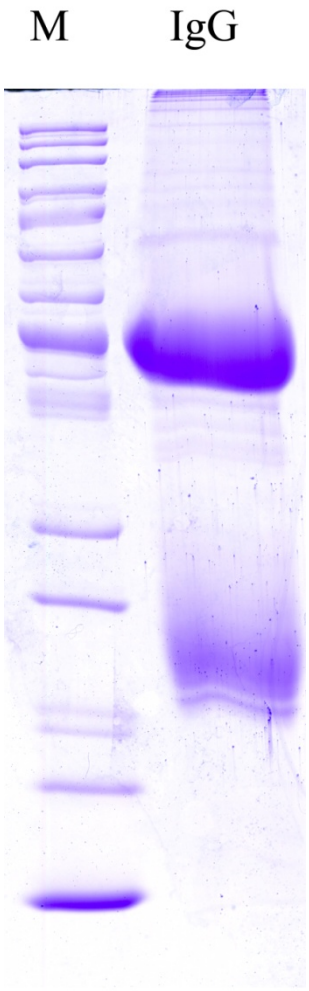

Figure 9. Rabbit IgG purified with recombinant SPA agarose (IgG). M is molecular weight marker (Fermentas Page Ruler) representing the migration of 200, 150, 120, 100, 52, 70, 60, **50**, 40, 30, 25, 20kd proteins in a 12% SDS PAGE.

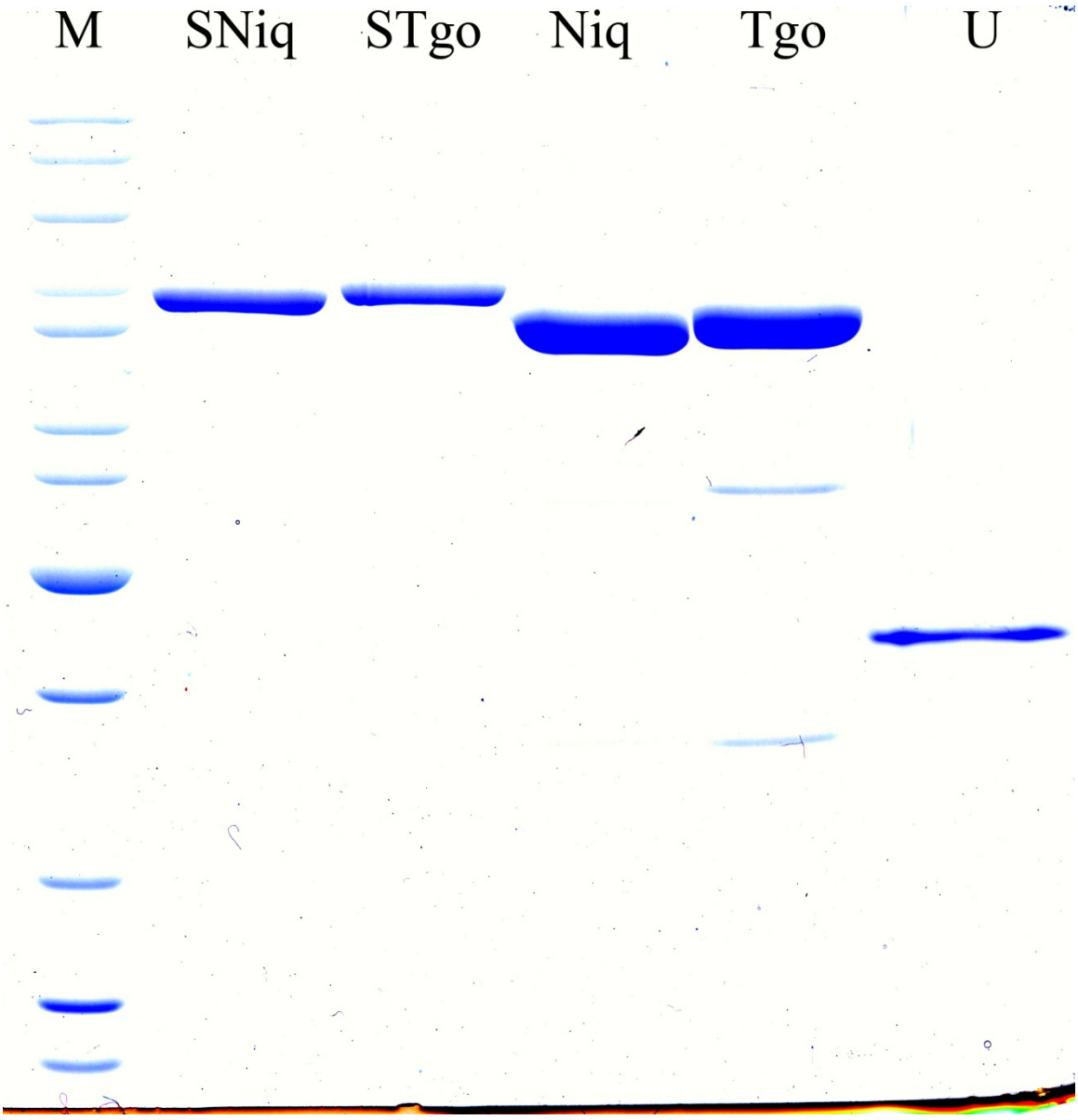

Figure 10. 12% SDS PAGE of thermal stable enzymes purified. M is molecular weight marker (Fermentas Page Ruler) representing the migration of 200, 150, 120, 100, 52, 70, 60, **50**, 40, 30, 25, 20kd proteins in a 12% SDS PAGE.

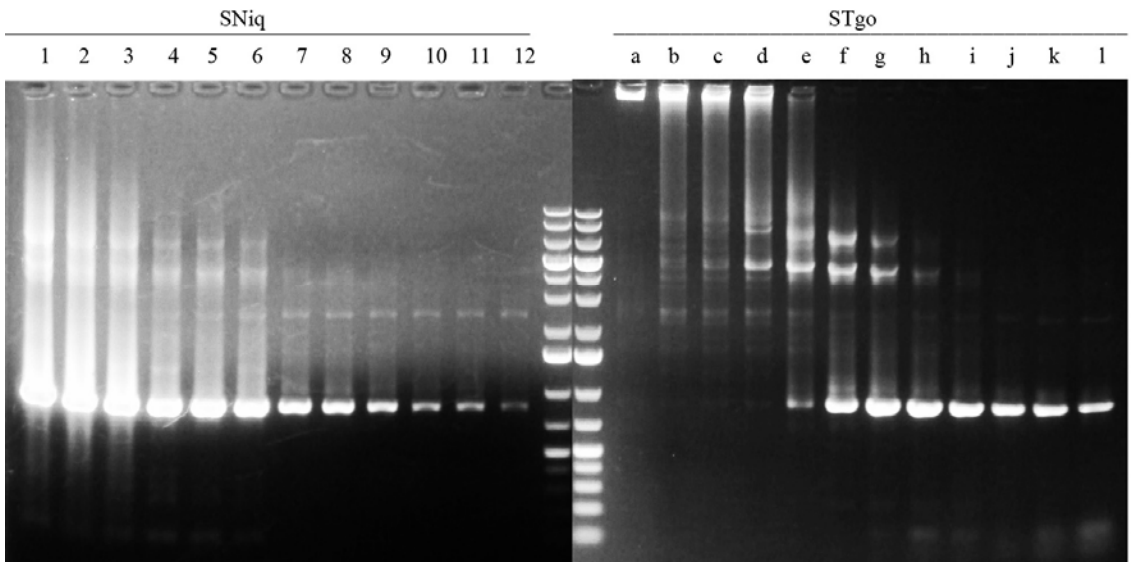

Figure 11. Titering of SNiq and STgo for optimal enzyme concentration for PCR. Lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 represent 10 µl of PCR catalyzed by 1.1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015, 0.008, 0.004, 0.002, 0.001, 0.0005 µg of SNiq in 50 µl reaction. Lane a, b, c, d, e, f, g, h, i, j, k and l represent 10 µl of PCR catalyzed by 1.4, 0.7 0.35, 0.18, 0.09, 0.05, 0.025, 0.012, 0.006, 0.003, 0.0015, 0.00075 µg of STgo in 50 µl.

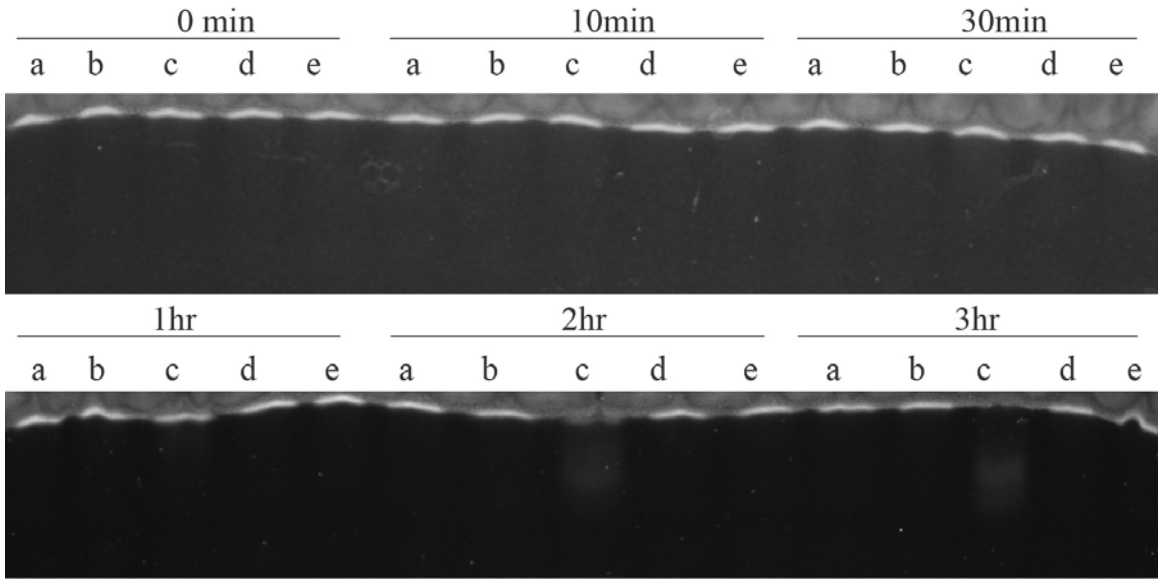

Figure 12. Non-radioactivity exonuclease activity assay by measuring the degradation of a 60kb single strand DNA catalyzed by no enzyme (a), Taq (b), STgo (c), Niq (d), SNiq (e).

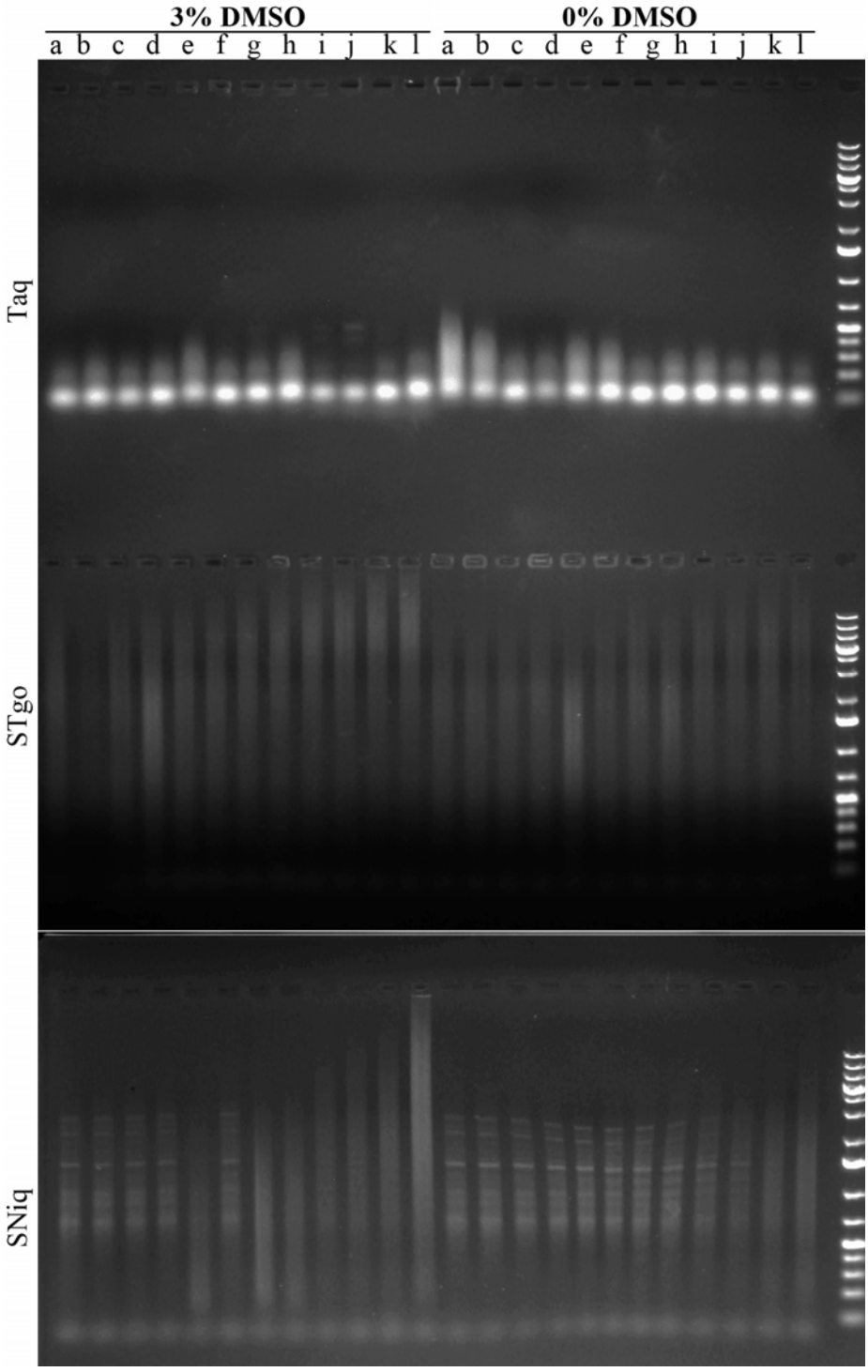

Figure 13. Volocity of DNA synthesis catalyzed by Taq, STgo, and SNiq in gradient PCR. (Legend is present next page)

Figure 13. Volocity of DNA synthesis catalyzed by Taq, STgo, and SNiq in gradient PCR. Gradient PCR from 58 (a) to 70°C (l) was used to compensate the difference of the annealing temperature. With the extension time of 48sec, Taq was able to produce DNA less than 500bp whereas SNiq catalyzed the synthesis of DNA fragments up to 3kb.

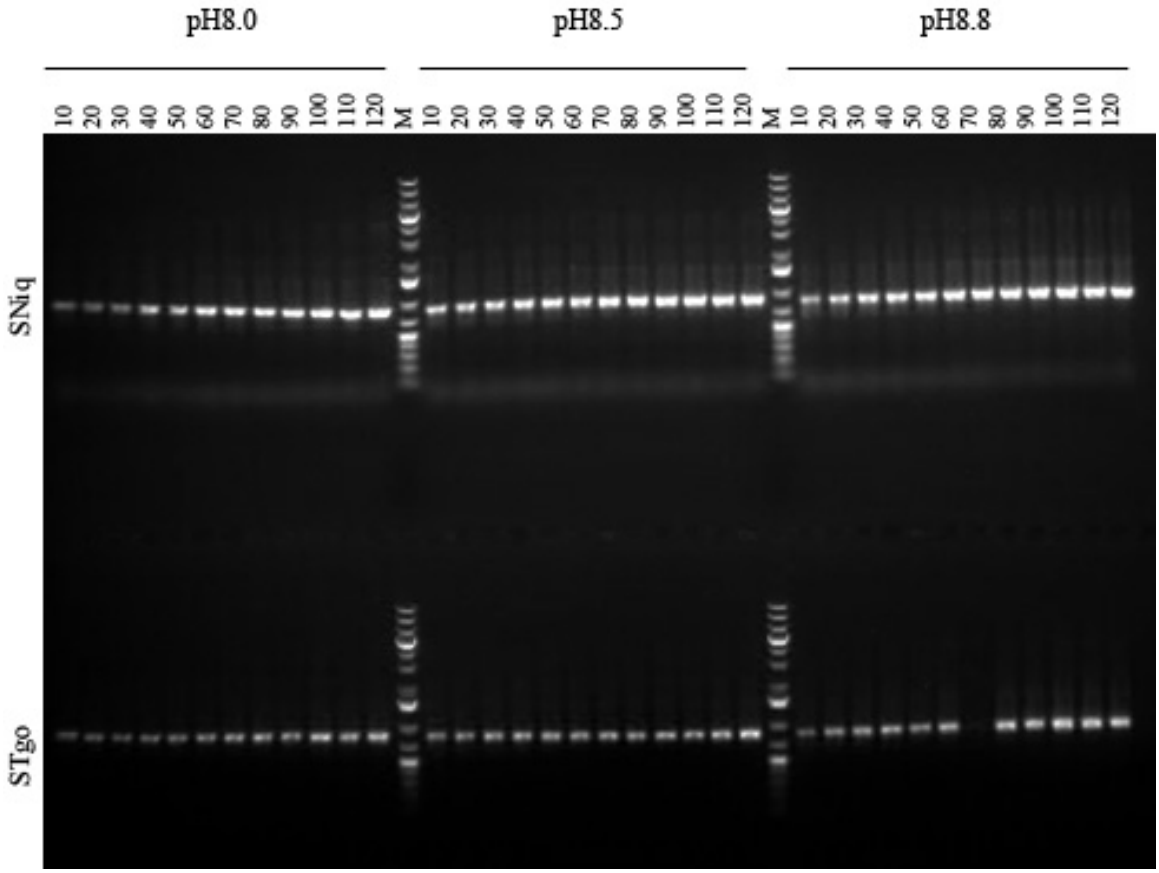

Figure 14. Titration of salt (Tris at its own pH) concentration that STgo and SNiq can tolerate.

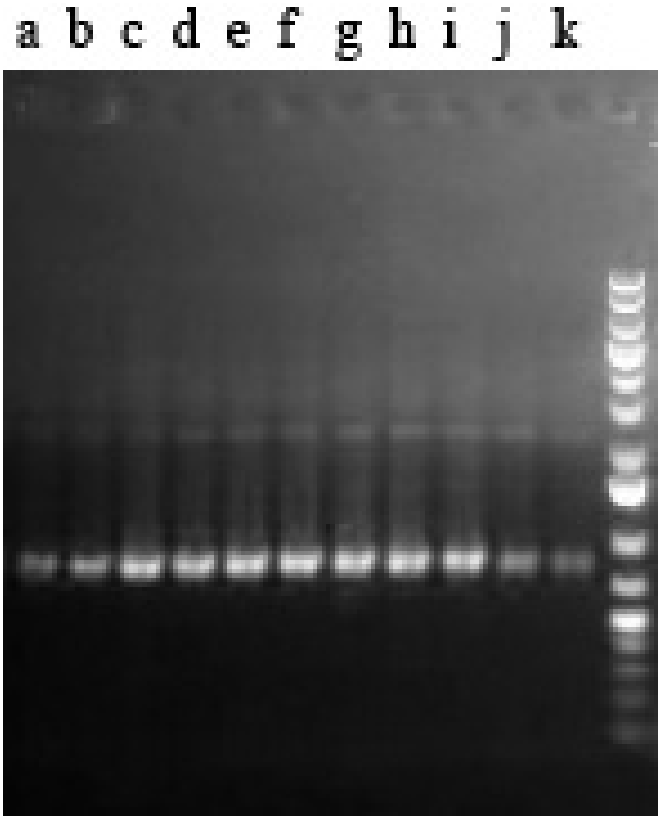

Figure 15. Titration of optimal enzyme concentration of dUTPase purified from *Pyrococcus furiosus*. 0 (a), 0.8 (b), 0.4 (c), 0.2 (d), 0.1 (e), 0.05 (f), 0.025 (g), 0.012 (h), 0.006 (i), 0.003 (j), 0.0015 (k) µg of dUTPase were added into a 50 µl of PCR reaction to see the enhances of PCR yield by the dUTP detoxification.

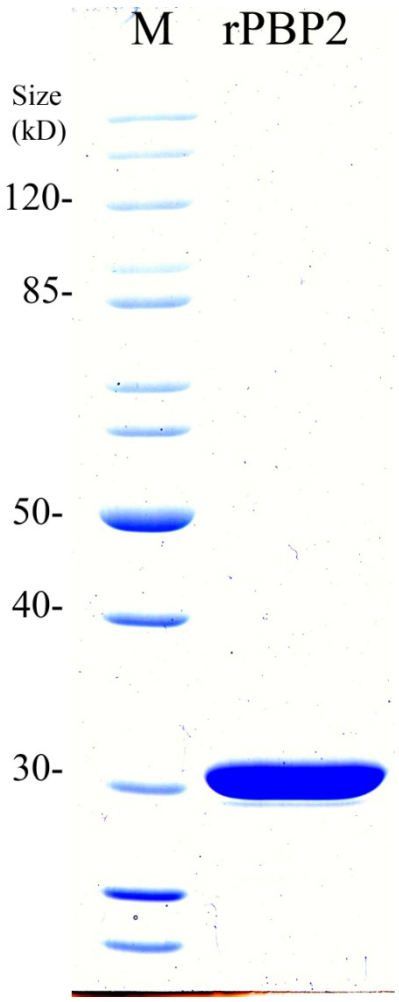

Figure 16. 12% SDS PAGE of recombinant pbp2a purified. M is molecular weight marker (Fermentas Page Ruler) representing the migration of 200, 150, 120, 100, 52, 70, 60, **50**, 40, 30, 25, 20kd proteins in a 12% SDS PAGE.

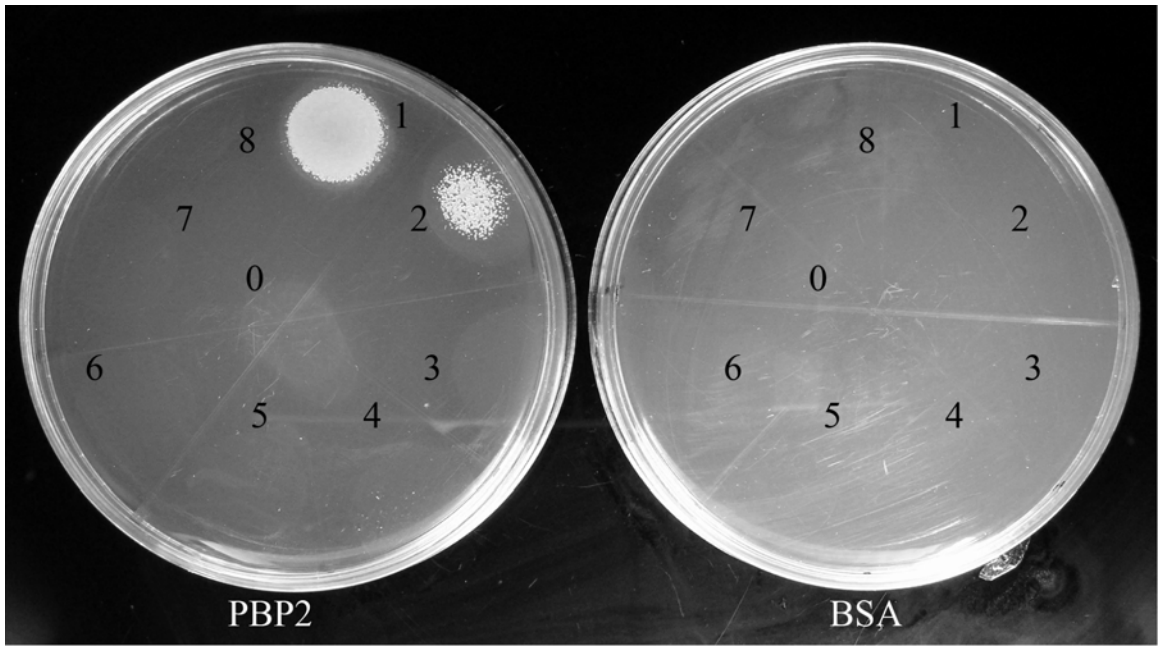

Figure 17. Penicillin binding assay for confirm the translation of rpbp2a. 10^3 penicillin sensitive *E. coli* (BL21 Gold tet^r) was suspended in 100 µl of LB broth with serial dilutions of rPBP2a or BSA. The mixtures were then pipetted on LB agar with 15 µg/ml of tetracycline and 50 µg/ml of penicillin G. The plates were placed in a biological hood until the liquid dried, and incubated at 37°C for 16 hrsto see if rPBP2 complemented *E. coli* to grow in penicillin environments. Spot 1: 560 µg; 2: 280 µg; 3: 140 µg; 4: 70 µg; 5: 35 µg 6: 17.5 µg; 7: 8.75 µg; 8: 4.3 µg. Spots 0 is 560 µg of PBP2 or BSA in LB broth without any bacteria to see if there's any contaminations in both proteins.

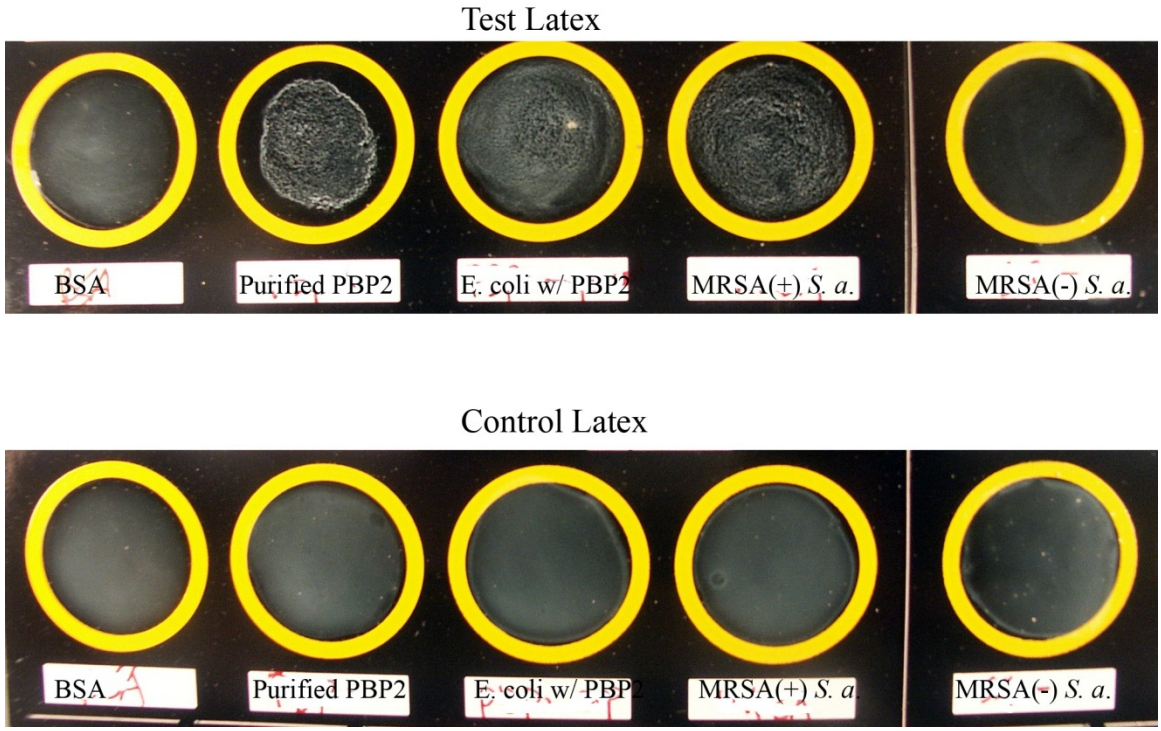

Figure 18. Latex Agglutination assay for confirm the translation of rpbp2a.

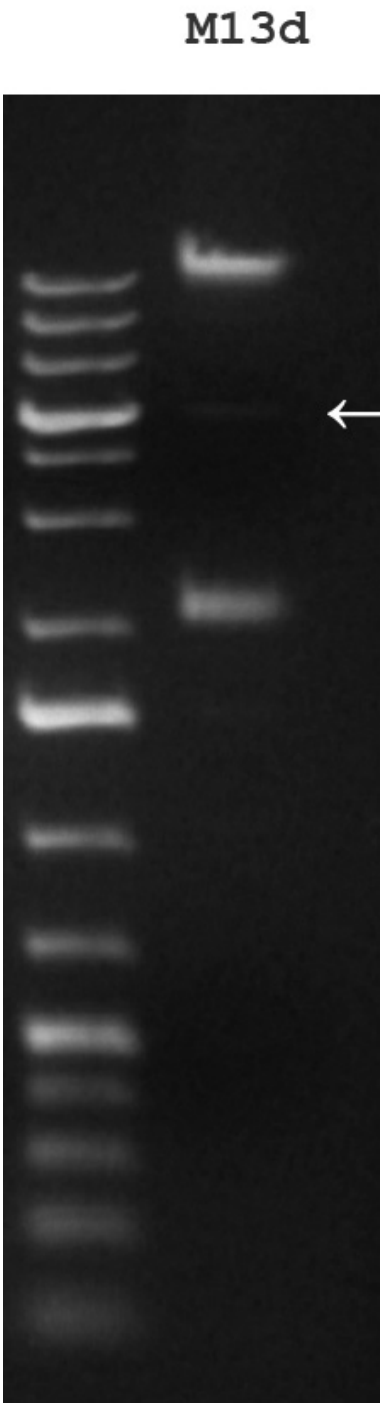

Figure 19. 1% TAE agarose electrophoresis of nucleic acid purified from the PEG8000 precipitation of the M13dp3A helper phage. Noticeable amount of the suspicious pKscFv (5kb, indicated by arrow) was copurified, as well as genomic DNA located slightly higher than the largest DNA ladder (20kb).

Phages	Nonfat	*B. anthracis*	*B. cerus*	*B. subtilis*
BF1	1.035	1.883	1.888	1.497
BF2	0.772	1.888	1.7	1.35
BF3	0.713	1.872	1.511	1.348
CB5	0.841	1.874	1.697	1.363
CB6	0.713	1.786	1.652	1.422
CB8	0.766	1.884	1.573	1.328
CC13	0.753	1.848	1.613	1.427
CF16	0.568	1.746	1.538	1.28
CF17	0.629	1.728	1.548	1.49
FB20	0.872	1.815	1.568	1.428
FF27	0.615	1.619	1.548	1.272
FF29	0.53	1.683	1.478	1.428
M13	1.207	1.773	1.659	1.513

Table 1. ELISA readings of antibody phages reacting with *B. anthracis, B. cerus* and *B. subtilis.*

scFv	BSA	*B. anthracis*	*B. cerus*	*B. subtilis*
HSA	0.204	0.279	0.171	0.141
CB27	0.183	0.245	0.17	0.179
CC4	0.167	0.261	0.185	0.147
FB1	0.208	0.359	0.184	0.169
FB4	0.178	0.245	0.17	0.151
FC3	0.209	0.332	0.2	0.174
FF1	0.179	0.288	0.168	0.137

Table 2. ELISA readings of scFv antibodies, produced by *P. methanolica,* reacting with *B. anthracis, B. cerus* and *B. subtilis.*

Figure 20. SDS PAGE of affinity purified recombinant INLA (1-9). The 2 red bands in MW standards (M) are 70 and 27kd. Those 2 blue bands in between is 55 and 35kd.

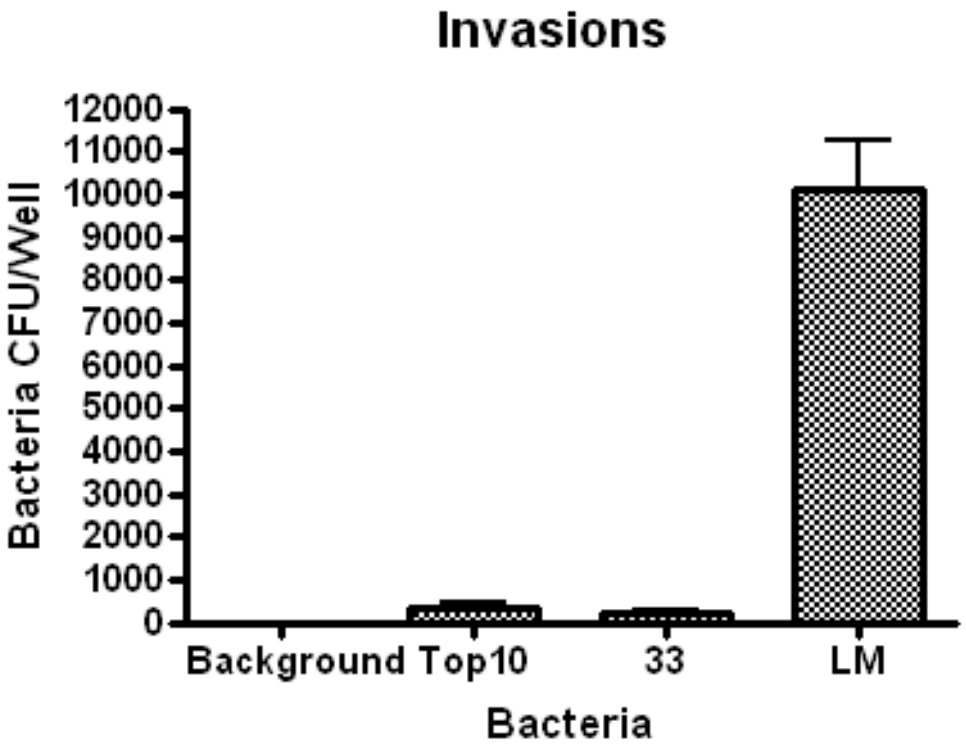

Figure 21. The comparison of bacterial invasion of *L. monocytogenes*, and *E. coli*. Surface expression of INLA on *E. coli* (33) did not enhance infection to COCA2 cells as compared to untransformed *E. coli* (TOP10).

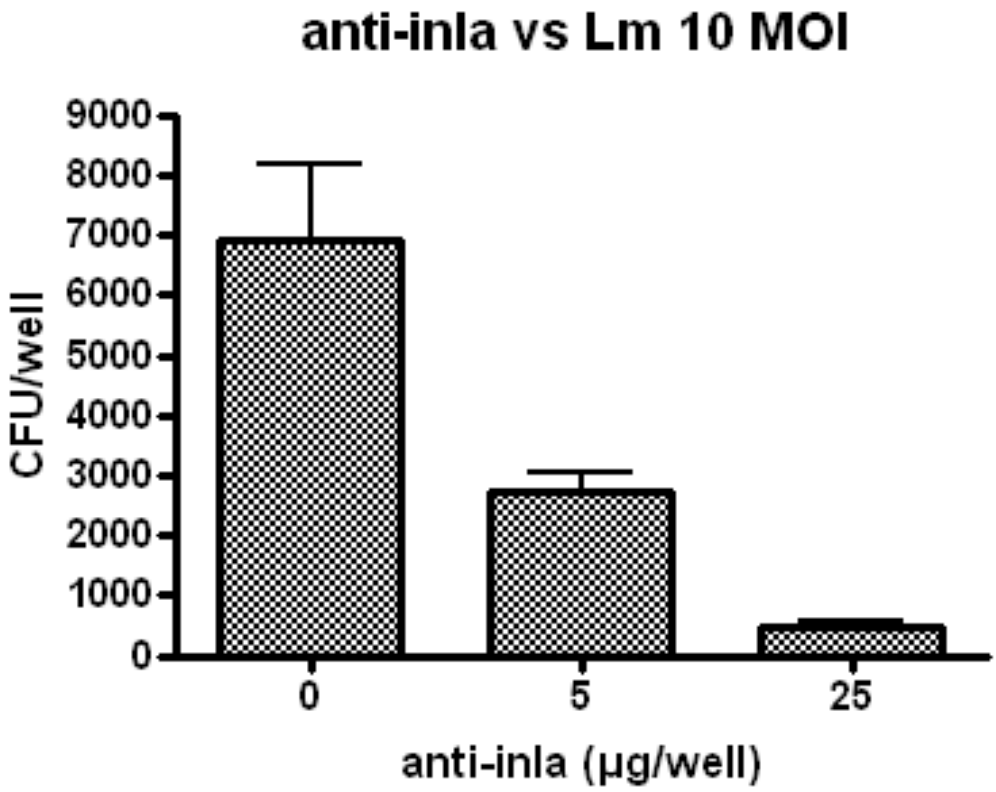

Figure 22. *In vitro* study of the anti-INLA antibody in protecting the COCA2 cells from invading by *L. monocytogenes* at the MOI of 10. Dosage effect showed anti-INLA IgG blocked the invasion of *L. monocytogenes* to COCA2 cell at a MOI of 10. The standard deviations represent the variance from triplicate invasion assays.

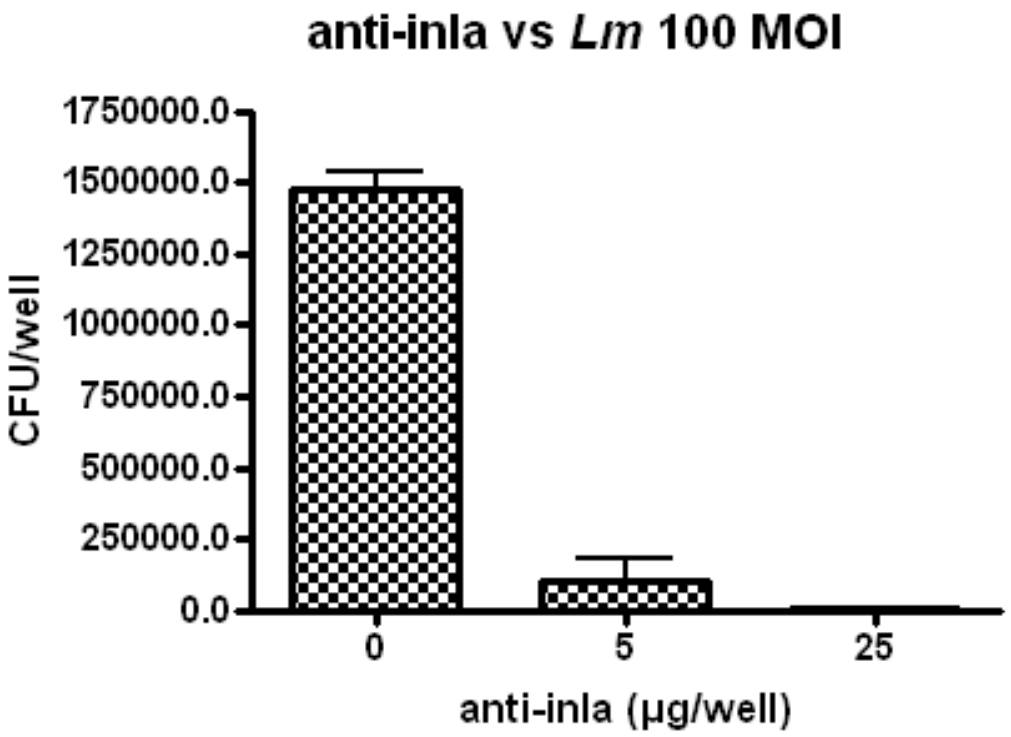

Figure 23. *In vitro* study of the anti-INLA antibody in protecting the COCA2 cells from invading by *L. monocytogenes* at the MOI of 100. Dosage effect showed anti-INLA IgG blocked the invasion of *L. monocytogenes* to COCA2 cell at a MOI of 100. The standard deviations represent the variance from triplicate invasion assays.

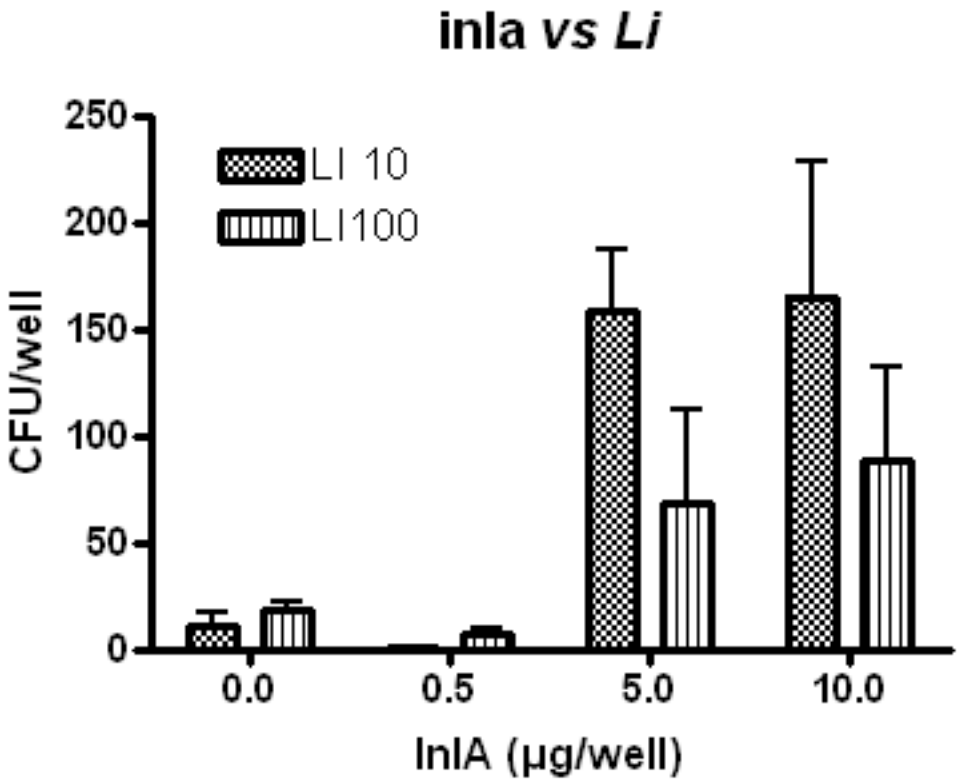

Figure 24. The invasions of noninvasive *L. innocua* facilitated by different quantity of INLA. The standard deviations represent the variance from triplicate invasion assays.

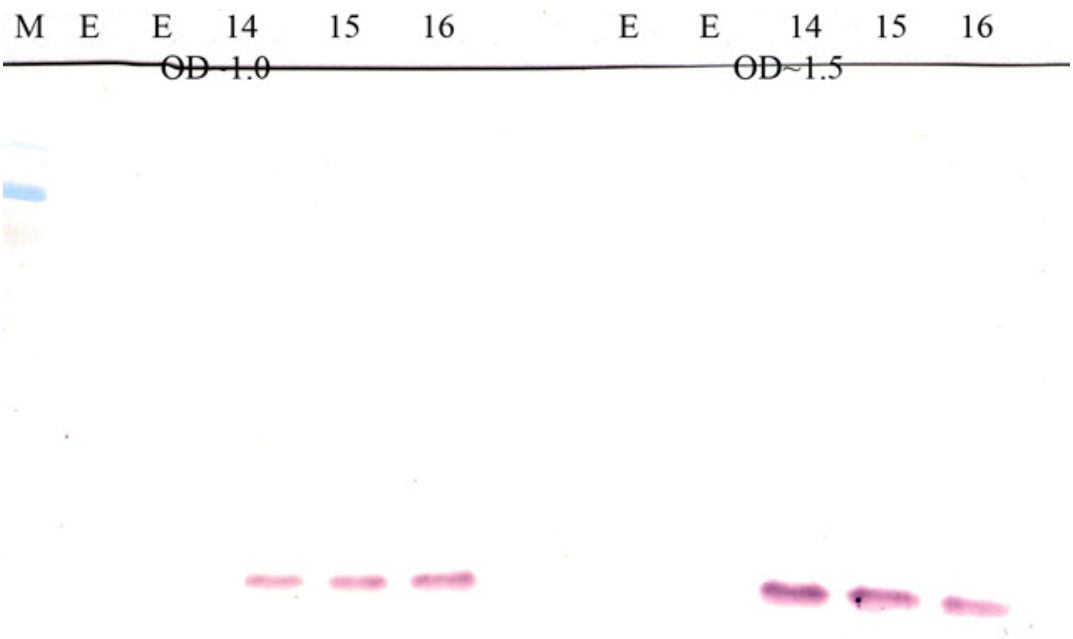

Figure 25. λCE6 screening of OSM inducible plasmids. λCE6 was used to bring the T7 RNA polymerase into non-DE3 strain of *E. coli* (TOP10) to induce the osmotin in pETOSM14, 15 and 16 (Lane 14, 15, and 16) and empty pET20b (E) as control at the bacterial density of OD_{600nm} 1.5 and 1.0. Recombinant protein was not able to detected by SDS PAGE staining with coomassie blue R-250, and hence, western blotting was used.

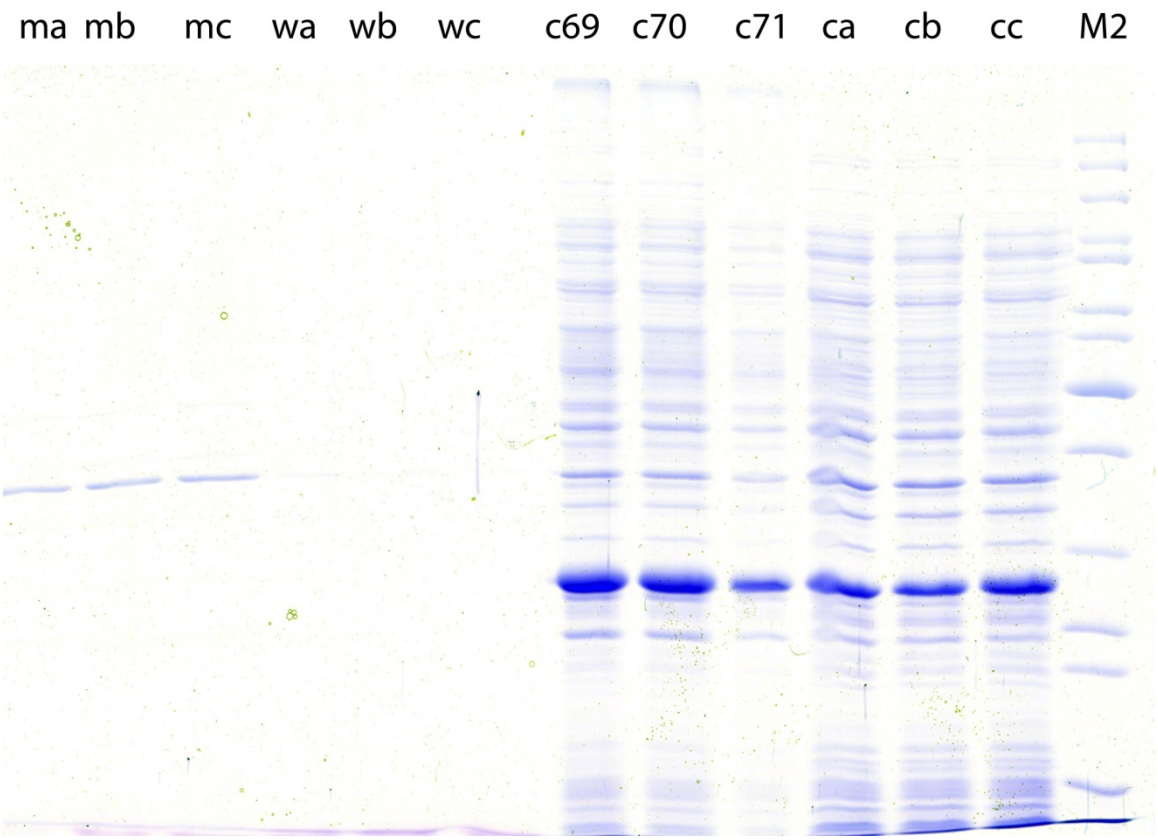

Figure 26. OSM induction pH optimization and recombinant protein content of fractions of large scale expression. C69, C70, C71, small scale expressions of OSM in ZYM5052 with pH of 6.9, 7.0 and 7.1. OSM was large scale expressed (ca, cb, cc) at pH 7.0, and no recombinant proteins existed in media (ma, mb, mc), or buffers (wa, wb, wc) after the washes of bacteria. M2 is molecular weight marker (Fermentas Page Ruler) representing the migration of 200, 150, 120, 100, 52, 70, 60, **50**, 40, 30, 25, 20kd proteins in a 12% SDS PAGE.

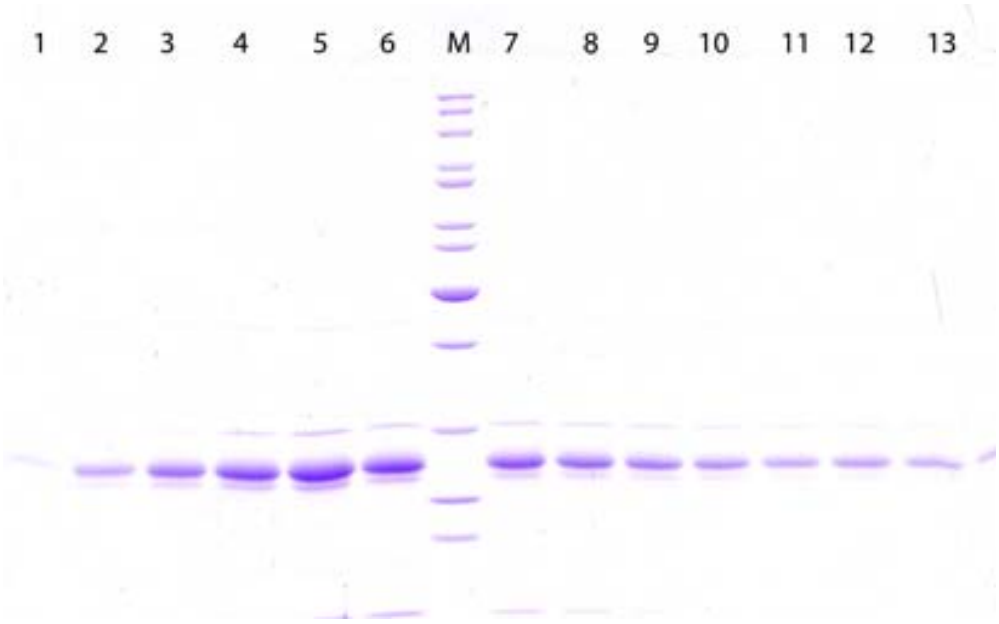

Figure 27. Affinity purification of osmotin with cobalt-immobilized matrix. Lanes 1-13 is the fractions collected from elutes. M is molecular weight marker (Fermentas Page Ruler) representing the migration of 200, 150, 120, 100, 52, 70, 60, **50**, 40, 30, 25, 20kd proteins in a 12% SDS PAGE.

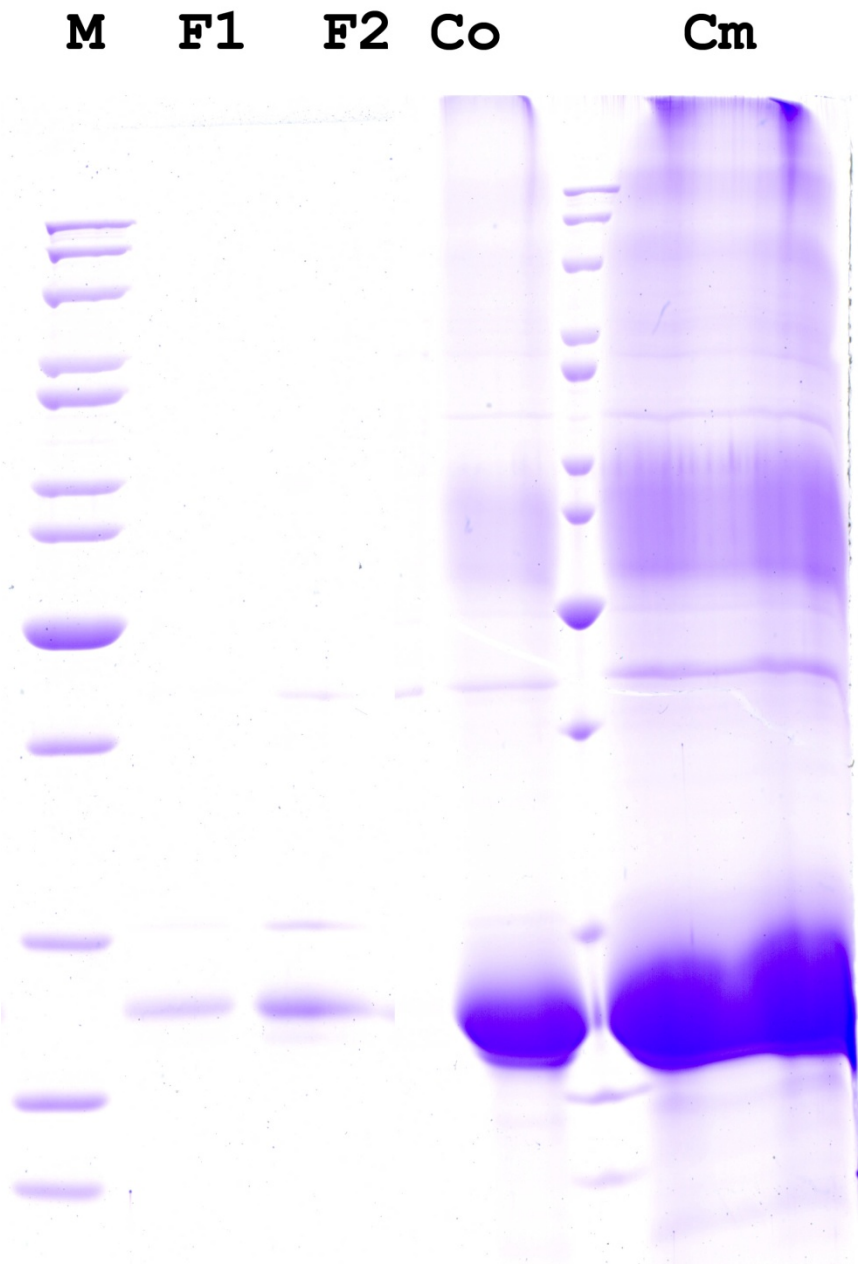

Figure 28. SDS elution of OSM on Co-agarose and CM-cellulose. Significant amount of OSM on Co-agarose (Co) and CM-cellulose (Cm) were not able to be eluted by mild elution buffers by standard procedures of protein purification. These recombinant proteins were eluted with SDS sample buffer, and load in a 12% SDS PAGE and compared with same volume of fractions (F1, F2) eluted with standard protocols. M is molecular weight marker (Fermentas Page Ruler) representing the migration of 200, 150, 120, 100, 52, 70, 60, **50**, 40, 30, 25, 20kd proteins in a 12% SDS PAGE.

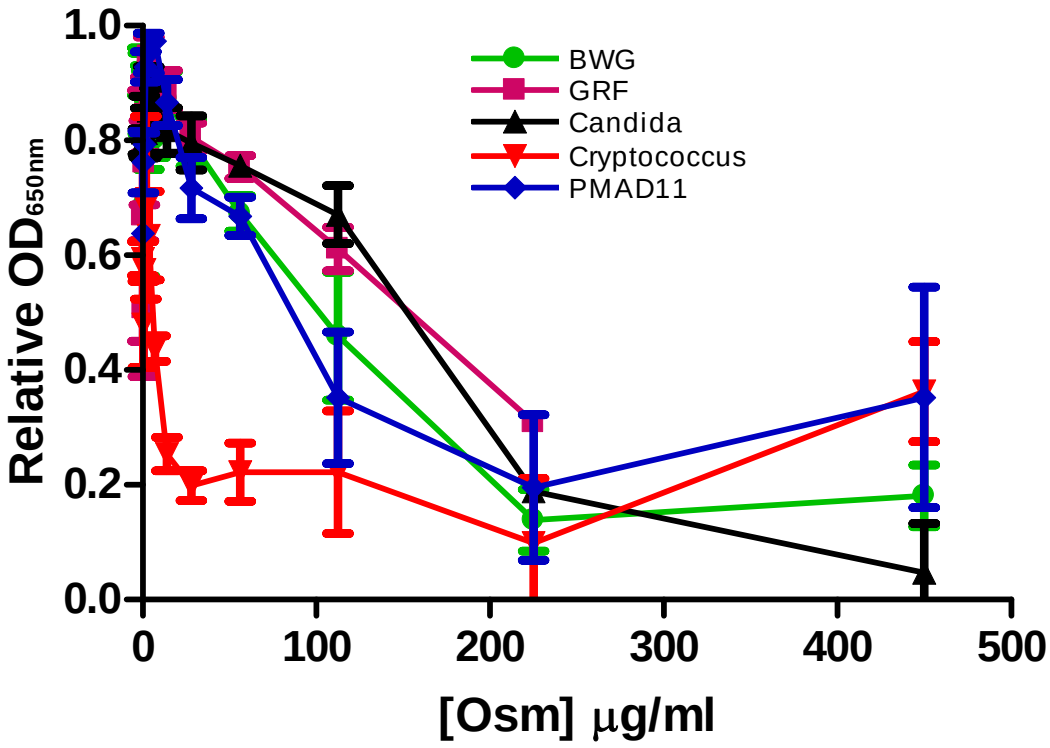

Figure 29. Relative growth of yeast strains in YPAD media with different OSM concentration.

	OSM Concentration (µg/ml)											
	450	225	112.5	56.25	28.12	14.06	7.031	3.51	1.76	0.88	0.44	0
BWG	a b	a	a b c	a b c	b c	c	c	c	c	b c	a b c	c
Grf	a	b	c d	c d e f	e f	f	f	f	d e f	c d e	c	c d
Can	a	a	b	b c	c	c	c	c	c	c	c	c
Cry	a	a b	b c	b c	b c	b c	c d	d	d	d	c d	d
11	a b	a	b c	c d	d e	d e	e	e	d e	d e	d e	d e
16	a	b	b	c	c	c	c	c	c	c	c	c
HSA	a	a	b	b c	c	c	c	c	c	c	b c	b c
	450	225	112.5	56.25	28.12	14.06	7.031	3.51	1.76	0.88	0.44	0
	OSM Concentration (µg/ml)											

Table 3. Turkey Post Hoc Tests of relative growth of yeast strains in YPAD medium with different OSM concentrations.

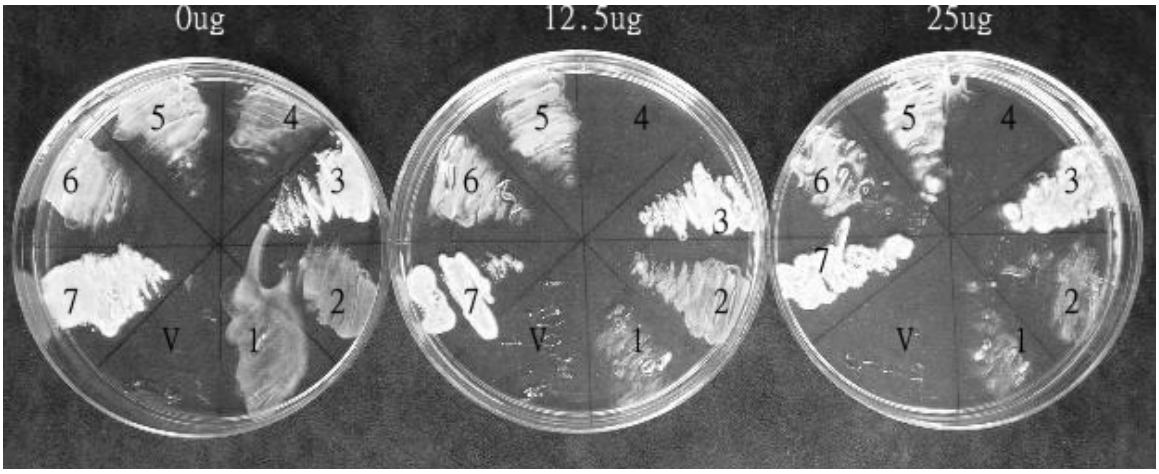

Figure 30. The inhibition of growth in yeast strains on 0.8% low-melting agarose YPAD plates with or without OSM. Spot 1: BWG7a; 2: Grf147; 3: *Candida albicans*; 4: *Cryptococcus neoformans*; 5: PMAD11; 6: PMAD16; 7: PMAD16HSA; V: *Vibrio cholera* (bacteria).

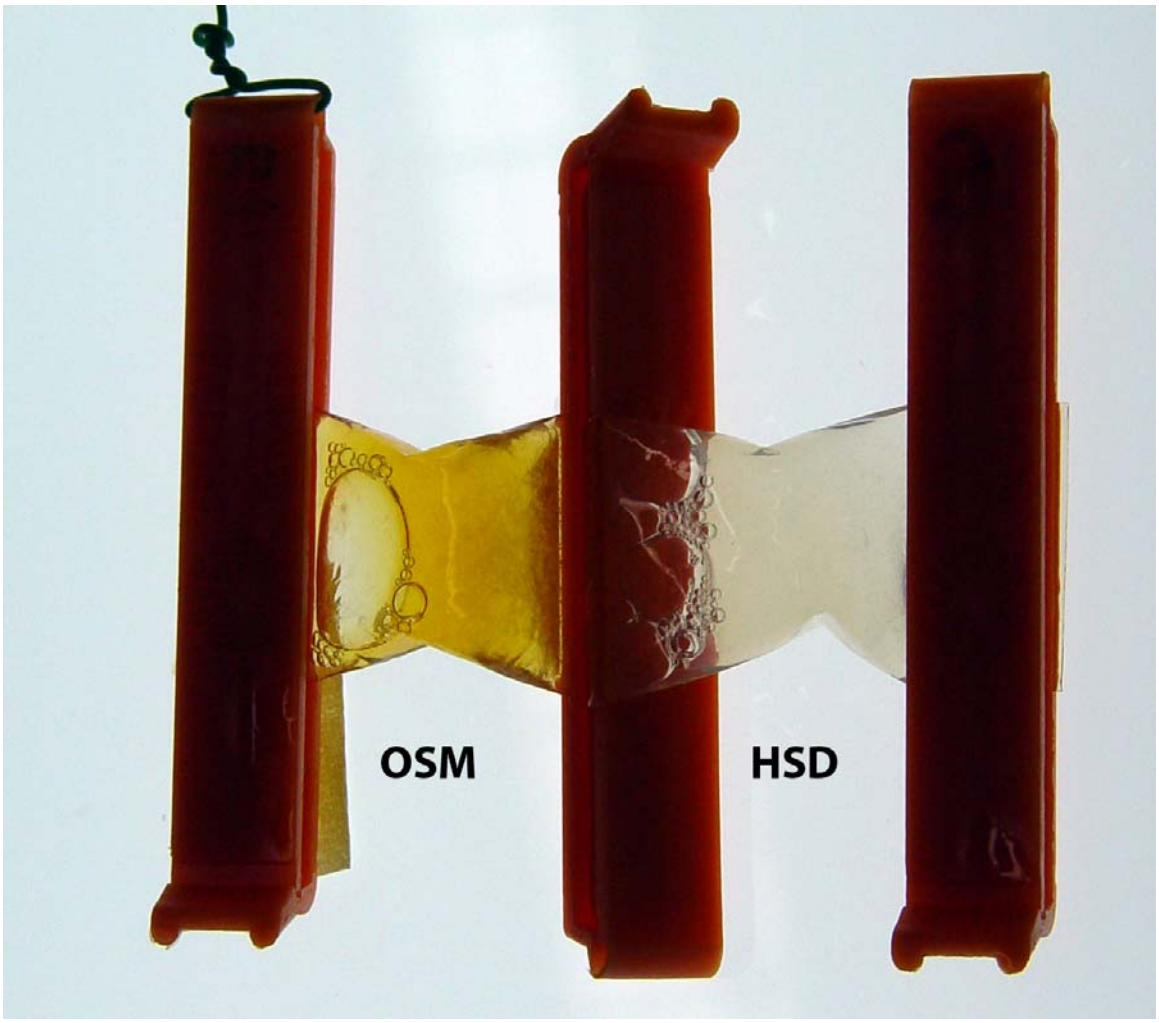

Figure 31. Iron (III) binding of OSM and heatshock protein 60 (HSD).

Binding condition		**1 mM Fe^{3+} /Na^{+}**		**10 mM Fe^{3+} /Na^{+}**	**1 mM Fe^{3+} /NH^{4+}**
Proteins	HSD1	OSM Supernatant	OSM precipitation	OSM supernatant	OSM supernatant
Fe^{3+} bound/protein	0.0087	0.191	0.257	0.104	0.003

Table 4. Fe^{3+} binding of OSM in various binding conditions.

Access#	From->To	Name
PS00001	225->229	ASN_GLYCOSYLATION
PS00005	157->160	PKC_PHOSPHO_SITE
PS00006	83->87 93->97 101->105 227->231	CK2_PHOSPHO_SITE
PS00007	173->181	TYR_PHOSPHO_SITE
PS00008	41->47 61->67 70->76 151->157 193->199	MYRISTYL
PS00009	24->28	AMIDATION

Table 5. Prosite DB for Patterns Search for OSM

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APPENDIX A.

Genotypes of *E. coli* strains used in this dissertation

Strains	Genotype
BL21(DE3)	F– *ompT hsdS*B(rB– mB–) *gal dcm* (DE3)
BL21-Gold (DE3)	F– *ompT hsdS*(rB – mB–) *dcm*+ Tetr *gal* λ(DE3) *endA* Hte
BL21(DE3)pLysS	F– ompT hsdSB(rB–, mB–) gal dcm (DE3) pLysS (CamR)
BL21(DE3)pLysE	F– ompT hsdSB(rB–, mB–) gal dcm (DE3) pLysE (CamR)
BLR(DE3)	F– *ompT hsdS*B(rB– mB–) *gal dcm* (DE3) Δ(*srl-recA*)*306*::Tn*10* (TetR)
C2925	ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10) TetS endA1 rspL136 (StrR) dam13::Tn9 (CamR) xylA-5 mtl-1 thi-1 mcrB1 hsdR2
DB3.1™	F– gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20(rB–, mB–) supE44 ara-14 galK2 lacY1 proA2 rpsL20(SmR) xyl-5 λ– leu mtl1
E. cloni *10G*	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 Φ80*dlac*ZΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L *nup*G λ- *ton*A
ED8739	F– *hsdS* (rK– mK–) *metB supE supF*
LE392	F– *hsdR514* (rK – mK+) *mcrA supE44 supF58 lacY1* or Δ(*lacIZY*) *galK2 galT22 metB1 trpR55*
Mach1™ T1R	F– Φ80lacZΔM15 ΔlacX74 hsdR(rK–, mK+) ΔrecA1398 endA1 tonA
MegaX DH10B™ T1R	F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu)7697 galU galK λ– rpsL nupG tonA
NEB Turbo	F' *proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ*lacZM15/fhuA2* Δ*(lac-proAB) glnV gal R(zgb-210::Tn10)*Tet^{S} *endA1 thi-1* Δ*(hsdS-mcrB)5*
OverExpress C41(DE3)	F – *ompT hsdSB (rB- mB-) gal dcm* (DE3)
OverExpress C41(DE3)pLysS	F – *ompT hsdSB (rB- mB-) gal dcm* (DE3) pLysS (CmR)
OverExpress C43(DE3)	F – *ompT hsdSB (rB- mB-) gal dcm* (DE3)
OverExpress C43(DE3)pLysS	F – *ompT hsdSB (rB- mB-) gal dcm* (DE3) pLysS (CmR)
OmniMAX™ 2 T1R	F´ (proAB+ lacIq lacZΔM15 Tn10(TetR) Δ(ccdAB)) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ(lacZYA-argF) U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD
Rosetta 2(DE3)	F– *ompT hsdS*B(rB– mB–) *gal dcm* (DE3) pRARE23 *(*CamR)
Rosetta 2(DE3)pLysS	F– *ompT hsdS*B(rB– mB–) *gal dcm* (DE3) pLysSpRARE23 *(*CamR)
Stbl3™	F– mcrB mrr hsdS20 (rB–, mB–) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20 (StrR) xyl-5 λ– leu mtl-1
Stbl4™	mcrA Δ(mcrBC-hsdRMS-mrr) recA1 endA1 gyrA96 gal- thi-1 supE44 λ– relA1 Δ(lac-proAB)/F´ proAB+ lacIqZΔM15 Tn10 (TetR)
TOP10	F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG
TOP10F'	F´(lacIq, Tn10(TetR)) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG

Key to Genotypes	
Genotype	Description
ara-14	Blocks arabinose catabolism
argF	Ornithine carbamoyltransferase mutation blocks ability to use arginine
dam/dcm	Abolishes endogenous adenine methylation at GATC sequences (dam) or cytosine methylation at CCWGG sequences (dcm). Used to propagate DNA for cleavage with certain restriction enzymes (e.g. Ava II, Bcl I)
DE3	Lysogen that encodes T7 RNA polymerase. Used to induce expression in T7-driven expression systems
endA	endA Mutation in the non-specific endonuclease Endonuclease I; eliminates non-specific endonuclease activity, resulting in improved plasmid preps
F´	A self-transmissible, low-copy plasmid used for the generation of single-stranded DNA when infected with M13 phage; may contain a resistance marker to allow maintenance and will often carry the lacI and lacZΔM15 genotypes
glnV	See ***supE.***
galK	Galactokinase mutation blocks catabolism of galactose—cells that are galK minus grow in the presence of galactose as the sole carbon source
galU	Glucose-1-phosphate uridylyltransferase mutation blocks ability to use galactose—cells that are galU minus can grow on media that contains galactose as the sole carbon source
gyrA96	DNA gyrase mutant produces resistance to nalidixic acid
hflA	Results in high frequency lysogenization by λ.
hsd	Mutations in the system of methylation and restriction that allow E. coli to recognize DNA as foreign. The hsd genotype allows efficient transformation of DNA generated from PCR reactions *hsdR–eliminates restriction of unmethylated EcoK I sites. (1) **hs
lacI	Encodes the lac repressor that controls expression from promoters that carry the lac operator; IPTG binds the lac repressor and derepresses the promoter; often used when performing blue/white screening or to control expression of recombinant genes
lacY1	Blocks use of lactose via β-D-galactosidase mutant
lacZ	β-D-galactosidase gene; mutations yield colorless (vs. blue) colonies in the presence of X-gal
lacZΔM15	Element required for β-galactosidase complementation when plated on X-gal; used in blue/white screening of recombinants; usually carried on the lambdoid prophage ϕ80 or F´
leuB	Requires leucine for growth on minimal media via β-isopropyl malate dehydrogenase mutation
lon	lon Deficiency in the Lon ATPase-dependent protease; decreases the degradation of recombinant proteins; all B strains carry this mutation
mcrA, mcrBC,or mrr	Mutations that allow methylated DNA to not be recognized as foreign; this genotype is necessary when cloning genomic DNA or methylated cDNA

Genotype	Description
nupG	Mutation for the transport of nucleosides
ompT	Indicates that the E. coli lack an outer membrane protease—reduces degradation of heterologous the strains and recovery of intact recombinant proteins is improved in ompT minus strains
P3	A 60-kb low-copy plasmid that carries the ampicillin and tetracycline resistance genes with amber mutations; used predominantly for selection of supF-containing plasmids; carries the kanamycin resistance gene for selection
pLys	pLys Plasmid that encodes T7 lysozyme; used to reduce basal expression in T7-driven expression systems by inhibiting basal levels of T7 RNA polymerase
proAB	proAB Requires proline for growth on minimal media
recA	Mutation in a gene responsible for general recombination of DNA; particularly desirable when cloning genes with direct repeats
relA	RNA is synthesized in absence of protein synthesis (relaxed phenotype) relA locus regulates the coupling between transcription and translation. In the wild type, limiting amino acid concentrations results in the shutdown of RNA synthesis (also known as th
rpsL	Confers resistance to streptomycin (this makes a mutant ribosomal protein, small subunit, the target of the drug)
supE,F	tRNA glutamine suppressor of amber (supE)(UAG) or tyrosine (supF)
thi-1	Requires thiamine for growth on minimal media
Tn10	Confers tetracycline resistance via a transposon
tonA	Confers resistance to the lytic bacteriophage T1, T5 and f80
traD, D36	Prevents transfer of F' episome via transfer factor mutation
tsx	Confers resistance to phage T6 and colicin K
xyl-5	Blocks catabolism of xylose

APPENDIX B.

Genotypes of yeast strains used in this dissertation

Species	Strains	Genotype
Candida albicans	ATCC11651	
Cryptococcus neoformans	ATCC13690	
Saccharomyces cerevisiae	BWG7a	*MATα ade1-100 leu2-3,112 ura3-52 his4-519*
Saccharomyces cerevisiae	GRF167	*MATα his3Δ200 ura3-167 (osmR)*
Pichia methanolica	PMAD11	*Ade2-11*
Pichia methanolica	PMAD16	*Ade2-11 pep4Δ prb1Δ*
Pichia methanolica	PMAD16/pMETαB/HSA	*Ade2-11 pep4Δ prb1Δ ADE2 HSA*

APPENDIX C.

List of Oligo-nucleotides used in this dissertation

Oligo	Sequence
1C5_60.3	CCATGGGATGTACAGCTTCAGGAGTCAGGACC
20b3_bgl2	TTGTTAGCAGCCAGATCTCAGTGGTGG
20b5_MFE	AACAATTGGCTTCCACATATGAAATACCTGCTGC
adipo_3_eco	CTCGAATTCGGGTTGGTGTCATGGTAGAGAAG
adipo_5_nco	TTTCCATGGAAACCACGACTCAAGGGC
adipo_5_nde	TTTCATATGAAACCACGACTCAAGGGCCC
ADP	CCGACTCGAGTCGACATCG
ADP_low	GACTCGAGTCGACATCG
adp2 (58.3)	CAATTGGATCCATGGCCATATGAGC
adp2 22	GGATCCATGGCCATATGAGCTCTTTTTTTTTTTTTTTTTTT
adp3bamh	GGAAGGATCCGTTGGTGTCATGGTAGAG
Adp5 K ECO	ATGGAATTCAAAATGTCTGAAACCACGACTCAAGGG
Adp5ECO	ATGGAATTCATGGAAACCACGACTCAAGGGCCC
ADPT17	CCGACTCGAGTCGACATCGTTTTTTTTTTTTTTTT
adt-tc	GACTCGAGTCGACATCGATTTTTTTTTTTCCCCC
amp_3	TTACCAATGCTTAATCAGTGAGGCACCTATCTC
amp_5	GTACAATCTGCTCTGATGCCGCATAGTTAAG
amp3BamH	GGTCGGATCCTTACCAATGCTTAATCAGTG
amp3nhe	GGTCGCTAGCTTACCAATGCTTAATCAGTG
amp5nhe	AAAAGCTAGCAGGAAGAGTATGAGTATTC
amp5sal	AAAAGTCGACAGGAAGAGTATGAGTATTC
At2_3_Eco	TAGAATTCCCTGGGTTAACCTTTTCTGAAG
AT2_5_bam	ATGGATCCCATGGGAACACATGTAGCACCATGG
B1	AGACATCTCCAAGATCAAGAAAGTATGGCG
B2	ACGACGAGGGCGGTGGCAAGACCGGCCGCG
B3	CGAAGGAGCTGCTGCAGATGCTGGAGAAGCA
cad3(18mer)	CTAACTGCATCACTAACC
cad3Xho	CTCGAGGTCGTCCTCGCCGCCTCCGTACAT
cad5nde	CATATGGGCCCTTGGAGCCGCAGCCTCTCG
CadRT(2910)	CTAACTGCATCACTAACCAGTCTC
CadRT(-70)	TTGCGGAAGTCAGTTCAGAC
colFP3BamH	GGATCCTCTAGAGTCGCGGCCGCT
DNTT3 Xho (61.6/76)	CTCGAGAGCATTTCTTTCCCATGGTTCAATGTAG
DNTT3 Xho (61.6/76)	CTCGAGAGCATTTCTTTCCCATGGTTCAATGTAG
DNTT5 n (63/76)	CATATGGATCCGCTGCAAGCAGTCCAC
DNTT5 n (63/76)	CATATGGATCCGCTGCAAGCAGTCCAC
dsRed5Sal	GTCGACATGGCCTCCTCCGAGAACGTC
ecad_3_1950	GGTCTGCATCAATGATGTTTATGACCTGAG
ecad_5_870	GATCACGGTAACCGATCAGAATGACAACAAG

Oligo	Sequence
EcDp3Sal	GTCGACGTGCGCCTGATCCCAGTTTTC
EcDp5Nde	CATATGGTTCAGATCCCCCAAAATCCAC
EcLig3Xho	CTCGAGGCTACCCAGCAAACGC
EcLig5Nde	CATATGGAATCAATCGAACAACAACTGACAG
EcRH3Xho	CTCGAGTTCAACTTGGTAGCCTGTATCTTC
EcRH5Nde	CATATGCTTAAACAGGTAGAAATTTTCACCG
egfp_er5_3	GATATCTGTTTCCTGTGTGAAATTGTTATCCGCTC
egfpN5	ATGACCATGATTACGCCAAGCTTGCATG
egfpN5_-ATG	ACCATGATTACGCCAAGCTTGCATGC
egfpN5-pro	CCGACCTTGATTACGCCAAGCTTGC
flag5_ecor1	GAATTCCATGAAAGTAAATACTAATATCATTAGC
GC_Bcl	CCTTATCGCATTGATCAGACTCCAGCGTAAC
GC_Xho	CTCGAGCTTCCTGTGTGAATTGTTATCCG
GFP_Sal	GTCGACATGGTGAGCAAGGGCGAGG
gfp1048-1030	GCGACCGGCGCTCAGTTGG
gfp1048-1030	GCGACCGGCGCTCAGTTGG
gfp1057-1080	GCAATTGCCAACTTGTCTGGTGTC
gfp1057-1080	GCAATTGCCAACTTGTCTGGTGTC
GFP3	TAATGGTAGCGACCGGCGC
gfp5-nde	CATATGACCATGATTACGCCAAGCTTGC
gfp5reverse	CTTGCTCACCATGGTGGCGAC
gfp5xho	ACCTCGAGGGATCCCCGGGTACC
gfpxho	ACCTCGAGGGATCCCCGGGTACC
GIII-96	CCCTCATAGTTAGCGTAACG
h_b5Sal66	AGGTCGACCAACTGCAGCAGTCTGGAACTG
H_bf_3nco65.7	AACCATGGAGGAGACGGTGACTGAGGTTCC
h_c_5sal65	TTGTCGACCAGCTTCAGGAGTCAGGACCTG
h_c3nco	AACCATGGAGGAGACTGTGAGAGTGGTGCCTTG
h_f_sal66	AAGTCGACGCACAGATCCAGTTGGTGCAGTC
HC_C	TCACAGTGAACACCTTCCCACC
HC1026	CAAGTGCACAGTTACCCATCCTGAGTC
HC3.22	AGGAGTAGGGACAGGCAGGGTGGCTCAG
Hc3.4	CTCAGTAGCAGATGCCATCTCCCTC
HC3_19mer	AGGAGTAGGGACAGGCAGG
HC3-1	ACTTTGGATGAGCACAGAG
HC3-1	ACTTTGGATGAGCACAGAG
HC3-3	GTGGCTCAGTAGCAGATGCC
HC3-3	GTGGCTCAGTAGCAGATGCC
hc5	TCTCCACAGTCACTGAACACACTGACTCTAACCATGG
HC5-1	CTGAACACACTGACTCTAACCATGG
HC5-1	CTGAACACACTGACTCTAACCATGG
HC5-2	CYTCTCMACAGWCHCTGAAVAC
HC5-2	CYTCTCMACAGWCHCTGAAVAC
HC52-1	YTCGAYTCHCARKTCYTYMBVTTC
HC52-1	YTCGAYTCHCARKTCYTYMBVTTC
HC52-2	TCYTYMBVTTCAGTGAYBA
HC52-2	TCYTYMBVTTCAGTGAYBA
HCRT3.11	GATGAGCACAGAGTTTATTTCAG
hspds1145-1122	GTTGTGACATCTAACTGCTCAATG
hspds545-566	TTGGCAATATCATCTCTGATGC

Oligo	Sequence
Igγ1 (49/57?)	CTCGAGTATGCAAGGCTTACAACC
Igγ2A (62/66?)	CTCGAGACAGGGCTTGATTGTGGGCCC
Igγ2B (62/66?)	CTCGAGACAGGGGTTCAGTGTTGAAATGG
inl_full_3-XHO-his	CTCGAGTGGTTTACTAGCACGTGC
inl_full_5-nde	CATATGAAGACGGTCTTAGGAAAAACG
inl_full_Surface_3-BAM	GGATCCTATTTACTAGCACGTGC
Inla_3_Ter_BamHI	GGATCCTAAAGTGGCTGCGTCACG
Inla_5'_ecorI,+Proline	GAATTCTGCCGGCAGCTACAATTAC
inla_full_surface_MFE	CAATTGAAGACGGTCTTAGGAAAAACG
inla_surface_3_xho	CTCGAGTGGCTGCGTCACGGTTC
InlA-3	TTCTCGAGCTTAAGTGGCTGCGTCAC
inlA-5	CATATGCAGGCAGCTACAATTACACAAG
Int-1	AATCTAGCACCACTGTCGG
Int-2	GCCAATGGTGTAAGATCGC
intl_485-510_forward	GGTGTCGGATATTAGTGTTCTGGC
intl_510-485_rev	GCCAGAACACTAATATCCGACACC
intl_70-92_forward	CGCTTCAGGCGGATAGATTAGG
intl_800-780_rev	GCTCTAAGTTAGTGAGTGCGGTT
INTL_det_3	GCCAATGGTGTAAGATCGC
INTL_det_5	AATCTAGCACCACTGTCGG
Intl_seq_forward	AGATATAGGCACATTGGCGAG
INTL_SEQ_reverse	ACTCGCCAATGTGCCTATATC
IntlA_sq1	AGCTCTAAGTTAGTGAGTGCGG
IntlA_sq2	GCACCACTGTCGGGTCTAAC
intla-5_ECOR1	GAATTCTGCAGGCAGCTACAATTAC
inv3	GGTATCTGCTGAAGTTGAGGATG
inv5	ACATCGACAGACGACGTAAGGAGG
inv5	ACATCGACAGACGTAAGGAGG
invA3	GGTATCTGCTGAAGTTGAGGATG
Kappa_299-317	GGCAGTGGATCAGGGACAG
kappa_3	ACACTCATTCCTGTTGAAGCTCTTGAC
kappa_5	TCTCCTCAGGTTGCCTCCTC
l_c_all_3Hind66	GTAAGCTTTATTTCCAGCTTGGTCCCCCCTCCG
LacI_19b3_mfe_er1	CAATTGCATCGGTCGAATTCCCGGTGCCTAATG
LacI_19b5	GGTGCTCAACGGCCTCAACCTAC
LB5 59	CCTCCATTGTGATGACCCAGTCTC
lb5sal	GGAGTCGACATTGTGATGACCCAGTCTCAAAAATTC
LC_3	TCCTCAAAATGATGAGTCCTGCCCAG
LC_C_5xho66	AGGTCGACATCTTGCTGACTCAGTCTCCAGCC
LC_RT_22mer	TAGGATGGAGCTGGGGAGCTGG
LC3.11	GAAGGAGGTGGGGAGGAGGTTTGGAGCACCG
LC31	AAGTGCAAAGACTCACTTTATTG
LC31	AAGTGCAAAGACTCACTTTATTG
LC3-2	AGCCAAGGAAAGGGAGGAGGAGGAG
LC3-2	AGCCAAGGAAAGGGAGGAGGAGGAG
LC432	ATCTTCCCACCATCCAGTGAGCAG
lc6g	TATTTCCAGCTTGGTCCCCCCTCCGAACGTG
lcb5xho66	GCTCGAGGCCTCCATTGTGATGACCCAGTC
lclfj5xho65	TGCTCGAGACATTGTGTTTGACCCAATCTCCAGC
LLO_242_forward	CGGAGATGCAGTGACAAATGTGCC

Oligo	Sequence
LLO_714_reverse	CCCTTCACTGATTGCGCCGAAG
Lm_flag3	CTCGAGGCTGTTAATTAATTGAGTTAACATTTG
Lm_flag5	CATATGAAAGTAAATACTAATATCATTAGC
M13_g3_1471-1492	CGCAACTATCGGTATCAAGCTG
M13_g3_1471-1492	CGCAACTATCGGTATCAAGCTG
M13_g3_1795-1774	CCGTAACACTGAGTTTCGTCAC
M13_g3_1795-1774	CCGTAACACTGAGTTTCGTCAC
m13p3_3_ter_bam	TTGGATCCTTAAGACTCAGACTCCTTATTACGCAG
M13P3_5-sig_xho	AACTCGAGACTGTTGAAAGTTGTTTAGCAAAA
m13p3_eco	AAGAATTCGAAACTGTTGAAAGTTGTTTAGCAAAA
m13p33bcl	GGTGATCATTAAGACTCCTTATTACGCAGTATG
m13p35eco	GAATTCATGCGTGGGCGATGGTTGTTGTCATTG
MCA3 Full Sal (56)	GTCGACATCGTATTTTTTATTACCGTTCTC
MCA3 Sal (51/61 /74)	GATGGTGGTCGACTGTATGTGCGATTGTATTGC
MCA3 Xho (61 /74)	CTCGAGTGTATTGCTATTATCGTCAACGATTGTGAC
MCA3 Xho (61 /74)	CTCGAGTGTATTGCTATTATCGTCAACGATTGTGAC
mca5 NBg (65/79)	CCATGGGCAGATCTGAAGTAGAAATGACTGAACGTCCG
mca5 NCO (63/79)	CCATGGGCGATAATGGTGAAGTAGAAATGACTGAACG
mca5 NCO (63/79)	CCATGGGCGATAATGGTGAAGTAGAAATGACTGAACG
METHB5MFE	AACAATTGCAACTGCAGCAGTCTGGAACTGAGC
METhC5mfe	CACAATTGGACCAGCTTCAGGAGTCAGGACCTGG
METHF5MFE	CACAATTGGACGCACAGATCCAGTTGGTGCAGTCTG
METLB3Bg2	GGAGATCTCTTGATTTCCAACTTGGTGCC
METLC3Bg2	GGAGATCTCTTTATTTCCAGCTTGGTCCCC
Mfe_20b	GGCAATTGGTCCACATATGAAATACCTGC
Mfe_20b	GGCAATTGGTCCACATATGAAATACCTGC
not_MSS_xho	GGCCGCTGGCCACCCGGGCCGGC
OligodT(18)	TTTTTTTTTTTTTTTTTT
OSM3_BAM	ATTGGATCCCTTAGCCACTTCATCACTTCCAGG
OSM5 K_ECO	ATGGAATTCAAAATGTCTGCGACTATCGAGGTCCG
OSM5_ECO	ATTGAATTCATGGCGACTATCGAGGTCCGAAACAACTG
osm5nde_bam	CATATGGGATCCGCGACTATCGAGGTCCGAAACAAC
OSM5ndeB	CACACATATGGGATCCGCGACTATCGAGGTCCG
osmo3eco	TCGAATTCTTAGCCACTTCATCACTTCCAGGC
p3_3_ter_bgl	GGAGATCTTAAGACTCCTTATTACGCAGTATG
p3d+nhe65	GCTAGCGGTTGAATGTCGCCCTTTTGTCTTTGG
p3d+nhe65	TATTGATCAGGTTGAATGTCGCCCTTTTGTCTTTGG
p3d-nhe63	AGGCTAGCTCCAAAAGGAGCCTTTAATTGTATCGG
p3d-xho63	TAGCTCGAGTCCAAAAGGAGCCTTTAATTGTATCGG
P3seq1	GAGCAAAACCCCGCTAATCC
p3seq2	ACCAGAACCACCACCAGAGC
pA 3xho (64/77)	CTCGAGTTCGCGACGACGTCCAGCTAATAAC
pA 3xho (64/77)	CTCGAGTTCGCGACGACGTCCAGCTAATAAC
pA 5Bam (61/76)	GGATCCGAACTTAAACGCTGATCAACGTAATGG
pA 5Bam (61/76)	GGATCCGAACTTAAACGCTGATCAACGTAATGG
PA3 Eco	GTGAATTCCCATCTTCTTTGCCAGGCTTGTTGCCGTCTTC
pa5nco	ATCCATGGCTGCGCAACACGATGAAGCTCAAC
pAV3XHO	CTCGAGCTCTCCTTCTTAAAGTTAAACAAAA
pAVbgl2	GAACTGTAATAAGATCTTAGGCCTCTAGTCTAG
pcap3	ACGAGTGCCGTAAACGACGATGGTTTTAC

Oligo	Sequence
pcap5	ATGGTACTTGGTAAACCTCAAACGGATCC
pCBER1TER1	AATTCGCCCTTAAGGGCG
PCRSCRIPT_5	AATAGGCCGAAATCGGCAAAATCC
pelb 5 nde (62)	TACATATGAAATACCTGCTGCCGACCGC
pet3_1XB	GAGGATCCTCTCGAGGTGGCACTTTTCGGGGAAATG
pet3_2XBNE	ACGAATTCGCATATGAGGATCCTCTCGAGGTGGC
pET5_ECO_Bg2	CGAATTCGAGATCTAACCTCTGACACATGCAGCTCC
pett7up nco (47/60)	CGCGCCATGGTATATCTCCTTCTTAAAGTTAAAC
pfu3_mfe	CAATTGGGGGATTTTTTAATGTTAAGCC
pfu3Mfe	CAATTGGGGGATTTTTTAATGTTAAGCC
pgex_His_tail_Xho_not	TCGACCTCGAGGCGGCCGCACACCACCACCACCACCACTA
pgex_His_tail_Xho_not_reverse	GGCCTAGTGGTGGTGGTGGTGTGCGGCCGCCTCGAGG
pgex5 nde	AACATATGGGATCCCCGAATTCCCGGGTCGAC
pgexup3 nde	CCCATATGTACTGTTTCCTGTGTGAAATTGTTATCC
pJet F	CGACTCACTATAGGGAGAGCGGC
pJet F	CGACTCACTATAGGGAGAGCGGC
Pjet R	GAAGAACATCGATTTTCCATGGCAGCTG
Pjet278-304	CCTGAACACCATATCCATCCGGCG
pjet2959-13	GGCGTATCACGAGGCCGCCCCTGCAGCCG
pLac3_mfe	ATTCAATTGGCACGACAGGTTTCCCGACTGG
pLyss3	CGTATGGCAATGAAAGACGGTGAGCTGGTG
pLyss5-er1	AAAGTCCGGATGAGCATTCATCAGGCGGG
POMC_3_SALI	GTCGACCTCGCCCTTCTTGTAGGC
POMC_5BAMHI	GGATCCATGCCGAGATCGTGCTGC
ppsp3nde	CCCATATGTTGTCCTCTTGATTTCTGCGTT
ppsp5eco	CCGAATTCTAGCGAGTTCATCAAGAAATAGC
Protaq5	CTCGAGCCTATGAGGGGGATGCTGCCCCTC
proteinA_xho	AACTCGAGTTCGCGACGACGTC
ProteinA5Er1	GGAATTCACCTAACTTAAATGCTGATCAACG
pspa 5	ATCAAGAGGACAACATTATGGGTATTTTTTCTCGC
pspA3 bam	AAGGATCCTTAGACGATGAGCGGCGGCG
pspa5 SSP	CAATATTATGGGTATTTTTTCTCGCTTTGCCG
ptrcscEcoBgl	CCGAATTCAGATCTGTTTTGGCGGATGAGAGAAGATTTTC
ptrcscnde_eco	ACGGAATTCCCCATATGGTTTATTCCTCC
ptrcscNE-mini	GGGAATTCCATATGTAATATATACCTCTTTAATTTTTAATAATAAAGTTAATCG
pTRCSCsal	ATGGTCGACTCTCAGTACAATCTGCTCTG
pTRCSCxho	GTCTCGAGAAAATACCGCATCAGGCGCTC
pTX101 (-500)	TATCACGAGGCCCTTTGGATAACCAGAAGC
pTX101 3-att	TTACCAATGCTTAATCAGTGAGGCACCTATCTC
pTX101 5 ORF	ATGAAAGCTACTAAACTGGTACTGGGCGCG
ptxNDE	CATATGAAAGCTACTAAACTGGTACTGGGC
ptxreverse	CAGAGTAGAACCCAGGATTACCGCG
PUC_3_ATC	ATCCACTGAGCGTCAGACCCCGTAG
PUC_3_CCA	CCACTGAGCGTCAGACCCCGTAG
PUC_3_up_cca	CCATTCTTTCCTGCGTTATCCCCTGATTC
PUC_5_ATC	ATCATGTGAGCAAAAGGCCAGC
PUC_5_CCA	CCACATGTGAGCAAAAGGCCAGC
PUC_frame3	TAGTTAAGCCAGCCCCGACACC
PUC_frame5	AGCTGTTTCCTGTGTGAAATTGTTATCCGC

Oligo	Sequence
PUC_seq1	AGCAGACAAGCCCGTCAGGG
PucSeq2	TATGTTGTGTGGAATTGTGA
rrn3sal	TTGTCGACGCAAAAAGGCCATCCGTCAGGATGG
rrn5bam	GCGGATCCATGTGTTTTGGCGGATGAGAGAAG
S1	GCTATGACTCACCCGGACGG
S2	CTTAAGAATTCGTCCCACGG
sal6h (60/65/?)	TAGTGATGGTGATGATGGTGGTCGACTGTATGTGCG
scfv_B1	CCACCGGGAATTCCACTACC
SCFV_P2	TCCCGGTGGCGGGTCTGGAGGTGGAGGTTCTCTCGAGGATAAGCTTGGGCCCATCGAGCACCACCACCACCACCACCACCCCGGGGGCCAATTGGGCCC
scfv3	TATGATCAAGATCTGGGGCCCAATTGGCC
SCFVP1	AAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCGTCGACTCCATGGGTGGAGGAGGTAGTGGAAT
sclb3hind	GTAAGCTTGATTTCCAACTTGGTGCCTCCACCG
sec1	ATCTTGGAGATGTCTACCTCTTTTTCTTCGCCTTTGTACTTGAACTTTACGGTTGC
sec2	CACCGCCCTCGTCGTAGGTGAAGGAGATCATCTTGCCCACACGCCATACTTTCTTG
sec3	TGCAGCAGCTCCTTCGGCGCGTCCTTTTCGCTTACCGCACCGCGGCCGGTCTTGC
sec4	ATCCAGTGCCTTGGGACTAGTGCCACCGCCGCCCTTTTTCTGCTTCTCCAGCATC
sefA_3_xho	AGCTCGAGCACCACCAGATACCGAAGC
sefA_5_nde	GCATCCGCACAGATAAATTGTGCATATGC
sefa3_xho	CTCGAGGTTTTGATACTGCTGAACGTAG
sefa5_e1_nde1	GAATTCACATATGCGTAAATCAGCATCTGC
SMP_forward	AATTCCTGCAGGGTTTAAACTGGCCAG
smp_reverse	GATCCTGGCCAGTTTAAACCCTGCAGG
spa20b_eco	CGGAGCTCGAATTCCCCATCTTCTTTGCCAGGC
spa6538_3_eco	TGGAATTCTTGCCAGGTTTGTTGTTGTCTTCCTC
SPA6538_5_nde	GCTCATATGGCGCAACACGATGAAGC
SPM_forward	AATTCCTGCAGCCCATGGCCAGTTTAAACG
SPM_reverse	GATCCGTTTAAACTGGCCATGGGCTGCAGG
SS03XHO	CTCGAGATCCAGTGCCTTGGGACTAGT
SSO5_ECOR1	GAATTCCGCAACCGTAAAGTTCAAGTAC
SSO5NDE	CATATGGCAACCGTAAAGTTCAAGTACAAAGGC
SSO5R1	GAATTCCCGCAACCGTAAAGTTCAAGTAC
SSO7D3(1)	ATCAAGAAAGTATGGCGTGTGGGCAAGATG
SSO7D5	CTTGGAGATGTCTACCTCTTTTTCTTCGC
ssoGFP3	TTCTGCTTCTCCAGCATCTGCAGCAGCTCCTTC
ssoGFP5	AAAGGGCGGCGGTGGCACTAGTCC
STN136_65	CCTTCAGCGTGGCGATACCGTCCATCTC
stn251_66	CGTCGCTCTGGCCTACAGTGGGC
STN371_F	ACTGGCAACCAGATAGTAAAGACC
STN371_F	ACTGGCAACCAGATAGTAAAGACC
STN371_F	ACTGGCAACCAGATAGTAAAGACC
STN744	CAAAGCAGAGAGATTCAGTTGAGA
STN744	CAAAGCAGAGAGATTCAGTTGAGA
STN744	CAAAGCAGAGAGATTCAGTTGAGA
sv3ter	CAAGATCTTTAGGGGCCCAATTGGCCCCCGG

Oligo	Sequence
t4Dp3BamH	GGATCCCCAAACAGGAAGTCTAACGAAG
t4Dp5Nco	CCATGGCGAAAGAATTTTATATCTCTATTG
T4k3	CTCGAGAAAATCTCCCGAAGC
T4k5	CATATGAAAAAGATTATTTTGACTATTGGCTG
T4lig3	CTCGAGACCAGTTACCTCATGAAAATC
T4lig5	CATATGATTCTTAAAATTCTGAACGAAATAGC
t7_terminator_rev	GCTAGTTATTGCTCAGCG
T7RNApol3	TTACGCGAACGCGAAGTCCGACTC
T7RNApol5	TGGCCAATGAACACGATTAACATCGC
Taq_3_sal	TAGTCGACCTTGGCGGAGAGCCAGTCC
taq3Eco	CAAGAATTCTCCTTGGCGGAGAGCCAGTCCTCC
TG_ECR1	GCGTGGCTAAAACCTAAGACAGGAATTC
tgo_die_5	ACGCTCTATCACGAGGGCGAGG
tgo_die3	CTGAATATTGAAGGCGAGCATCTTAAGTTCCTCG
TGO3_ECR1	GAATTCGGTGTCTTAGGTTTTAGCCACG
TOP3bamHI	ACGGATCCCCATCCGTAGATGATTTAACGTGATC
top5nde	CCCCATATGCGTGCACTTTTTTATAAAGATGG
topo_ecoI modify	AATTCGCCCTTAAGGGCATATGCCCTTAAG
UTPase_sal3'	CAGTCGACTTTCTTTCTCTTTGAAAACGC
Xho_SSM_not	TCGAGCCGGCCCGGGTGGCCAGC
Z for	CGCCAGGGTTTTCCCAGTCACGAC
Z Rev	AGCGGATAACAATTCACACAGGAAAC
1C5_60.3	CCATGGGATGTACAGCTTCAGGAGTCAGGACC

APPENDIX D.

Lists of IACUC Approved Protocols

Purpose of animal uses	IACUC approvals	
Obtaining anti-INLA antiserum	2002-0179	
Obtaining anti-*Listeria monocytogenes* antiserum	2007-1228	

APPENDIX E.

Trainings of Individuals that Performed the Treatments to Animals in this Dissertation

Trainee	Training Courses	Trainer
Tung-Shi Huang	Rat Basic Handling & Techniques	Auburn University College of Veterinary Medicine
	Mouse Basic Handling & Techniques	
	Rabbit Basic Handling & Techniques	
Ywh-Min Tzou	Rat Basic Handling & Techniques	
	Mouse Basic Handling & Techniques	
	Rabbit Basic Handling & Techniques	

APPENDIX F.

A fail-safe protocol for T7 induction of toxic recombinant in *E. coli*

Although the T7 promoter has a strong expression level and has many benefits over Lac, Tac or BAD, many researchers have experienced inconsistency with this expression. Many people believe that once the insertion sequence is correct, and with appropriated bacteria strain, the recombinant will be produced. However, in the T7 expression system, this is not quite true. Here, we share some of our observations, and give some logical explanations to them.

The most common misunderstanding about the T7 system is the stability of plasmids in expression strains of *E. coli*. Many of the researchers also believe that the mutation rate of *E. coli* is only 10^{-7} and this tiny number would not have significant effects. This is not true and could be very wrong in the T7 expression system, especially when the protein of interest is toxic. Indeed, the 10^{-7} is a tiny number. However, this number is really tiny only when the expressed bacteria can grow as fast as mutated. Thus, the real situation is the opposite. Because of the very high level of expression, when T7 RNA polymerase is turned on, bacteria grow slowly because

many resources are occupied for this "unnecessary" protein synthesis. Thus, those bacteria capable of expressing are eventually taken over. Data indicate that over expression kills bacteria even when the target proteins are innocuous (Studier, 1991; Studier and Moffatt, 1986; Studier and others 1990). In fact, only certain portions of bacteria carry the expressing plasmid after induction (Studier, 2005).

In our laboratory, we discovered similar observations. We have tried to background express green fluorescent protein (GFP) *E. coli* strain C41 (DE3, Lucigen) for it to be distinguished from *L. monocytogenes* under the microscope. While the green color of colonies to be developed on the LB plates at room temperature for days, we found that the colonies were not solid green. We picked several of these colonies and some freshly transformed colonies to streak on plates with IPTG to see the capability of expression. Most of the bacteria lost the capability to express GFP in the old colonies. However, in the plates from the fresh colonies, significantly greater portion of colonies were found to be green. The theory behind this phenomenon is that there is trace amount of lactose present in tryptone. The induction of this trace amount of lactose was inhibited by the amino acids in the media (Studier, 2005) while the colonies are young. However, in old colonies, starvation turns on the c-AMP pathway and makes lactose capable of induction (Grossman and others, 1998). Once induced, toxic proteins start to kill or inhibit the growth of *E coli* capable of expression. The other example we can provide is in the case when we tried to help one of off-campus immunologist to express PC2 (an ER peptidase) with pET19B in *E coli* (Rosetta2, DE3, pRare2). After several failures from "standard protocol", none of his single

colonies worked. Then he induced his target proteins successfully from his transformation mixture. We suggested to him not to let his plates spread from transformation mixture stay too long. He picked the colonies immediately after they were visible (about 12 hrs) and 100% of them were expressed at 10 mM phosphate pH 7.5 buffered LB (Tzou and others 2008).

Another misunderstanding is the reason for the induction of T7 at log phase is due to of quorum sensing. This misunderstanding was proven incorrect when expression was observed in ZYM5052 at OD 4.0 (Studier, 2005). We believe that pH is much more important than culture ages. The pH in an unbuffered LB mixed by components is roughly measured to be 6.5. After the growth of bacteria it might reach pH 5.5, in which lactose permease no longer active (Page and others 1988). Induction of toxic protein in later log phase is important and is suggested by many successful cases. However, the pH could be too low for induction because *E. coli* produces acids during the growth. Adjusting the pH above 7.0 significantly facilitate the Maillard reaction during the autoclave but created additional problems. Here, we developed fail-safe protocols to induce T7 driven genes in *E. coli* based on two strategies: To minimize the T7 polymerase activities and shorten the duration of un-expressing period.

1. Always verify and propagate plasmid in non-DE3 strains. It will be better and safer in strains with *recA*$^{-}$ and *endoA*$^{-}$.
2. Transform the verified plasmid into DE3 strains, and plating on LB Lennox agar plates, with 20 mM MgSO4 and appropriate antibiotics, for colony

formation. Do not add antibiotics for the resistance genes are located at the bacterial chromosome. Too many antibiotics slow the growth of bacteria, and make the bacteria stay longer on plate.

3. Pick colonies immediately after they are visible (12hr for 100 μg/ml ampicillin only plates or 14hr for ampicillin + 34 μg/ml chloramphenicol plates). If the colonies are too old, there's a chance that the Lac promoter will turn on by the induction of the trace amount of lactose and c-AMP accumulation due to starvation. When picking the colonies, do not pick only large ones. For our routine, we picked two large and two small colonies. Usually, small colonies gave the highest expression levels, and this is very reasonable when the target proteins are toxic. Transfer the bacteria to 500 μl of LB Lennox with 0.25% glucose, 10 mM $MgSO_4$, and appropriate antibiotics in 2.0 ml centrifuge tubes. Incubate the bacteria in 37˚C with 300 rpm shaking. Culture should be harvested in less than 6 hr or when turbidity is evident. The bacteria need to be spun down to replace fresh medium with all antibiotics that the bacteria are resistant to and cultures can be store in a refrigerator as master cultures. Verification of plasmids is usually not necessary, however, if recycled electroporation cuvettes are used in the transformation, use 1-10 μl of master culture and grow the bacteria in LB or other media that is suitable for plasmid isolation.
4. Screening for inducible clones with standard methods would be difficult for this protocol because the master cultures are not evenly saturated, and hence we suggest overnight expression medium (ZYM5052) developed by Studier

(Studier, 2005). This medium is more difficult to make. Some similar medium are available from Invitrogen (MagicMedia) and Novagen (Overnight Express) and were determined to be as good as ZYM5052. Pipette 10 µl of master cultures into 50 ml screw-capped tubes with 2-3 ml of auto-inducing media with appropriate antibiotics. Place the tubes in a 45˚ tilted rack fixed in the 37˚C incubator with 300 rpm, and grow the culture overnight. The matured cultures will have an OD_{600} >10, and hence, dilution is required to measure this OD for normalization in SDS PAGE.

5. 8% of glycerol was added to master cultures of the clones capable of expression and store at -80°C.
6. Grow the master culture for large scale expression in LB Lennox with 0.25% glucose, 10 mM $MgSO_4$, and appropriate antibiotics at 30°C or lower for overnight. Dilute this overnight culture 100 times in ZYM5052 for overnight expression. For slightly better yield, we diluted the overnight culture in ZYN505 (ZYM5052 without lactose) and induced the culture at OD 3.0-5.0 with isopropyl β-D-1-thiogalactopyranoside (IPTG), and adjusted culture pH to 6.5-6.6 for every 2hrs.

We put this protocol as an appendix because some observations are difficult to explain very well. These observations are also considered to be very rough or not directly ours. However, this protocol robustly helped us and our collaboratories to

consistently obtain protein of interest, including mice PC2 (Tzou and others, 2008) and tobacco osmotin which have never been expressed in *E. coli* successfully.