2013

An Anti-Pyruvate Kinase Monoclonal Antibody And Translocated Intimin Receptor (tir) For Specific Detection Of Listeria Species And Shiga-Toxigenic Escherichia Coli

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Graduate School ETD Form 9
(Revised 12/07)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By TITIKSHA DIKSHIT

Entitled AN ANTI-PYRUVATE KINASE MONCONAL ANTIBODY AND TRANSLOCATED INTIMIN RECEPTOR (TIR) FOR SPECIFIC DETECTION OF LISTERIA SPECIES AND SHIGA-TOXIGENIC ESCHERICHIA COLI

For the degree of Master of Science

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Approved by: DR. BRIAN FARKAS 10/11/2013
Head of the Graduate Program Date

AN ANTI-PYRUVATE KINASE MONOCLONAL ANTIBODY AND TRANSLOCATED INTIMIN RECEPTOR FOR SPECIFIC DETECTION OF *LISTERIA* SPECIES AND SHIGA-TOXIGENIC *ESCHERICHIA COLI*

A Thesis
Submitted to the Faculty
of
Purdue University
by
Titiksha Dikshit

In Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

December 2013

Purdue University

West Lafayette, Indiana

To my parents.

ACKNOWLEDGEMENTS

I would like to thank:

Dr. Arun K. Bhunia, my research advisor, for his guidance, advise and patience. I have learnt much during my research and I sincerely appreciate this opportunity. Thank you for your time and continuous encouragement.

Dr. Bruce M. Applegate, Dr. John A. Patterson and Dr. Ramesh Vemulapalli for serving in my graduate committee and for their insights and support.

Dr. Mary A. R. Amalaradjou, Dr. Marcelo Mendoça, Dr. Atul K. Singh and Dr. Krishna M. Mishra for helping me learn various techniques. I am grateful to you for your assistance in problem solving, for lending a patient ear and for motivating me to give my best, particularly when the going got tough.

My lab mates of Molecular and Microbiology Lab, former and present; and my wonderful friends; it has been great working with you and your friendship means a lot to me. I wish you all the best in your future endeavors.

David La, Dr. Daisuke Kihara, Dr. Chittaranjan Das, Gwen Shoemaker, Deb Livengood and Linda Webster for their assistance.

My wonderful friends.

Last but not the least, my family. My parents Mumukshu and Nirupama Dikshit, for their unconditional and endless love and support. Thank you for making me the person I am today. And finally, Saattwik Dikshit, for being the best little brother one could wish for.

Funding:

This research was supported through a cooperative agreement with the Agricultural Research Service of the US Department of Agriculture project number 1935-42000-035 and the Center for Food Safety Engineering at Purdue University.

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ABSTRACT

Dikshit, Titiksha. M.S., Purdue University, December 2013. An Anti-Pyruvate Kinase Monoclonal Antibody and Translocated Intimin Receptor (TIR) for Specific Detection of *Listeria* Species and Shiga-Toxigenic *Escherichia coli*. Major Professor: Arun Bhunia.

Foodborne illnesses pose a significant health concern and economic impact worldwide. In this study, we aimed at developing alternate and improved methods for Shiga toxigenic *E. coli* (STEC) and *Listeria* species detection. In *Listeria monocytogenes*, an auxiliary secretory system, SecA2, plays an important role in translocating virulence and housekeeping proteins to cell surface to aid bacteria to maintain saprophytic and intracellular life styles. Here we investigated if pyruvate kinase (PyK), present in both pathogenic and nonpathogenic *Listeria,* is translocated by SecA2 system and determined its potential application in immunologic detection of these bacteria. Additionally, cell surface localization and enzymatic activity of PyK were examined. Enzyme immunoassay with anti-PyK antibody, MAb EM-7H10, indicated the presence of PyK in all *Listeria* species except *L. roquortiae*. Immunofluorescence assay confirmed surface localization. Analysis of *L. monocytogenes* Δ*secA2* mutant revealed the absence of PyK in cell wall and the supernatant fractions along with reduced levels in the intracellular fraction indicating that PyK translocation to cell

surface is SecA2-dependent. Reverse transcriptase PCR (RT-PCR) confirmed reduced levels of PyK transcript in the Δ*secA2* mutant indicating SecA2-dependent regulation of *pyk*. Furthermore, PyK expression was found to be 10-fold higher in *L. monocytogenes* cultured in Brain-Heart Infusion Broth (BHI), Tryptic Soy Broth (TSB) and buffered *Listeria* enrichment broth (BLEB) than in University of Vermont medium (UVM) or Fraser Broth (FB). In summary, PyK is determined to be a SecA2-dependent surface displayed glycolytic enzyme present in both pathogenic and nonpathogenic *Listeria*, which could serve as a strong immunologic target for *Listeria* species detection. Shiga toxigenic *E. coli* (STEC) has been implicated in several foodborne outbreaks exhibiting severe hemolytic uremic syndrome (HUS) and fatalities. Here, we focus on a novel approach for STEC detection. Translocated Intimin Receptor (TIR) binds exclusively with intimin, a STEC adhesion protein which mediates intimate attachment of the bacteria to the host cell. This receptor-ligand system is unique to STEC and can be used for its detection on biosensor platforms. Collectively, data provide strong evidence for the use of anti-PyK antibody and TIR and for specific detection of *Listeria* species and STEC, respectively.

CHAPTER 1. INTRODUCTION

1.1 Implications of foodborne illnesses

Over the last century, foodborne illness has acquired a new dimension that has made it a major global health crisis and economic burden. In the United States, episodes of foodborne incidences have increased consistently and significantly over the last fifteen years (1996-2011) (CDC-2011). According to the Center for Disease Control and Prevention (CDC), in the United States alone, annually, foodborne pathogens have caused approximately 47.8 million incidences of foodborne illness resulting in 127,839 hospitalizations and 3,037 deaths. About 20% of these episodes were caused by the 31 known foodborne pathogens, which accounted for 55,961 hospitalizations and 1,351 deaths. Almost 90% of these incidences were attributed to norovirus, nontyphoidal *Salmonella* spp., *Clostridium perfringens*, and *Campylobacter* spp. [1].

Akin to the CDC in the US, the European Food Safety Association (EFSA) holds records and estimates for foodborne infections in the 27 European Union member states. For the year 2010, EFSA and the European Center for Disease Prevention and Control (ECDC) jointly reported that there were a total of 43,473 incidences resulting in 4,695 hospitalizations and 25 deaths. Similar to the trend

in the US, *Salmonella*, *Campylobacter*, verotoxigenic *Escherichia coli* (VTEC) and *Listeria monocytogenes* were involved in the majority of the outbreaks [2].

Thus, recognizing the gravity of foodborne pathogen-associated illnesses, the World Health Organization (WHO) established the Foodborne Disease Burden Epidemiology Reference Group (FERG), in 2006, to help estimate the statistics pertaining to global foodborne diseases [3]. However, accomplishing this colossal task requires significant planning, considerable economic resources, and global communication and partnerships along with an extensive infrastructure. The group began pilot studies in 2011 in six WHO regions. The results are awaited.

Due to the massive number of foodborne cases, the economic implications of these diseases are tangible. The United States Department of Agriculture - Economic Research Service (USDA-ERS) has been providing comprehensive cost estimates for the various pathogens since 1989. In 2003, the department launched an interactive online tool- 'Foodborne Illness Cost Calculator' to estimate the expenditure associated with *Salmonella* and *E. coli* O157:H7 outbreaks and diseases. This calculator provides an annual estimate of approximately $2.3 billion for *Salmonella* and $488 million for *E. coli* O157:H7 [4].

Besides health related expenses, both domestic and international food trade also are impacted due to outbreaks. Foodborne illness may cost billions of dollars in recalls and food import and export [5, 6]. Food recalls are not only costly, but may also eventually lead to loss of reputation, trustworthiness of the company and the brand. So far in 2013, there have been nearly 20 recalls reported by the FDA and about 8 recalls by the USDA Food Safety Inspection

Service (FSIS), owing to possible *Salmonella* or *E. coli* O157:H7, *Listeria* contamination (FDA recalls and withdrawals: http://www.fda.gov/animalveterinary/safetyhealth/recallswithdrawals/default.htm & USDA-FSIS recalls: http://www.fsis.usda.gov/Fsis_Recalls/open_federal_cases/index.asp).

1.2 Food diagnostics: Conventional and rapid detection techniques

Detection of a pathogen is essential in identifying the source and possible means of contamination and for ensuring food safety. Over the last few decades, tremendous progress has been made in developing methods for foodborne pathogen and toxin detection. Subsequently, detection methods have evolved from the traditional culture involving nucleic acid (DNA/RNA) or protein based assays to the modern rapid high throughput systems and biosensors. While traditional methods are reliable and accurate, they are time consuming and lack the sophistication, speed and sensitivity of new and upcoming methods such as Surface Plasmon Resonance (SPR), micro-fluidic biochips, mammalian cell based sensors, fiber optics sensors, microarray based systems and nanotechnology based approaches [7, 8].

Polymerase chain reaction (PCR) is one of the most widely used pathogen detection techniques [9, 10]. It has been used for the detection of various foodborne bacteria including *Listeria* spp., *E. coli* O157:H7, *Salmonella* spp., *Vibrio* spp., *Yersinia* spp. among others [11-13]. Today, DNA based techniques have developed further, enabling easy visualization of probe hybridization or

amplification of target DNA/RNA with high accuracy and speed. Some of the new DNA based molecular techniques include single phase hybridization assays and oligonucleotide arrays (DNA disc technology)[14, 15]. Microarray platforms are highly efficient and are being used extensively today for detection of various bacteria [16, 17]. The US Food and Drug Administration (US-FDA) has developed an array based tool for identification of *E. coli*, *Shigella* and *Salmonella* species [18].

Immunologic methods are the next most popular pathogen detection tool. These are powerful techniques for highly specific pathogen detection. These techniques include enzyme-linked immunosorbent assay (ELISA), lateral flow “dip stick” method and antibody-coated latex or magnetic beads [19].

Conventional methods are time consuming and cumbersome, and require extensive sample preparation and product specific working protocol. This hinders the use of these methods in modern day high speed and performance of the food industry. Biosensors, on the other hand, are highly sensitive and use minimal amount of sample and show potential for their application in pathogen detection. Some of the commonly used biosensor platforms are: Surface Plasmon Resonance sensor (SPR) [20], evanescent wave fiber optics platform [21], cytometric bead array biosensors [22], DNA-based biosensors or genosensors [23] and lab-on-a-chip microfluidic device [24].

What unites the various biosensors is the basic principle behind their development: the use of capture and receptor biomolecules. Why and how certain proteins recognize and interact with each other, both spatially as well as

temporally, to carry out the various essential cellular functions is an intriguing question. Katritis and Bonvin [25] defined the binding of two proteins '*as a reversible and rapid process in an equilibrium that is governed by the law of mass action. The binding affinity is the strength of the interaction between two (or more than two) molecules that bind reversibly (interact). It is translated into physico-chemical terms in the dissociation constant (Kd), the latter being the concentration of the free protein that occupies half of the overall sites of the second protein at equilibrium*.' Biomolecular recognition is conceptually carried out by one of these three methods: (i) lock and key e.g. trypsin with bovine pancreatic trypsin inhibitor (BPTI) [26], (ii) induced fit and (iii) conformational selection (dynamic fit).

When protein (ligand) is applied to a biosensor platform, the participating molecule is termed a bioreceptor. This molecule can be a DNA or RNA aptamer [27-30] or a protein molecule such as an antibody, an enzyme, a membrane protein or a binding protein [31-35]. Antibody based immunological methods have been the most popular. Even though they lack the level of sensitivity provided by the DNA or RNA based methods, they are easy to use and can be generated in large quantities rendering immunological techniques as the preferred choice [36, 37]. Subsequently, over the last two decades, antibody based bacterial detection systems gained significant impetus.

At the same time, several challenges remain to be addressed. For robust detection, the specificity and affinity of the biorecognition molecules must be improved. In the case of antibodies, it is imperative to develop means for faster

production and purification. Rapid antibody generation and more specific target identification will mark a major step towards the application of immunological techniques and biosensors on a commercial scale. It is critical to develop and cultivate enhanced pathogen detection techniques to catch up with the rapidly growing food industry.

Besides these molecular methods, many other innovative and out of the box technologies have also been developed. One example of such a novel technique is BActeria Rapid Detection using Optical scattering Technology (BARDOT)-a label free bacteria detection system [38-41], which identifies pathogens based on a characteristic scatter pattern obtained by shining a laser beam on a bacterial colony. Researchers have also successfully used nanotechnology based strategies [42] and immobilized metal hydroxides for bacterial capture [43] as label free high throughput screening methods.

In summary, food safety is evidently of utmost concern today. The research objectives stated in this thesis address some of the short-comings of the current antibody based detection systems, with the ultimate goal of developing unique, improved platforms for fast, sensitive and specific detection of pathogens.

1.3 Research goal and objectives

Developing novel techniques and strategies for foodborne pathogen detection is crucial for maintaining safer food supply, ensuring public health and reducing food recalls and economic losses. Despite tremendous advances in science and technology, Enterohaemorrhagic *E. coli* (EHEC) / Shigatoxigenic *E. coli* (STEC)

and *Listeria* species continue to be two of the most potent food borne pathogens that have been implicated in several outbreaks in recent years. This study aims at improving the existing technology and developing better detection tools for more rapid, specific and sensitive capture and identification of EHEC and *Listeria* species.

For *Listeria* species detection, we demonstrate the use of a novel monoclonal antibody, MAb EM7H10. This antibody reacts with all the *Listeria* species and is capable of being used on multiple detection platforms, such as ELISA and the evanescent wave fiber optic biosensor. MAb EM7H10 is, thus, a significant improvement over several existing antibodies and holds tremendous potential for large scale industrial application. In addition, this antibody also could be used for further characterization of antibody-reactive antigen in *Listeria*: (i) Determine identity of the protein through mass-spec analysis (the protein is identified as Pyruvate kinase); (ii) Monitor distribution of the protein in different cell fractions such as cell surface and cytosolic fractions, and (ii) Investigate if the surface translocation mechanism of the protein is dependent on SecA2, a lesser known bacterial protein secretory system.

For STEC detection, a novel approach that does not involve traditional antibodies was pursued. Bacterial own extracellular protein, Intimin, and its corresponding receptor in the host, Translocated Intimin Receptor (TIR) was used as the biorecognition molecule for detection of STEC. Intimin and TIR interaction has been found to be highly specific in STEC and we employed this receptor-ligand system for STEC detection. The advantages of using such

system is that both the proteins are bacterial (produced by STEC itself) and therefore relatively easier to produce in large quantities economically. Furthermore, the proteins can be immobilized on multiple detection platforms. The specific objectives are:

1. To investigate the cell surface displayed pyruvate kinase as a potential target for antibody based detection of *Listeria* species (**Chapter 3**).
2. To capture and detect EHEC/STEC using immobilized receptor, translocated intimin receptor (TIR), on a biosensor platform. (**Chapter 4**)

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

The World Health Organization (WHO) defines foodborne illnesses as "diseases, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food." These agents can be biological or chemical in nature. The biological agents, or pathogens, usually virus, fungi, bacteria and parasites, are disease causing microorganisms that are transmitted by food.

WHO estimates that in 2007, over 1.8 million people died worldwide due to foodborne diseases. This alone highlights the magnitude of the implications of foodborne incidences. Both economically prosperous as well as poor countries suffer from millions of foodborne diseases. The United States Center for Diseases Control and Prevention (CDC) estimated that in 2011, around 48 million individuals, or nearly one in six Americans, were sickened by foodborne disease. These illnesses resulted in approximately 128,000 hospitalizations and 3,000 deaths (CDC), culminating in billions of dollars in economic losses due to healthcare cost, and product recalls and tarnished brand reputation. The most common manifestation of a foodborne infection is gastroenteritis; however, certain pathogens may also lead to severe long term consequences such as

abortion, stillbirth, and other neurotic and paralytic diseases. Currently, 31 food-borne pathogens are known to be responsible for the majority of diseases caused by 4 viruses, 3 parasites and remaining 24 bacteria [1] (Table 2-1).

Table 2-1 Known foodborne pathogens

Virus	**Bacteria**	**Parasite**
Astrovirus	*Bacillus cereus*	*Toxoplasma gondii*
Norovirus	*Brucella* spp.	*Giardia intestinalis*
Rotavirus	*Campylobacter* spp.	*Trichinella* spp.
Hepatitis A virus	*Clostridium perfringens*	*Cryptosporidium* spp.
	Clostridium botulinum	*Cyclospora cayetanensis*
	Shigatoxigenic *E. coli* (STEC) O157	
	STEC non-O157	
	Enterotoxigenic *E. coli* (ETEC)	
	Diarrheagenic *E. coli* non-STEC/ETEC	
	Listeria monocytogenes	
	Mycobacterium bovis	
	Salmonella, nontyphoidal	
	Salmonella enterica serotype Typhi	
	Shigella spp.	
	Staphylococcus aureus	
	Streptococcus spp. Group A	
	Vibrio cholerae, toxigenic	
	Vibrio vulnificus	
	Vibrio parahaemolyticus	
	Vibrio spp., other	
	Yersinia enterocolitica	

The origin of these foodborne pathogens can be traced back to sources that may vary from animals or insects to natural habitats like soil and water to

humans. Thus they can be classified as zoonotic, if they are transmitted to humans via animals or insects (*Staphylococcus aureus*, STEC O157, *Campylobacter* spp., *Salmonella enterica* serovar Typhimurium); as geonotic, if they are acquired from soil, water or decaying plant matter (*Listeria monocytogenes*); and as pathogens of human origin if they are transmitted from person to person (*Salmonella enterica* serovar Typhi, *Shigella* spp., *Vibrio cholerae*, Hepatitis A virus).

Foodborne pathogens can cause diseases by three mechanisms: food intoxication, toxicoinfection and foodborne infection. Food intoxication occurs due to ingestion of pre-formed toxin. Some examples include *Staphylococcus aureus*, *Clostridium botulinum* and *Bacillus cereus*. Toxicoinfection is caused when the toxin is produced by the bacteria following ingestion into the host. *Clostridium perfringens*, enterotoxigenic *E. coli* (ETEC) and *Vibrio cholerae* are known to cause toxicoinfection. Finally, foodborne infection is caused due to ingestion of the infective pathogen, for instance, *Salmonella enterica*, STEC. *Listeria monocytogenes*, *Shigella* spp, *Yersinia enterocolitica* and viruses and parasites.

2.1.1 Foodborne pathogens and illnessees

Foodborne pathogens cause innumerous illnesses and deaths globally. One of the first initiatives to quantify the number and impact of foodborne incidences on human health was undertaken by Paul S. Mead and his group [44]. They concluded that food borne pathogens cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each

year. It was observed that *Salmonella*, *Listeria* and *Toxoplasma* were responsible for most deaths. Over the last fifteen years, from 1996-2011, the number of recorded incidences of foodborne episodes has remained either constant or decreased, except in the case of *Salmonella*, wherein the number of reported cases has increased. Figure 1a shows the total number of laboratory-confirmed bacterial and parasitic infections, and post-diarrheal hemolytic uremic syndrome (HUS), by year and pathogen (Source: CDC; Foodborne Diseases Active Surveillance Network (FoodNet), United States, 1996–2011); Figure 1b represents the incidence of laboratory-confirmed bacterial and parasitic infections, and post-diarrheal hemolytic uremic syndrome (HUS), by year and pathogen (Source: CDC; Foodborne Diseases). However, it must be noted that the number of population surveyed for these data has also increased significantly over the years. Therefore, the increase in reported cases may be due to higher population and also better detection tools employed.

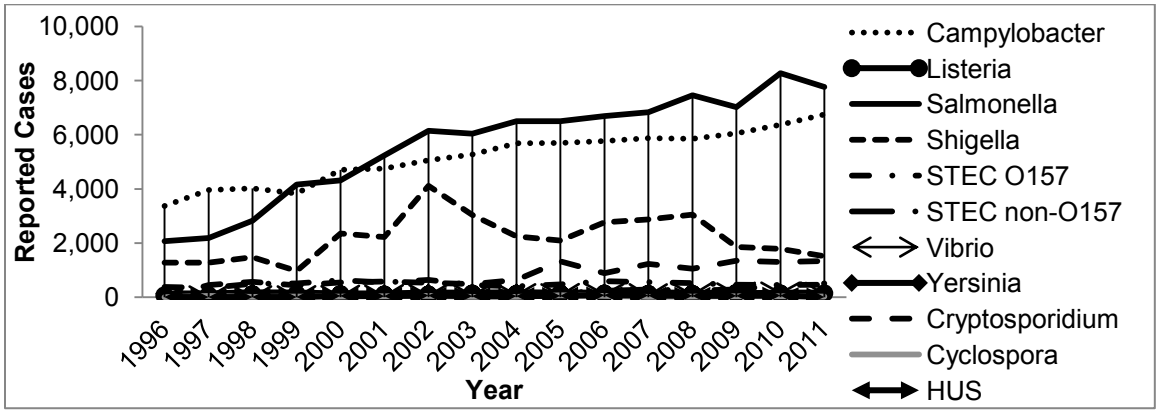

Figure 2-1 Number of laboratory-confirmed bacterial and parasitic infections, and post diarrheal hemolytic uremic syndrome (HUS), by year and pathogen (Source: CDC; Foodborne Diseases Active Surveillance Network (FoodNet), United States, 1996–2011)

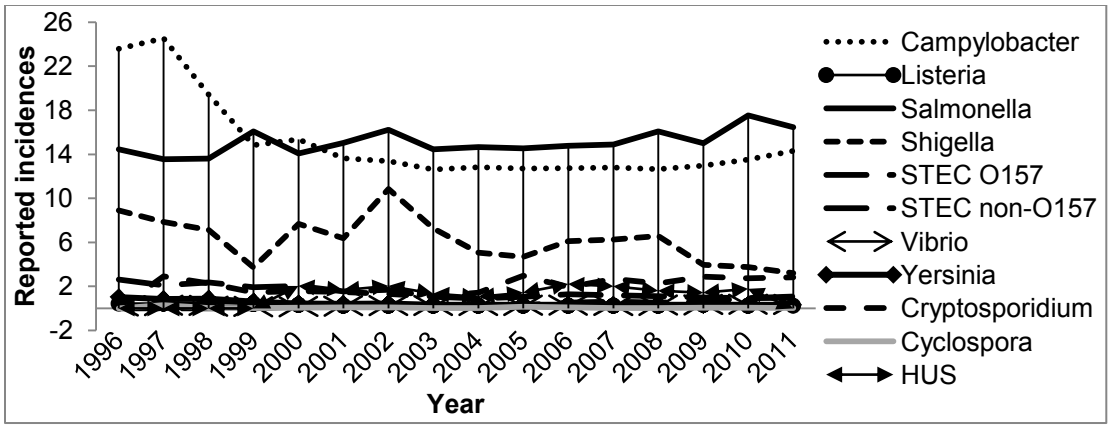

Figure 2-2 Incidences of laboratory-confirmed bacterial and parasitic infections, and post diarrheal hemolytic uremic syndrome (HUS), by year and pathogen (Source: CDC; Foodborne Diseases Active Surveillance Network (FoodNet), United States, 1996–2011)

For the year 2011, CDC estimated that the total number of foodborne illnesses to be a staggering 47.8 million cases. Today, 31 of these foodborne pathogens are known, which are responsible for about 9.4 million incidences throughout the country. Figure 2-1 shows the CDC estimates of the total foodborne illnesses and subsequent hospitalizations and deaths caused by known as well as unknown pathogens.

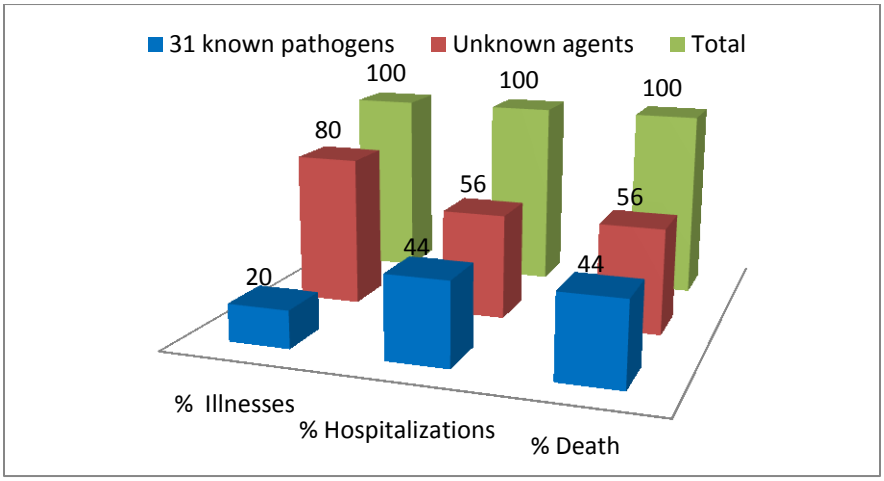

Figure 2-3 Illnesses, hospitalizations and deaths caused by the known and unknown food borne agents as a percentage of total episodes. (Source: CDC)

While the known pathogens are reported in only about 20% of the total foodborne illness cases, they are highly potent and thereby result in about 44% of the total hospitalizations and eventual deaths. Of these 31 known pathogens, *Salmonella*, *Clostridium perfringens*, *Campylobacter, E. coli* O157:H7 and *Listeria monocytogenes* are highly infectious and were responsible for the majority of the hospitalizations and deaths. Figure 3 summarizes the bacterial pathogens surveyed by CDC that posed maximum health risk to the population in 2011.

Pathogen	Illness	%
Norovirus	5,461,731	58
Salmonella nontyphoidal	1,027,561	11
Clostrudium perfringens	965,958	10
Campylobacter spp.	845,024	9
Staphylococcus aureus	241,148	3

Pathogen	Hospitalization	%
Salmonella nontyphoidal	19,336	35
Norovirus	14,663	26
Campylobacter spp	8,463	15
Toxoplasma gondii	4,428	8
E.coli O157:H7 (STEC)	2,138	4

Pathogen	Death	%
Salmonella nontyphoidal	378	28
Toxoplasma gondii	327	24
Listeria monocytogenes	255	19
Norovirus	149	11
Campylobacter spp	76	6

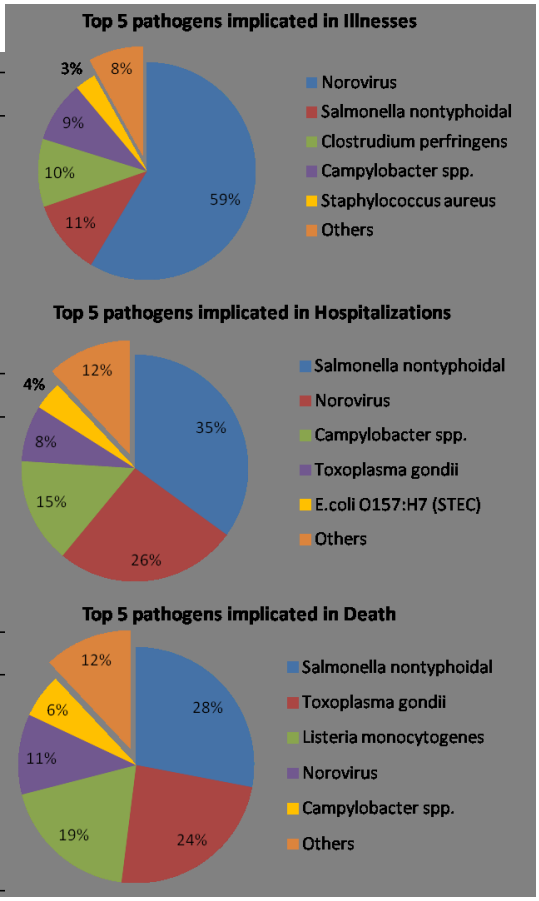

Figure 2-4 Top 5 foodborne pathogens responsible for Illnesses, Hospitalizations & Deaths in 2011

As mentioned previously, the 31 known foodborne pathogens are highly infectious and health hazardous and they were responsible for almost 50% the total hospitalizations and deaths. Some examples of the known foodborne bacteria are *Salmonella*, STEC, *Listeria monocytogenes*, *Staphylococcus aureus*, *Campylobacter* and *Clostridium perfringens*. Each of these bacteria has unique characteristics that aid their survival by evading the host immune system, thereby making them more efficacious. A brief description of these pathogens, their food carriers and infection mechanisms is provided below.

Salmonella

Salmonella, the 'model enteric pathogen' is a gram negative, rod shaped bacteria that causes various gastrointestinal diseases. *S. enterica* serovars Typhi and Typhimurium are implicated in numerous incidences of enteric fever all over the world, particularly in the developing nations of Asia and Africa [45, 46]. The key virulence factors in *Salmonella* pathogenesis are transported by the type III secretion system (T3SS) and the genes for which are located in the *Salmonella* Pathogenicity Island 1 (SPI-1) [47]. *Salmonella* serovars responsible for most outbreaks have been traced back to both fresh produce and food animals [48]. The contamination of fresh produce could happen during cultivation or handling and processing [49]. Recent *Salmonella* outbreaks have been caused by a varying food sources such as peanut butter, hedgehog, mangoes, cantaloupes, tuna fish, ground beef, live poultry and even dry dog food (source: CDC).

Shiga toxigenic *E. coli*

Shiga toxigenic *E. coli* (STEC), also known as vero-toxigenic *E. coli* (VTEC), is a sub group of enterohaemorrhagic *E. coli* (EHEC) and is one of the most virulent *E. coli* strains till date [50]. It causes bloody diarrhea and hemolytic uremic syndrome (HUS) among other severe gastrointestinal diseases. Key virulence factors for STEC pathogenesis are Shiga-toxin (*stx*), intimin and translocated intimin receptor (TIR), and T3SS, and the genes are encoded by the locus of enterocyte effacement (LEE) pathogenicity island[51] and an F-plasmid, O157 [52]. Even though *E. coli* O157:H7 is widely regarded the most virulent serovar, other STEC serovars such as O145, O26, O104 and O111 have also been implicated in several STEC outbreaks (source: CDC). While ground beef is the most common carrier for STEC as the bacteria can easily survive in cattle gut, it has also been found in turkey, some amphibians and produce such as lettuce and sprouts [53].

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive bacterium that causes listeriosis, which may prove fatal for the more vulnerable or the at-risk population - young, old, pregnant and immuno-compromised [54]. *Listeria* uses several virulence factors to adhere and invade host cells and trigger a series of escalating events that often leads to fatal consequences. Critical virulence factors include *Listeria* adhesion protein (lap), internalin A and B (InlA and InlB), actin A (ActA), listeriolysin O (LLO), cystic fibrosis transmembrane conductance regulator (CFTR), among many others [55]. Recent listeriosis outbreaks have

involved cantaloupes, cheese, ready-to-eat foods and several dairy products (Source: CDC).

Staphylococcus aureus

Staphylococcus aureus, a Gram-positive coccus, is another common foodborne pathogen. *S. aureus* food poisoning causes emesis and toxic shock syndrome (TSS) which may prove to be fatal. The major virulence factors include the *S. aureus* enterotoxins (SEs) [56]. SEA and SEB are the two most notable toxins out of the more than twenty different SEs that have been identified so far [57]. Formation of these enterotoxins also depends on the food matrix [58]. *S. aureus* outbreaks often associated with bakery products (cakes and ice creams), ready to eat foods, meat products and dairy products [59].

Campylobacter

Campylobacter is a Gram-negative, spiral shaped bacterium which is one of the top five agents of foodborne illness, hospitalizations and deaths in the US. The immediate clinical effect of *Campylobacter* infection is gastroenteritis; however it may cause severe long term sequelae such as Guillain-Barré syndrome (GBS), and reactive arthritis (ReA) [60, 61]. The major virulence factors for *Campylobacter jejuni* are Campylobacter invasive antigens (Cia) and a set of toxins called cytolethal distending toxin (CdtA, CdtB,CdtC) [62, 63]. The main source of infection is poultry [64, 65]; however, recent outbreaks have also involved unpasteurized milk and water (Source: CDC).

Clostridium perfringens

Clostridium perfringens is a Gram-positive, rod shaped bacterium. *Clostridium* infection results in watery diarrhea and abdominal cramps. The prominent virulence factors include *C. perfringens* enterotoxin (CPE), alpha, beta, iota, delta and theta toxins [66-68]. What makes this bacterium so infectious is its ability to form spores. Spore formation enables survival under unfavorable conditions, especially in food that has been kept heated for a long time prior to serving [69, 70]. Common sources of *C. perfringens* contaminations are beef, poultry, dry and pre-cooked food (Source: CDC).

Food Outbreaks and Recalls

CDC defines a foodborne outbreak as the occurrence of two or more cases of a similar illness resulting from the ingestion of a common food. All the aforementioned bacteria are responsible for the bulk of the foodborne outbreaks and recalls. In 1985, a California based *Listeria* outbreak from Jalisco Cheese resulted in one of the deadliest outbreaks ever, infecting 86 people and killing almost 50. More recently, in 2011, another *Listeria* outbreak from Jensen Farm cantaloupes infected 146 people, culminating in 33 deaths. In 2008, a Canadian Listeriosis outbreak from cold cut meats from Maple Leaf Farms infected over 50 people and killed 22. In 2011, a STEC O104:H4 outbreak from sprouts in Germany affected close to 4,000 individuals, killing 53. This is the worst recorded *E. coli* outbreak in the world. In 2008, a US *Salmonella* outbreak from peanuts

(Peanut Corporation of America) infected over 200 people, eventually killing 9. More notable outbreaks that occurred in 2012 are listed in Table 2-2.

Table 2-2 Food borne outbreaks in 2012. [1]

Source	**Pathogen**	**Cases**	**Hospitalizations**	**Deaths**	**States**
Peanut Butter	*Salmonella* Bredeney	35	8	0	19
Cheese	*Listeria monocytogenes*	18	18	3	13
Hedgehogs	*Salmonella* Typhimurium	14	3	0	6
Mangoes	*Salmonella* Braenderup	121	25	0	15
Cantaloupes	*Salmonella* Typhimurium and Newport	270	101	3	26
Ground Beef	*Salmonella* Enteritidis	46	12	0	9
Live Poultry	*Salmonella* Hadar	37	8	0	11
Live Poultry	*Salmonella Montevideo*	76	17	1	22
Multistate Outbreak	*Escherichia coli* O145	18	4	1	9
Live Poultry	*Salmonella* Infantis, Newport, Lille	163	33	2	26
Dry Dog Food	*Salmonella* Infantis	49	10	0	20
Ground Tuna	*Salmonella* Bareilly, Nchanga	425	55	0	28
Small Turtles	*Salmonella* Sandiego, Pomona, Poona	196	36	0	31
Clover Sprouts	*Escherichia coli* O26	29	7	0	11
Restaurant Chain A	*Salmonella* Enteritidis	68	-	-	10

Clearly, food borne pathogens create hundreds of illnesses and cost millions of dollars in health care and product recalls. Therefore, there is a necessity of developing tools for rapid and sensitive pathogen or toxin detection.

2.1.2 Detection of foodborne pathogens

Conventional methods for pathogen detection include molecular techniques such as DNA/RNA based polymerase chain reaction (PCR), real time PCR and antibody based immuno-assays like enzyme linked immunosorbent assay (ELISA). Modern techniques have evolved to enable rapid, high throughput and sensitive detection. Biosensors are devices that combine biomolecules with electronic transducers, converting any physicochemical change in the biomolecule to a detectable electric, optic or chemical signal which is displayed on a biosensor reading device. Some of the biosensor based methods currently used for pathogen detection are surface Plasmon resonance (SPR) sensor, cell based biosensors and evanescent wave or fiber optics based biosensors. Other technologies include microarrays, immuno-magnetic separation and BActeria Rapid Detection using Optical scattering Technology (BARDOT). Apart from these, newer and better methods are continuously been developed which will permit even more sensitive and rapid bacterial detection.

2.1.2.1 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a molecular biology technique used for pathogen identification by DNA amplification. Developed in 1983 by Kary Mullis [71], PCR has become a standard tool for identification of known pathogens and toxins. PCR is based on the principle of thermal cycling and exponential amplification of a DNA template with the help of a DNA polymerase enzyme. A typical PCR requires the following reagents: (i) DNA template to be

amplified, (ii) forward and reverse primers that are complementary to the 3' strands of the target DNA, (iii) Thermostable DNA polymerase enzyme (generally Taq polymerase), (iv) deoxynucleoside triphosphates (dNTPs; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand, (v) buffer solutions, and (vi) monovalent ions (potassium; K^+) and/or divalent cations (usually magnesium; Mg^{++}) for enhanced specificity and polymerase activity.

A multiplex PCR is advancement over the regular PCR as it allows detection of a deletions or duplications in a large gene. First developed and used by J.S. Chamberlain in 1988 [72], it is now a popular method for identifying a pathogen. A multiplex PCR makes use of multiple primers, specific for different genes, in a single PCR reaction. These primers produce amplicons of varying lengths depending on the gene, thereby indicating the presence or absence of these and thus combining multiple PCR reactions in a single run. More recently, this method has been used to detect single nucleotide polymorphisms (SNPs) [73]. This technique saves valuable time and reagents; however optimization of run conditions, in particular, adjustments of the annealing temperature, may cause complications.

Real-time PCR, also known as quantitative real time polymerase chain reaction (Q-PCR/qPCR/qrt-PCR) or kinetic polymerase chain reaction (KPCR) is an evolved version of the traditional PCR as it allows visualization of the increase in the amount of the DNA as it amplifies in real time. It is rapid and robust and is

widely replacing the traditional methods for detection and identification of genes of target bacteria [74].

PCR, multiplex PCR and real time PCR techniques have been widely used to identify a vast range of pathogens and associated toxins, including but not limited to *E. coli stx* gene subtypes [75], *Brucella* spp. [76], rotavirus [77] and other pathogens such as *Salmonella*, *Campylobacter*, STEC O157, *Staphylococcus aureus*, *Clostridium perfringens* and *Bacillus cereus* [78-80] and *Clostridium botulinum* botulinum toxin types A and B [81].

2.1.2.2 Immunoassays

Immunoassays are biochemical tests that are generally performed to determine the presence of a particular substance in a complex mixture using antibodies. The most popular form of an immunoassay is the Enzyme-linked ImmunoSorbent Assay (ELISA). ELISA is based on the principle of high avidity binding between an antigen and its corresponding antibody. The analyte (substance to be detected, for instance a mix containing the test pathogen) is coated on a microtiter plate in an indirect ELISA technique. It is then exposed to the primary antibody, which is specific for the targeted pathogen. The primary antibody is then detected with the help of a secondary antibody (detection antibody) that is tagged with an enzyme. Finally, a substrate specific to the enzyme is added and as it changes color upon reaction, the presence of the pathogen is confirmed. This type of ELISA is termed ‘Indirect ELISA’. Some less common variations, such as Sandwich ELISA and competitive ELISA, also exist,

and while they all involve the same basic principle, alterations may occur in the order of addition of the analyte(s) or antibodies.

Other kinds of immunoassays include latex agglutination assay and microbead agglutination assay. In a latex agglutination assay, latex beads are coated with an antibody and exposed to antigens. If the targeted antigen is present, it will bind to the antibody, causing the latex beads to clump together forming a network. The same idea applies to microbead agglutination test . Specially designed polymer based microbeads (varying in size from 0.5 to 500 microns) can be used as attachment surfaces for antibodies. Once the antigen (toxin) is bound to the antibody, the clumped beads can be easily separated.

Immunoassays are widely popular and have been used to detect a diverse range of toxins including staphylococcal enterotoxins [82, 83], algal brevetoxins (responsible for red tide) [84], beta, epsilon and iota$_b$ toxins from *Clostridium perfringens* [85], botulinum neurotoxin (BoNT) serotypes from *Clostridium botulinum* [86-89] and Stx from *E. coli* O157:H7 [90].

2.1.2.3 Immuno-Polymerase Chain Reaction (iPCR)

Immuno-Polymerase Chain Reaction (immuno-PCR or iPCR) is a novel technique developed in 1992 by T. Sano, C.L. Smith and C.R. Cantor [91, 92]. This method combines the advantages of immunoassays (ELISA) and PCR resulting in an exceedingly sensitive antigen detection tool. The characteristic of this method is the use of a specific DNA molecule as the marker. As opposed to ELISA, which utilizes enzyme-substrate reaction for signal generation, immuno-

PCR uses amplification of a DNA marker. The detection antibody is conjugated with a DNA molecule, which is PCR amplified and the products are analyzed to indicate the presence or absence of antigen. This technique bears the advantage of combining the amplification power of PCR and specificity of ELISA, thereby enabling it to achieve a 100-100,000-fold increase in sensitivity as compared to ELISA. This method is capable of detecting as few as 580 antigen molecules (9.6 x 10^{-22} moles). However, despite its high detection power, immuno-PCR is remarkably underutilized; improvements in developing ready-to-use reagents and faster protocol may increase the usage of this method for routine diagnostics [92]. Immuno-PCR has been used for the detection of *E. coli* Shiga-toxin 2 [93, 94], *Clostridium botulinum* neurotoxin type A [95], staphylococcal enterotoxins [96] and *Bacillus thuringiensis* [97].

2.1.2.4 Immuno-Magnetic Separation

Immono-magnetic separation (IMS) is a rapid and reliable assay for pathogen detection. The method involves paramagnetic beads, usually composed of iron oxide, coated with a biomolecule, such as an antibody, for the capture and detection of a target pathogen. It utilizes the unique characteristic of paramagnetic materials, which exhibit magnetic properties only when exposed to a magnetic field. Thus, once the pathogen is bound to the coated antibody, the bead-antibody-pathogen complex can be easily separated by using a magnetic particle concentrator (MPC). This complex can subsequently be used with any other technique, like PCR, ELISA, plating and other methods, for further analysis.

IMS has been used for the detection of many pathogens like *Listeria monocytogenes* [98], STEC O157 [99], *Yersinia enterocolitica* [100] and *Salmonella* Typhimurium [101]. IMS-PCR has been used to detect *Campylobacter* [102] and *Salmonella* [103] among other pathogenic bacteria. IMS-ELISA has been used for detection of *Staphylococcus aureus* [104].

2.1.2.5 Cell culture based assays

Mammalian cells, tissues or organs serve as excellent model systems for studying functional aspects of pathogens and toxins. Cell culture refers to the growth and maintenance of cells usually derived from eukaryotes. These cells are maintained under specific temperature, humidity and gas mixture (O_2, CO_2, N_2 concentrations) conditions which mimic *in vivo* state. Pathogens and toxins can damage the cells by altering the cell morphology or physiology and eventually lead to apoptosis or necrosis. These changes can be visualized and/or monitored to indentify the presence of a pathogen. For example, loss of fluorescence in a Vero-cell line constitutively producing enhanced green fluorescence protein (EGFP) may be an indicator of the presence of protein-synthesis inhibiting Stx produced by *E. coli* O157:H7 [105]. A similar approach, based on a macrophage culture system method was used to detect endotoxins [106]. Neuroblastoma cell cultures have been used for the detection of ciguatoxins, brevetoxins, saxitoxins, and seafood extracts [107].

2.1.2.6 Mammalian cell based biosensors

A cell based biosensor (CBB) refers to a system in which bacteria or prokaryotic or eukaryotic cells detect a specific physiological phenomena and act as transducers for generating signal, which in turn is converted to an optical or electric signal by a secondary transducer [8, 108]. The physiological phenomena detected can be related to cellular metabolism, impedance, intracellular or extracellular potentials, or a receptor-ligand type interaction between the cellular receptors and the analyte. Novel cell lines can be developed to sense a particular change, for example a B-lymphocyte based sensor for detection of bacteria and virus within seconds [109]. CBBs have also been used for detection of *Listeria monocytogenes* [110] and toxins [111].

More recently, three dimensional cell culture systems have also been developed for pathogen and toxin detection. Cell lines are cultured on collagen or alginate based biocompatible matrices and are then exposed to appropriate analyte [112]. This technique has been used for *Listeria* and *Bacillus* detection [113].

2.1.2.7 Microarrays

The principle behind microarrays is the hybridization of a target cDNA or RNA to the corresponding probe on the microarray chip. Microarrays require minimal amounts of probes (pico-molar in quantity) to hybridize with and detect the target gene. The probe-target hybridization is then visualized by fluorophore-, silver- or chemiluminiscence- based signals. This high throughput method is

sensitive enough to accurately detect up to nano-grams of DNA. Microarrays have been used for a range of applications, most notably, for profiling gene expression, identifying single nucleotide polymorphisms (SNPs), alternative splicing and comparative genome hybridization.

More recently, DNA microarrays have been applied extensively for detection of foodborne pathogens [17, 114-117]. Specific examples are detection of STEC O157 [118], ETEC [119], *Yersinia pestis* [120] and *Toxoplasma gondii* [121]

2.1.2.8 Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance (SPR) sensor is an established method for measuring protein-protein interactions [122]. The implementation of SPR and other biosensors for detection purposes has increased considerably over the last few years and continues to surge [123-126].

SPR is a label free technique for highly specific detection of an analyte. This device measures the change in resonance frequency of photons prior to and after the binding of the analyte (pathogen or toxin) to the surface of the sensor, which is coated with a biomolecule exhibiting affinity towards the analyte. The sensor surface is usually gold and the immobilized biomolecule is an antibody or a receptor for the target analyte [127].

An SPR biosensor is rapid and involves easy sample preparation, requiring fewer reagents and a simple protocol, with minimal risk of

contamination. It can be used for high-throughput screening of a large number of samples.

SPR has been used for the detection of pathogens and toxins such as STEC O157 [128], *Yersinia enterocolitica*, *Salmonella enterica* [129], *Listeria monocytogenes* [125, 127], *Vibrio cholerae* [130], botulinum neurotoxin A [131], *E. coli* heat labile enterotoxin [132] and various other endotoxins [133].

2.1.2.9 Fiber optics

Optical fiber-based biosensors have evolved rapidly over the last decade. This biosensor is based on the principle of total internal reflection (TIR). A probe, usually a biomolecule like an enzyme, an antibody or a DNA oligo reacts with the target analyte to generate a signal that is captured by the fiber optic device, which serves as the signal transducer [134, 135], and displayed on a screen. This technique is non-destructive, specific, and sensitive. Fiber optic biosensors have been used for the detection of *Clostridium botulinum* toxin A [136], *Salmonella* [21, 137], *Yersinia pestis* [138], STEC O157 [139, 140], staphylococcal enterotoxin B [141], *Salmonella* (Valadez et al 2009) and *Listeria monocytogenes* [142, 143].

2.1.2.10 BARDOT

BActerial Rapid Detection using Optical light-scattering Technology (BARDOT) is truly a label free, rapid detection method for food borne pathogens [41]. A laser beam at 635 nm is scattered by a bacterial colony to generate a signature scatter pattern which is captured by a CCD (charged coupled device).

The scatter pattern obtained is characterized by a multitude of features which are quantified and analyzed by specific algorithms and thereafter classified as distinct bacterial patterns [144].

This is a highly sensitive method that is capable of detecting species level differentiations for the tested pathogens: *Listeria*, *Staphylococcus*, *Salmonella*, *Vibrio*, and *Escherichia* [40] and even at the serovar level. This sensor has been used for the identification of the pathogenic *Vibrio* spp. from oysters [145] and *Listeria* spp. [38].

2.1.2.11 Other new and developing techniques

New technologies for pathogen detection are continuously being developed that are more rapid and sensitive than the existing methods. Some of the technologies are: flourogenic DNAzymes based pathogen detection [146]; Raman scattering based detection and enumeration of *E. coli* [147]; peptide nucleic acid (PNA) based pathogen detection [148]; capillary electrophoresis (CE) and capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) based approach [149]; “sloppy” molecular beacon melting temperature signature technique for high throughput analysis [150]; and finally, a loop mediated isothermal amplification (LAMP)-based detection system [151].

2.2 Pathogenic *E. coli*

Escherichia coli are Gram negative, rod shaped bacteria belonging to the *Enterobacteriaceae* family. While most *E. coli* are harmless and are a part of a healthy gut microbiome of warm blooded animals, some are pathogenic and

cause gastrointestinal diseases ranging from mild diarrhea to severe hemorrhagic colitis and hemolytic uremic syndrome (HUS), septicemia, pneumonia and urinary tract infection (UTI). Over the last few decades, widespread foodborne outbreaks due to enterohaemorrhagic *E. coli* (EHEC) have raised concerns regarding food safety. Sources of *E. coli* outbreak vary from meat products such as ground beef to fresh produce like sprouts, lettuce and spinach.

Pathogenic *E. coli* strains have a been serotyped (O:H:K typed) based on the three main surface antigens- lipopolysaccharide (LPS) based O antigen, flagellar H antigen and capsular K antigen [152]. As K antigens are difficult to type, O and H antigens are most commonly used to distinguish *E. coli* strains [152]. The O antigen determines the serogroup, whereas the K antigen identifies the serotype. Strains under the same O serogroup may have multiple H sub-types. Overall, there are 174 O antigens (O1-181, with the omission of 31, 47, 67, 72, 93, 94 and 122), 53 H antigens (H1-53) and about 80 K antigens. Overall, more than 200 *E. coli* serotypes have been identified (CDC).

Based on the surface antigens and virulence factors possessed, Kaper et al [153] have classified the diarrheagenic *E. coli* into 6 main pathotypes: (i) Enterotoxigenic *E. coli* (ETEC), (ii) Enteropathogenic *E. coli* (EPEC), (iii) Enteroaggregative *E. coli* (EAEC), (iv) Enteroinvasive *E. coli* (EIEC), (v) Diffusely adhering *E. coli* (DAEC) and (vi) Enterohaemorrhagic *E. coli* (EHEC). Table 2-2 lists the important serovars of each pathotype and certain key virulent factors.

Table 2-3 Important serovars of *E. coli* pathotypes and key virulent factors

Pathotype	Serovars	Virulent factors	Mechanism	Reference
ETEC	O6, O8, O11, O15, O20, O25, O27, O78, O128, O148, O149, O159, O173	Heat labile toxin (LT) or Heat stable toxin (ST) and colonization factors (CFs)	Adheres to host cells and produces toxins	[154] (Isidean, Riddle et al. 2011) [155]
EPEC	O18, O20, O55, O86, O111, O119, O125, O126, O127, O128, O142,O158	Intimin (eae), Bundle forming pili (bfpA), and various secreted proteins (espA/B/C/D/F)	Attaches to and invades host cells, but does not produce any toxins	[156] Robins-Browne and Hartland 2002, Humphries and Armstrong 2010, [157-159]
EAEC	O3, O7, O15, O77, O86, O111, O126, O127	Adherence fimbrae for attachment, cytotoxins and enterotoxins like plasmid-encoded toxin (Pet) and enteroaggregative ST (EAST)	Adheres to cells, forming a brick like pattern, and produces toxins; does not invade.	[155] [160, 161]
EIEC	O28, O29, O112, O124, O136, O143, O144, O152, O159, O164, O167	IpaA, IpaB, IpaC and IpgD, are secreted by the T3SS	Adheres and invades host cells but without the production of toxin	[155] [153]
DAEC		Fimbrial adhesion protein, F1845; afimbrial adhesins (Afa) and the locus of enterocyte effacement (LEE)	Diffused adherence pattern	[153] [162]
EHEC	O4, O5, O16, O26, O55, O84, O104, O111ab, O113, O117, O145, O157, O172, O176, O177, O178, O180, O181	Shiga-like toxins (*stx*s) or the verocytotoxin, intimin (eaeA), translocated intimin receptor (espE) and other effector proteins (espA/B/C/D/F/G) that are secreted by the T3SS	Binds intimately with the host cells, causing effacement of the brush border villi, and also releases toxins	[155] [153] [163]

Besides these major classes, other pathogenic *E. coli* have also been classified based on the diseases they are implicated in. For example, UTI (urinary tract infection) causing extraintestinal *E. coli* are termed uropathogenic *E. coli* (UPEC); the pathotype that causes meningitis is called meningitis-associated *E. coli* (MNEC); pathotypes implicated in extraintestinal diseases are classified as ExPEC; and finally, *E. coli* strains that are found in and cause diseases in animals (specially poultry), but not humans, are called avian pathogenic *E. coli* (APEC) [153].

Recalls and Outbreaks

Pathogenic *E. coli* have been responsible for multiple foodborne outbreaks over the last few decades. While STEC O157 is the main cause of HUS cases in the US, non-STEC O157 strains have also been implicated in outbreaks. The CDC Food Outbreak Online Database (FOOD) provides a comprehensive list of all the foodborne outbreaks over the last 12 years (1998-2010). It reveals that EPEC, ETEC, EAEC and EHEC have all induced outbreaks, but EHEC heavily dominates the list with most reported cases. A table of all *E. coli* outbreaks in the US over the last 5 years (2007-2012) is given below (Table 2-4). Most of the outbreaks resulted in recalls of thousands of pounds of contaminated food, costing millions of dollars (CDC).

Table 2-4 *E. coli* associated outbreaks from 2007-2012 (CDC/FOOD) in US.

Year	Pathogen	Source	Cases	Hospitalizations	Deaths
2007	O157:H7	Frozen pizza	21	8	0
2007	O157:H7	Ground beef patties	40	21	0
2008	O157:H7	Beef	49	27	0
2009	O157:H7	Cookie dough	72	34	0
2009	O157:H7	Beef	23	12	0
2009	O157:H7	Beef	26	19	2
2010	O157:H7	Beef	21	9	0
2010	O145	Romaine lettuce	30	12	0
2010	O157:H7	Cheese	38	15	0
2011	O157:H7	In-shell hazelnuts	8	4	0
2011	O157:H7	Lebanon bologna	14	3	0
2011*	O104:H4	Sprouted foods	6	Not available	1
2011	O157:H7	Romaine lettuce	58	23	0
2012	O26	Raw clover sprouts	29	7	0
2012	O145	Not identified	18	4	1

* This outbreak was associated with individuals travelling to Germany. In 2011, Germany suffered one of the largest foodborne STEC O104:H4 outbreaks, which caused about 4,000 illnesses and 53 deaths.

2.2.1 STEC/VTEC/EHEC

Shigatoxigenic *E. coli* (STEC), also called Enterohaemorrhagic *E. coli* (EHEC) and Vero-toxigenic *E. coli* (VTEC), were discovered 35 years ago [164] and cause microvascular endothelial damage leading to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [165-170]. Severe HUS cases can also face the elevated risks of diabetes mellitus due to reduced insulin [171]. STEC infections can also manifest failure of the central nervous system [172] and other neurologic symptoms such as hyperreflexia, attenuated cognitive abilities and impaired consciousness [173].

The key virulence factors in STEC are the Stxs. Stx toxins are family of structurally and functionally related toxins, secreted by the *Shigella dysenteriae* serotype I and Stx1 and Stx2 by STEC [174, 175]. STEC produces several variants of Stx1 (Stx1 and Stx1c) and Stx2 (Stx2, Stx2c, Stx2d, Stx2e, Stx2f), either alone or in multiple combinations [174]. The Stx binds specifically to the glycosphingolipid globotriaosylceramide (Gb3) receptor on the host cells [176]. This toxin is in a heterohexamer (AB_5) configuration consisting of one 32 kDa A subunit and five 7.7 kDa B subunits [177, 178]. The B subunits bind to the Gb3 receptor while the A subunit forms the catalytic and enzymatic domain [179]. Once the B subunit binds to its receptor, the A subunit undergoes endocytosis within the cell and gets activated. It is transported to the endoplasmic reticulum (ER) [180] where it blocks mRNA translation, thereby preventing protein synthesis and inducing ribotoxic stress which, in turn, trigger apoptosis [181-183].

2.2.2 Infection mechanism

The pathogenesis of STEC involves four major steps: (i) loose or non-intimate attachment of bacteria to the host cells, (ii) intimate attachment mediated by intimin and translocated intimin receptor (TIR), (iii) formation of attachment and effacement lesion (A/E lesion) which triggers cytoskeletal rearrangement leading to loss of microvilli structure and function and finally, and (iv) cell apoptosis.

Most proteins participating in the above steps are encoded by the locus of enterocyte effacement (LEE). LEE is a 35 kb pathogenicity island in EPEC,

EHEC and EAEC which contains a total of 33 virulence genes and two virulence regulators and T3SS [184]. These genes are organized in five operons (Figure 2-5) named *LEE1* through *LEE 4* and *TIR* [185, 186].

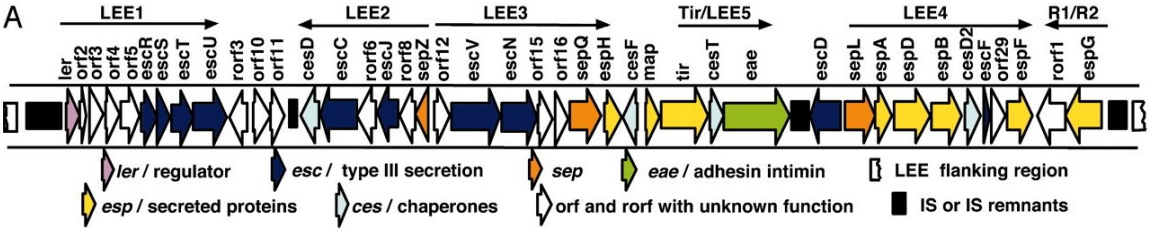

Figure 2-5 Organization of LEE operon

Important virulence factors include a regulator (Ler), intimin (Eae) and TIR (EspE) for intimate bacterial adhesion, chaperones such as CesT, Sep and *esc* genes that encode the T3SS [187], translocators like EspA, EspB, and EspD and effector proteins like EspG, EspF, Map, and EspH [184, 188, 189]. Non LEE encoded effector proteins include Esp I-O, EspR-T, Esp V-Y, Nle B-K, Cif, Tccp and Ipe [189].

2.2.2.1 TIR-intimin mediated A/E lesion formation

An attachment/effacement (A/E) lesion is characterized by destruction (effacement) of the brush border villi followed by intimate attachment of the bacterium to the host cells and cytoskeletal rearrangements for the formation of a pedestal like structure [190-192]. For EPEC, LEE encodes all the genes that are necessary and sufficient for formation of the A/E lesion [193, 194], however, for EHEC, LEE is necessary but not entirely sufficient [195].

The initial non-intimate attachment of the bacterium with the host cells is also mediated by the long polar fimbriae (lpf1 and lpf2) and the EspA protein secreted by the T3SS. Lpf1 (5.9kb) and Lpf2 (6.8 kb), are unique to STEC and help in intestinal colonization [51]. EspA forms large filamentous extracellular structures which form a bridge between the bacterium and the surface of host cell and are imperative for intimate attachment and for translocation of other effector proteins (EspB and TIR) into the host cell [192, 196]. Besides Lpf and EspA, STEC possesses many other fimbriae and pili for assistance in bacterial adherence: Type 4 pili (T4P), *E. coli* common pilus (ECP), F9 fimbriae, *E. coli* YcbQ laminin-binding fimbriae and sorbitol fermenting fimbriae (sfp) [51]. STEC also contains several auto-transporters: enterohemorrhagic *E. coli* autotransporters (EhaA, EhaB, EhaJ), EspP rope-like fibers, Sab and Cah autotransporters; and other adhesions like flagella, immunoglobulin binding protein G and *E. coli* factor for adherence 1(Efa1/ToxB/LifA) protein [51].

Intimin is a 94 kDa EPEC and EHEC adhesion protein, encoded by the *eae* gene located in the LEE, which is critical for virulence, for mediating intimate bacterial attachment and for triggering downstream events for actin pedestal formation [193, 197-201]. There are about 18 intimin types and 9 subtypes identified by performing a heteroduplex mobility assay of *eae* gene positive *E. coli s*trains and are denominated as: α, α2, β1 to -3, γ1, γ2, δ, ε, ε2 to 4, ζ, η, η2, θ, ι, ι2, κ, λ, μ, ν, ξ, ο, π, ρ, and σ [202].

Intimin binds to the translocated intimin receptor (TIR), a 78 kDa protein, produced in *E. coli* and translocated to the host cell membrane by the T3SS [203,

204]. EPEC and EHEC intimin-TIR are not functionally interchangeable and exhibit significant differences such as absence of tyrosine phosphorylation of TIR in EHEC and vice-versa in EPEC [205] and in pathways adopted for actin polymerization [206]. It has also been shown that while EPEC and EHEC intimins are interchangeable, there are significant differences between the binding affinity of EHEC TIR [205]. Intimin has also been shown to bind directly to uninfected host cells via β-integrins [207].

Intimin-TIR interaction has been studied extensively. The C-terminal region of intimin (Int190) has been shown to be the TIR-binding region [208]. Similar structural and biochemical analysis of TIR protein indicates that the C-terminal and N-terminal domains of TIR (called C-TIR and N-TIR) are membrane associated whereas the 55 amino acid long middle extracellular region (M-TIR) contains the intimin binding domain (TIR-IBD) [209-211]. The crystal structure of TIR-intimin complex was analyzed by Luo et al [212] (Figure 2-6) and they determined that the intimin-TIR-IBD binding affinity constant (K_a) was $3.2x10^6$ M^{-1} at 37°C, and it was similar to the binding constant of the full length TIR with intimin. More specifically, the binding occurred between the lectin-like D3 domain of intimin and β-hairpin and N-terminal of the helix HB of TIR-IBD [212].

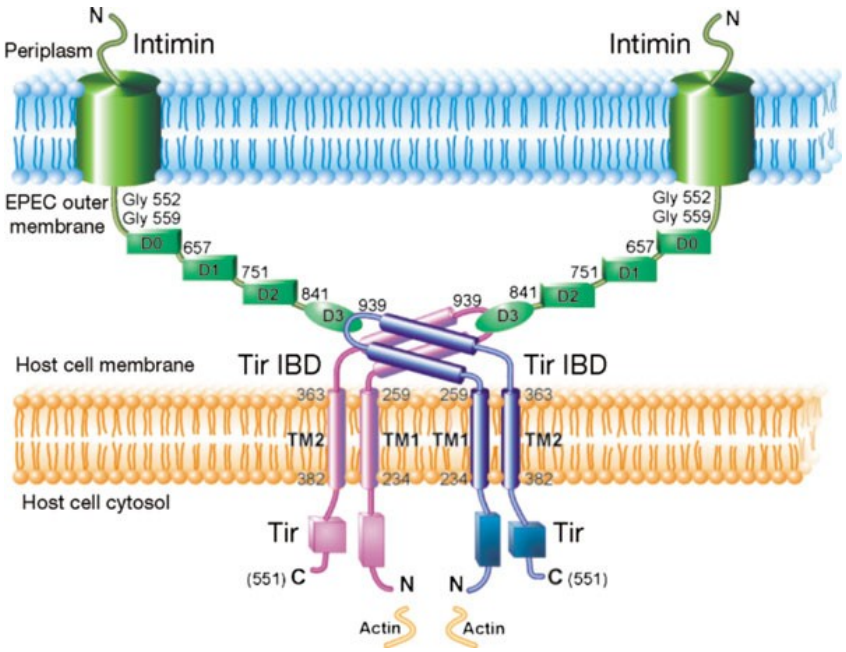

Figure 2-6 TIR-Intimin bindin [212]

EPEC TIR undergoes phosphorylation at a tyrosine residue (Y_{474}) by host cell tyrosine kinase [213, 214]. It then recruits host cell adapter protein, Nck, which in turn activates the neural Wiskott-Aldrich syndrome protein (N-WASP) via both WIP/WH1 and Nck/PRD interactions to initiate actin pedestal formation [189]. EHEC TIR, however, does not contain this tyrosine residue, and therefore uses an alternative Asn-Pro-Tyr ($N_{456}P_{457}Y_{458}$) motif for phosphorylation and recruitment of downstream proteins [215, 216]. It secures another translocated effector protein, $EspF_U$ or the TIR cytoskeleton coupling protein (TccP) which triggers actin polymerization in an Nck independent pathway [217, 218].

2.2.2.2 TIR-CesT

CesT, or chaperone for *E. coli* secreted TIR, is encoded by a 15 kDa locus (previously known as OrfU), located between the *TIR* and *eae* (intmin) genes on

the LEE [185, 219]. CesT is a cytoplasmic protein which has been shown to be essential for stable TIR production [219]. CesT also plays an important role in guiding TIR to the T3SS for secretion by interacting with a specific T3SS ATPase, EscN [220]. The CesT binding domain (CBD) of TIR is on the N-terminal of the protein and is distinct from its IBD [219, 221]. Therefore, CesT binding with TIR does not interfere with TIR-intimin interaction. Apart from TIR, CesT also assists in production and secretion of other T3SS proteins, most notably, Map [222] and NleA [223].

This robust infection mechanism renders STEC a considerable threat. The first STEC related HUS case was reported in 1982 [224]. From 1982 to 2002, there were a total of 350 STEC outbreaks in 49 states [225]. While food, usually beef, is the most common cause of infection, other known sources of transmission are person-to-person, laboratory associated, waterborne bacteria and animal contact. Today, CDC estimates that STEC associated food borne outbreaks cause over 265,000 illnesses in the US every year, leading to more than 3,600 hospitalizations and approximately 30 deaths [1].

Evidently, there is a pressing need for development of sensors that can effectively detect STEC and other pathogenic *E. coli*. A novel approach for this could be utilization of the TIR-intimin interaction. TIR, immobilized on a biosensor platform, would have the ability to bind specifically with its receptor, intimin. This high-affinity, intimate, ligand-receptor association would enable highly specific STEC capture and detection on multiple bacterial detection platforms, such as ELISA, SPR and evanescent wave based biosensor.

2.3 *Listeria monocytogenes*

Listeriosis, caused by the foodborne bacteria *Listeria monocytogenes*, is a potentially fatal disease which mainly affects a select population group comprising of the young, old, pregnant and immunocompromised individuals [226]. The *Listeria* genus consists of nine species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri, L. grayi* [226] and recently identified *L. marthii* [227], *L. rocourtiae* [228] and *L. weihenstephanensis* [229]; of which, *L. monocytogenes* and *L. ivanovii* are considered pathogenic [230]. While the total number of incidences of listeriosis are relatively few, infecting only about 0.1-11/1,000,000 individuals across the world [231], it has one of the highest mortality rate, ranging between 20-30% of all cases, among the known foodborne pathogens [232]. It is one the leading causes of stillbirth in women and infant encephalitis in newborns [233, 234]. The CDC affirms that *Listeria* is primarily propagated by contaminated ready-to-eat (RTE) foods such as uncooked or improperly prepared meats and vegetables, dairy products such as soft cheeses and unpasteurized milk, smoked seafood, and more recently, fruits such as cantaloupes (Source: CDC).

Critical virulence factors in *Listeria* include hemolysin (*hly*) gene which encodes for listeriolysin O (LLO); a range of flagella and adhesins like Listeria adhesion protein (*lap*), internalin A and B (*inl*A and *inlB*) and fibronectin binding protein (*fbp);* and actin polymerization protein A (*actA*). Many of these virulence genes are regulated by a transcriptional regulator- positive regulatory factor A (*prfA*) [226].

Recalls and outbreaks

The U.S. records approximately 1,600 *L. monocytogenes* cases every year (CDC). Between 1998 and 2003, the rate of *Listeria* infections dropped dramatically by about 38% (CDC). This drop can be attributed to increasing awareness about the infection and the necessity of cooking foods completely prior to eating. Despite precautionary measures, there have been 29 listeriosis outbreaks in the U.S. since 1998. (CDC-FOOD). One of the largest *Listeria* outbreaks occurred in 2002 due to consumption of contaminated deli turkey meat, which led to 54 illnesses, 8 deaths, and 3 fetal deaths. More recently, in 2011, a multi-state listeriosis outbreak due to consumption of contaminated cantaloupes resulted in 146 infections, 33 deaths and one miscarriage [235]; and in 2012, a ricotta cheese associated outbreak caused 20 illnesses culminating in 4 deaths [CDC]. Table 5 lists some of the recent *Listeria* outbreaks (Source: CDC, FOOD [Foodborne Outbreak Online Database]). These outbreaks, though rare, beget grave economic implications in terms of health and food recall related costs, which together may exceed 4 billion USD annually [236]. Many countries have hence established a ‘zero tolerance policy’ towards *L. monocytogenes* in RTE food [237]. There is, therefore, a compelling need to develop and establish reliable and accurate detection methods which may help mitigate these incidences.

Table 2-5 *L. monocytogenes* outbreaks in the last 5 years (2007-2012) (CDC)

Year	Source	Illnesses	Hospitalizations	Deaths
2007	Milk	5	5	3
2008	Tuna salad	5	5	3
2008	Cheese	8	4	0
2008	Sprouts	10	16	0
2009	Not identified	6	1	0
2009	Mexican style cheese	2	2	0
2009	Mexican style cheese	8	3	0
2010	Not identified	4	4	0
2010	Not identified	10	10	5
2010	Queso fresco	4	4	0
2010	Meats	8	7	2
2010	Mexican style cheese	5	5	1
2010	Mexican style cheese	6	4	1
2010	Sushi	2	1	0
2011	Cantaloupes	147	Not available	33
2012	Ricotta salata cheese	20	19	4

2.3.1 Infection mechanism

The pathogenesis of *Listeria* is complicated. Listeriosis can be manifested either as perinatal listeriosis or as adult listeriosis. Perinatal listeriosis, which represents about 17% of all *Listeria* infections (CDC), results in abortion, still birth or birth of an infected fetus. While it is not as fatal (10-20% mortality rate), it causes hydrocephalus or psychomotor retardation in the event of an early onset of neonatal listeriosis [238]. On the other hand, adult listeriosis, mostly in non pregnant immuno-compromised adults, affects the central nervous system (CNS) leading to septicemia, meningitis, encephalitis and ataxia among other

complications [226]. In fact, *L. monocytogenes* is the most common cause of bacterial meningitis in cancer-recovering patients [239].

Listeria pathogenesis comprises of two phases: the intestinal phase, which involves adherence and colonization of the bacteria on the host cells, invasion, intracellular replication and translocation to the mucosal barrier for systemic circulation; and the systemic phase, in which the bacteria is disseminated to various organs, like liver, spleen, lymph nodes, brain and placenta (in pregnant women).

L.monocytogenes uses the M-cells as the primary entry site into the host intestinal cells [240, 241]. To assist colonization, *L. monocytogenes* has several adhesion molecules, such as Lap which binds to Hsp60 on host cell surface [242], InlA which binds to E-cadherin [243] and InlB which binds to Met [244], gC1q-r [245] and other proteoglycan receptors [246]. Other adhesion proteins include a surface protein, autolysin amidase (Ami), a fibronectin binding protein (Fbp) and p60 [226].

Following colonization, *L. monocytogenes* invades the cell by getting engulfed inside a phagocyte [247]. The bacteria survive within the phagocyte by preventing its maturation into a phagolysozome [248]. It lyses the phagosome with a hemolytic toxin, listeriolysin O (LLO) (encoded by the *hly* gene), a key virulence factor [249, 250]. This toxin is a sulfhydryl (SH)-activated multifunctional protein, which is active at low pH (optimum at 5.5) and disrupts the phagosome membrane. In the absence of LLO, a phospholipase (PC-PLC or phosphatidyl choline PLC) can lyse the phagocytic vacuole [251]. Nevertheless,

LLO is indispensable for *Listeria* pathogenesis as it also has several other functions like activation of the nuclear factor κβ (NF-κβ) pathway [226, 252], activation of Raf-Mek-mitogen activated protein (MAP) kinase pathway [253, 254], calcium signaling for regulation of internalization [255], cytokine expression [256] and induction of dendritic cell apoptosis [257].

Once inside the cell, the bacterium multiplies rapidly, with a doubling time of 1 hour [258]. It utilizes host glucose as a carbon source by expressing hexose phosphate translocase (Hpt) for scavenging various sugar-phosphate salts within the cell [251].

The intracellular spread of *L. monocytogenes* is mediated by an actin polymerization protein (ActA), which assists in bacterial movement inside the cytoplasm [259-262]. The N-terminus of ActA interacts with the Arp2/3 complex and initiates actin polymerization; the central domain binds with the vasodilator-stimulated phosphoprotein (VASP) and Mena to help in directional actin assembly and finally, the C-terminal anchors itself to the bacterial cell wall [226]. All these lead to the formation of an actin-tail, in which actin is continuously deposited to create a support structure of actin monomers behind the cell, thereby providing the propulsion force that enables bacterial movement [226]. The moving bacteria develop a protruding structure, which is recognized and engulfed by the neighboring host cell, thus facilitating cell-to-cell transfer.

In the systemic phase of the infection, *Listeria* crosses the intestinal barrier, and is carried to the lymph nodes, spleen and liver by the lymph or blood. Liver (hepatocytes) is the main site for *Listeria* multiplication [226]. The invasion

across intestinal epithelial barrier may occur via an intracellular or paracellular route. In the intracellular route, *Listeria* invades cells with the help of invasins internalin A and B (InlA and InlB) [263]. InlA binds with receptor E-cadherin [263] while InlB accelerates bacterial internalization by activating its receptor, c-Met [264]. This triggers an intense immune response, mediated by NF-κβ activation, gamma interferon (IFN-γ) activated macrophages, interleukin 12 (IL-12) and tumor necrosis factor alpha (TNF-α) produced by the natural killer (NK) cells [226]. If *Listeria* proliferation is not checked in the liver, it may lead to liver abscess, septicemia and passage of the bacteria to the uterus and the CNS. Alternatively, in the paracellular route, *Listeria* undergoes transepithelial translocation with the help of Listeria Adhesion Protein (LAP), a bifunctional acetaldehyde-alcohol dehydrogenase homologue [265-268]. LAP uses Heat-shock protein 60 (Hsp60) as a receptor [110] and mediates bacterial translocation by loosening intestinal tight junction [269, 270].

Most of the virulence genes of *Listeria* are encoded and regulated by a 9 kb pathogenicity island (LIPI) [271]. This gene cluster encodes: *prfA*, *plcA*, *hylA, mpl*, *actA* and *plcB*. These genes are regulated by the positive regulatory factor A (*prfA*) gene [272, 273]. Apart from these 9 genes, PrfA also influences the expression of 145 different proteins within the bacteria [272]. It comes as no surprise, therefore, that PrfA itself is tightly regulated by stringent combination of temperature, pH, osmolarity, stress (σ^B), iron concentration and presence of fermentable sugars [274] and its own feedback loop [271].

2.3.2 *Listeria* surface proteins

L. monocytogenes exhibits a plethora of virulence proteins [55, 271], many of which can and have been targeted for nucleic acid or antibody based selective capture and detection [275]. The ability of *L. monocytogenes* to survive in a diverse range of environmental conditions, such as pH (4.3-9.6), temperature (1-45°C), salt concentrations and water activity (Aw to 0.93) [276], varying from food matrices to human and animal eukaryotic cells, can in part be attributed to its complex surface proteome. This has subsequently resulted in various attempts at the analysis of *Listeria* cell wall subproteome and at identification of the various factors contributing to bacterial survival under adverse conditions [277-282]. The analysis of *L. monocytogenes* genome (strain EGDe) revealed a total of nearly 3000 proteins, of which 133 were predicted to be associated with the cell wall [278]. This list includes a variety of proteins which perform diverse functions such as adhesion and invasion associated proteins, InlA and InlB [283] and the *Listeria* adhesion protein (LAP) [270], actin assembly inducing protein ActA [284], heat-shock protein DnaK and glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase [281]. Several of these surface proteins have also been targeted for *Listeria* detection and examples include the LAP [24, 285] and InlA [286].

These surface associated proteins can be broadly classified based either on the type of their anchoring mechanism or their function on the cell wall and relevance in pathogenesis. Under the first classification, there are four categories: (a) proteins covalently linked to the peptidoglycan, mainly the sortase substrates possessing the LPXTG motif or the NXXTX sorting signal, for instance, InlA; (b)

proteins non-covalently linked to the cell wall, containing the GW module, WxL domain, LysM domain or the peptidase peptidoglycan binding domain (example InlB); (c) membrane bound proteins, such as the lipoproteins and proteins with hydrophobic tails (example ActA); and finally (d) nonconventional secreted surface proteins (glycolytic enzymes, heat shock proteins and chaperones and some proteins involved in detoxification, nucleic acid transcription and translation and metabolism) [278, 279]. Most proteins belonging to the last category lack the conventional anchoring motifs or signaling peptides [281].

2.3.3 SecA2 based *Listeria* surface protein transport

The mechanisms by which so many proteins are transported to the surface of the bacteria have generated considerable interest. Currently, for Gram-positive bacteria there are six established protein secretion systems: (i) the Sec pathway (Secretion); (ii) the Tat pathway (Twin-arginine translocation); (iii) the FEA (Flagella Export Apparatus); (iv) the FPE (Fimbrilin-Protein Exporter); (v) the holins (hole forming); and (vi) the Wss (WXG100 secretion system) [287, 288]. SecA2, an ATPase and a SecA paralogous protein, has also been identified in certain Gram-positive bacteria, including *Listeria* spp.[289-291]. Similar to SecA, SecA2 also mediates protein transport across the cell membrane by utilizing structural changes induced by the ATP hydrolysis; however, unlike SecA, SecA2 is not vital to cell viability, and functions only to further facilitate protein translocation and increase the overall transport efficiency

[287, 292]. Some of the virulence proteins secreted by the SecA2 pathway include FbpA, lipoprotein LpeA and LAP [287, 293].

SecA2 was identified as an accessory protein to SecA [289] in all *Listeria* species but *L. rocourtiae* [291]. Similar to SecA, SecA2 also couples ATP hydrolysis based conformational changes with stepwise translocation of proteins to and across the cell wall [289, 292, 294, 295]. Since the deletion of this transport protein does not affect cell viability, it is evident that SecA2 mainly serves the purpose of improving the overall transport efficiency of the SecA system, thereby contributing to cellular virulence [292, 296]. Key *Listeria* proteins transported by the SecA2 system is summarized in Table 2-6.

Table 2-6 *Listeria* proteins transported by the SecA2 system

Protein Name	**Reference**
Fimbrial adhesins	[297]
Platelet binding protein	[298]
Invasion associated protein/Cell wall hydrolase A (Iap/p60/CwhA)	[289, 299]
N-acetylmuramidase A (NamA/MurA)	[299, 300]
Fibronectin binding protein A (FbpA)	[301]
Superoxide dismutase A (SodA)	[296]
Listeria adhesion protein* (LAP)	[270, 287, 293]
6-Phosphofructokinase* (PFK)	[302]
Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH)	[302]
Thioredoxin*	[302]
30S ribosomal protein S1*	[302]
Pyridoxamine 5′-phosphate oxidase*	[302]

* Cytoplasmic proteins, lacking conventional surface transport motifs.

The proteins indicated by an *asterisk* (*), such as LAP, PFK and GAPDH do not possess the conventional signal sequences. Further investigation is required to understand why and how these proteins are targeted for export and

the exact mechanism pertaining to this transport. The purpose of exhibiting these proteins on the surface also needs to be addressed.

2.3.4 Pyruvate kinase

Pyruvate kinase [EC: 2.7.1.40] is an essential glycolytic enzyme, which catalyzes the rate limiting step of conversion of phosphoenol pyruvate (PEP) to pyruvate with the production of ATP (Figure 2-7). The sequence of pyruvate kinase is highly conserved and does not display any alterations among bacterial species. While uncommon, it is not unusual to find pyruvate kinase enzyme on the surface of bacteria. *Streptococcus pyogenes* [303, 304], *Streptococcus suis* serotype 9 [305], *Streptococcus iniae* [306] *Clostridium deficille* [307] and certain lactic acid bacteria such as *Lactococcus lactis* IL1403 [308] all exhibit this enzyme on the surface. The tertiary structure of this protein consists of homo-tetramers of identical subunits which contribute to the allosteric regulation of the enzyme [309]. The exact reason behind the presence of this enzyme on the bacterial surface remains to be identified; however, studies pertaining to a two-component regulatory system in *Streptococcus iniae*, Siv S/R, indicates possible involvement of the surface displayed pyruvate kinase in malate metabolism [306]. In *L. lactis*, pyruvate kinase present on the bacterial surface has been found to bind with the yeast mannin [308, 310].

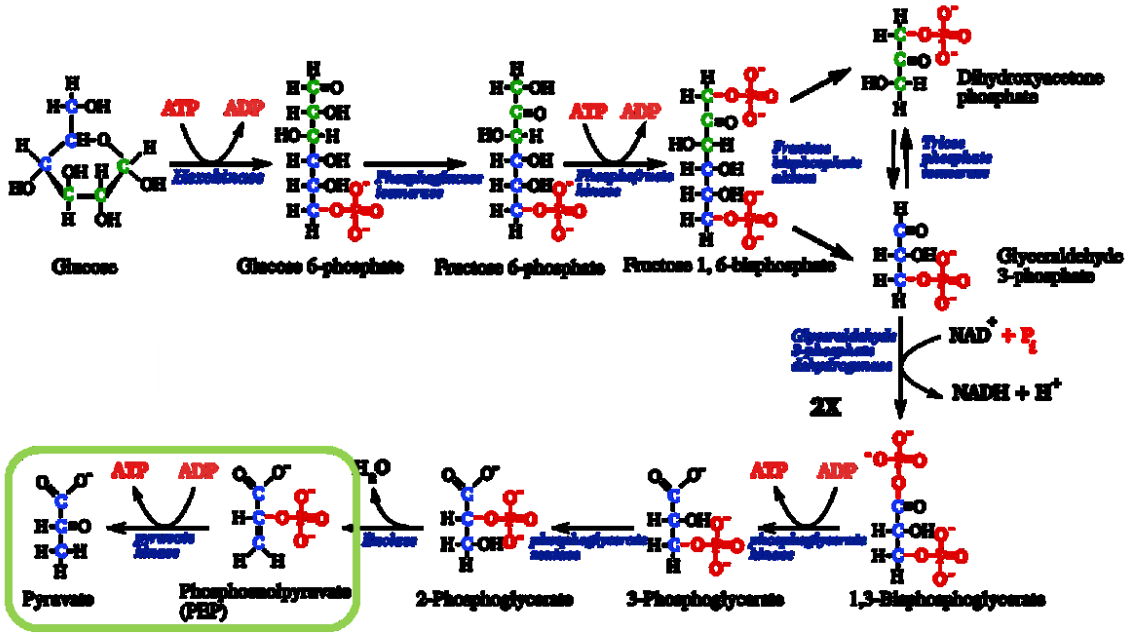

Figure 2-7 Overview of glycolysis pathway

2.3.5 Immunologic techniques for *Listeria* detection

Immunologic techniques are widely used for pathogen detection [311]. While several anti-*Listeria* antibodies are commercially available, many suffer from issues of non-specificity, low affinity towards the antigen as well as cross reactivity and poor detection limits [312]. Environmental variations, such as alterations in temperature and humidity, may induce physiological stress conditions which, in turn, adversely impact the antigen expression and thereby negatively impact the detection ability of the antibody [313, 314]. Antibody affinity is of particular importance, so that the bacteria are still bound to the antibody allowing detection and retention for further downstream experiments [315]. Certain antibodies are incompatible with the different bioassay platforms, which gravely limit their application [7, 275]. Some antibodies are highly specific

towards one specific species of *Listeria* spp. only, and/or within a species, lack the ability to react with the various serotypes, which once again makes those antibodies unsuitable for true application [316, 317]. There is, therefore, a pressing and ongoing need to develop new antibodies that are specific, exhibit high affinity towards the targeted antigen, and are also capable of broad-spectrum application for *Listeria* spp. detection.

CHAPTER 3. PYRUVATE KINASE, A SECA2-DEPENDENT SURFACE ASSOCIATED PROTEIN, IN *LISTERIA* SPECIES

3.1 Introduction

Listeria monocytogenes is an opportunistic foodborne pathogen causing systemic listeriosis in individuals with immunosuppressed conditions such as the elderly, infants, pregnant mothers and malignant cancer. A complex array of proteins helps this bacterium to maintain saprophytic life style when present in the environment and food and an intracellular life style in host [318].

All living organisms contain housekeeping enzymes which are expressed constitutively to perform vital life functions. Glycolytic enzymes, protein transport enzymes and tricarboxylic acid (TCA) cycle enzymes are all housekeeping enzymes. Over the last two decades, the concept of moonlighting proteins, which are capable of performing multiple functions, has become well established [310, 319-321]. The identification several housekeeping enzymes as moonlighting proteins added an novel twist to the conventional outlook towards the housekeeping proteins as ordinary, conserved, run-of-the-mill enzymes [322]. Several bacterial glycolytic enzymes such as phosphoglucose isomerase (PGI), adolase, hexokinase (HK), phosphofructokinase (PFK), triose phosphate isomerase (TPI), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), enolase (ENO) and pyruvate kinase (PK) have

been shown to moonlight [310]. In *Listeria monocytogenes*, four proteins are currently known to perform multiple functions including enhancement of bacterial virulence. These proteins are: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Internallin B (InlB), alcohol acetaldehyde dehydrogenase (AAD) or Listeria adhesion protein (LAP) and IspC [321]. Of these, GAPDH and LAP are housekeeping enzymes. *Listeria* GAPDH (*lmo2459*) has been shown to regulate a small GTPase Rab5a which controls the pahogosome and lysosomal fusion [323]. This enzyme also exhibits similarity with *Pseudomonas aeruginosa* ExoS toxin, thus suggesting further involvement in virulence and bacteria-host interaction [324]. LAP (*lmo1634*) was identified as an essential glycolytic enzyme, alcohol acetaldehyde dehydrogenase, which binds with the host Heat-shock protein 60 (Hsp60), and acts as a key bacterial adhesin [110, 267, 325]. Further analysis showed that LAP is present on the surface of only the pathogenic *Listeria* spp. and that the protein secretion and translocation from the cytoplasm to the bacterial membrane is mediated by the SecA2 transporter system.

SecA2 was identified as an accessory protein to SecA [289] in all *Listeria* species but *L. rocourtiae* [291]. Similar to SecA, SecA2 also couples ATP hydrolysis based conformational changes with stepwise translocation of proteins to and across the cell wall [289, 292, 294, 295]. Since the deletion of this transport protein does not affect cell viability, it is evident that SecA2 mainly serves the purpose of improving the overall transport efficiency of the SecA system, thereby contributing to cellular virulence [292, 296]. A recent study investigated the influence of absence of the SecA2 transporter on the expression

of *L. monocytogenes* exoproteins and identified 20 proteins, including 6 primarily cytoplasmic proteins, whose expression was modified [302]. Table 3-1 provides a list of certain key proteins transported by the SecA2 system, including the cytoplasmic proteins (indicated by a *) mentioned above.

Table 3-1 *Listeria* proteins transported by the SecA2 system

#	**Protein Name**	**Reference**
1	Fimbrial adhesins	[297]
2	Platelet binding protein	[298]
3	Invasion associated protein/Cell wall hydrolase A (Iap/p60/CwhA)	[289][299]
4	N-acetylmuramidase A (NamA/MurA)	[300][299]
5	Fibronectin binding protein A (FbpA)	[301]
6	Superoxide dismutase A (SodA)	[296]
7	Listeria adhesion protein* (LAP)	[270, 287, 293]
8	6-Phosphofructokinase* (PFK)	[302]
9	Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH)	[302]
10	Thioredoxin*	[302]
11	30S ribosomal protein S1*	[302]
12	Pyridoxamine 5′-phosphate oxidase*	[302]
13	Sporulation stage V, protein G*	[302]

PyK (EC: 2.7.1.40) is an essential glycoytic enzyme which is normally present in the cytoplasm and catalyzes the rate limiting step of glycolysis, the conversion of phospho-enol pyruvate (PEP) to pyruvate with the production of ATP. Here we investigated if PyK, present in all *Listeria* species, is translocated by SecA2 system in both pathogenic and nonpathogenic *Listeria* and determine its potential application in immunologic detection of these bacteria using a monoclonal antibody, EM-7H10. MAb EM-7H10 (immunoglobulin subclass IgG1) has been shown previously to react with all *Listeria* spp, except *L. roquortiae*

[326]. In this study we display the potential of this MAb to be used on multiple platforms for *Listeria* spp. detection.

3.2 Materials and methods

3.2.1 Bacterial cultures, plasmids and primers

All cultures, plasmids, and primers used in this study are listed in Table 3-2. All cultures were grown at 37°C, except *L. rocourtiae* which was grown at 25°C. All cultures were grown in aerobic conditions in the Brain Heart Infusion (BHI) broth, with the exception of the *Lactobacillus* spp. which were grown in de Man, Rogosa and Sharpe (MRS) medium under anaerobic conditions. Recombinant *E. coli* BL21 expressing PyK from *L. monocytogenes* F4244 was grown in presence of ampicillin (Am^R 50 µg/mL) and *L. monocytogenes* F4244 SecA2 complemented strain (*secA2*$^+$) was grown in the presence of erythromycin (Em^R 10 µg/mL).

Table 3-2 List of bacterial cultures, plasmids and primers used

Bacteria	**Strains**	**Description**	**Source**
Listeria monocytogenes	F4244	Wild type, serotype 4b	Our collection
L. monocytogenes	ΔSecA2	F4244, SecA2 deficient strain	Our collection
L. monocytogenes	SecA2+	F4244, SecA2 complemented strain (Em^R 10 µg/mL)	Our collection
L. innocua	F4248	Wild Type	Our collection
L. grayi	ATCC19120	Wild Type	Our collection
L. ivanovii	KC1714	Wild Type	Our collection
L.welshimeri	ATCC35877	Wild Type	Our collection
L. seeligeri	LA15	Wild Type	Our collection
L. marthii	BAA1595	Wild Type	Our collection

Table 3-2 continued

L. rocourtiae	CIP109804	Wild Type	Our collection
E. coli O157:H7	EDL933	Wild Type	Our collection
E. coli	ATCC 51739	Wild Type	Our collection
Salmonella Enteritidis	PT21	Wild Type	Our collection
Staphylococcus aureus	ATCC25923	Wild Type	Our collection
Staphylococcus epidermidis		Wild Type	Our collection
Pseudomonas aeruginosa		Wild Type	Our collection
Streptococcus mutans	ATCC25175	Wild Type	Our collection
Lactobacillus plantarum	NCDO955	Wild Type	Our collection
Lactobacillus acidophilus	NRRL31910	Wild Type	Our collection
Enterococcus faecalis	CG110	Wild Type	Our collection
Bacillus cereus	UW85	Wild Type	Our collection
Bacillus subtilis	P3-79	Wild Type	Our collection
E. coli BL21	PyK (AKB 701)	*E. coli* BL21 expressing PyK from *L.monocytogenes* (Am^R 50 µg/mL)	This study
Plasmids			
pGEM-T easy		Cloning vector (Am^R 50 µg/mL)	Promega
pET 32(a)		Expression vector (Am^R 50 µg/mL)	Promega
pET 32(a)-PyK		pET 32(a) carrying *PyK* (Am^R 50 µg/mL)	This study
Primers			
PyK (1758 bp)	Forward (NotI)	5'GCGGCCGCATGAAAAAA ACGAAAATT3'	This study
	Reverse (XhoI)	5'CTCGAGATGTGTTGCTG TTTTTGC 3'	This study
PyK-qPCR (86bp)	Forward	GCGCTGAAGCAAGTGACG TA	This study
	Reverse	TCACCGGACAACATAATT GCA	This study

3.2.2 Purification of anti-PyK antibody, EM-7H10

Frozen-stored hybridoma cell line EM-7H10 [326] was grown in Dulbecco's modified Eagles medium (DMEM) (Sigma Chemical Co., St Louis, MO, USA) with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) in a cultivation chamber of CELLine 1000 (Integra Biosciences, East Dundee, IL, USA) at 37°C in a humidified incubator with 7% CO_2. The medium was aspirated at 7-day intervals, centrifuged (300×***g*** for 20 min) and partially purified by ammonium sulphate precipitation [327]. A Protein G column (ActaPrime, Pharmacia-Amersham, Uppsala, Sweden) was used for affinity purification of antibodies and the final concentration was adjusted to 0.26 mg/ml [328].

3.2.3 Identification of PyK in *Listeria* by MALDI-TOF MS/MS

Bacterial cell lysate was prepared from *Listeria* cultures ($A_{595\ nm}$ ~ 1.2) that received heat treatment (95°C for 10 min), followed by sonication on ice for 5–7 cycles of 15 sec each using a Sonifier 150D (Branson, Niantic, CT). The sample was centrifuged and the SN was collected and stored at −20°C. The proteins were first separated by SDS-PAGE (7.5% acrylamide gel) and then transferred to Immobilon P membranes (Millipore). Membranes were blocked using 5% non-fat dry milk and 0.05% Tween-20 at room temperature (RT) for 1 h, washed with 20 mM phosphate buffered saline (pH 7.0) containing 0.5% Tween 20 (PBST) for 15 min at RT. Membranes were then reacted with purified EM-7H10 (250 ng/ml) for 1 h at RT and subsequently washed with PBST for 15 min before adding the

secondary antibody, HRP-conjugated anti-mouse antibody (1:4000 dilution; Jackson Immunologicals). Membranes were finally developed using Pierce enhanced chemiluminescence substrate (Thermo Scientific) on X-ray film. The antigenic protein to which EM-7H10 binds was determined by the western blot experiment. The corresponding band was cut and sent for identification and sequencing by MALDI/TOF and MALDI/TOF-TOF (Applied Biomics, Inc.).

3.2.4 Surface localization of PyK

3.2.4.1 ELISA

Bacterial cell pellets from freshly grown bacterial cultures ($A_{595\ nm}$ ~ 1.2) was resuspended in equal volume of 0.05 M sodium carbonate coating buffer, pH 9.6, immobilized in 96-well Immulon 4HBX plates (Thermo Scientific, Waltham, MA), and stored at 4°C for 48 h. Following bacterial immobilization, the plate wells were sequentially reacted with EM-7H10 (250ng/ml) and anti-mouse HRP-conjugated antibody (1:4000 dilution; Jackson Immunologicals). For all steps, plates were held at RT for 1 h and washed 3 times with PBST between steps. Finally 100 µL of a fluorescent substrate, either Super Red (10-acetyl-3,7-dihydroxyphenoxazine; Virolabs, Chantilly, VA; Ex: 540 nm, Em: 600 nm) or Quanta Blu (Ex: 320 nm, Em: 460 nm), was added to each well and fluorescence was measured using a Spectramax fluorescent reader (Gemini, Sunnyvale, CA) every 15 min for 1 h. To determine nonspecific protein binding, control reactions without bacteria and EM-7H10 were included. Fluorescent readings obtained from these controls were subtracted from the test results to obtain true values.

3.2.4.2 Immunofluorescence staining

Freshly grown (18 h) bacterial cultures were first reacted with EM-7H10-MAb (2 µg) in phosphate-buffered saline [PBS]) for 1 h followed by FITC-labeled anti-mouse monovalent secondary Fab fragment (diluted 1:50 in PBS; Jackson Immuno Research) for 1 h. Cells were washed between antibody treatments with PBS containing 1% bovine serum albumen. After the final wash, cells were examined under a fluorescence microscope (Leica, model DFC 310 FX, Wetzlar, Germany) equipped with Leica Application Suite (LAS) software (version 4.2).

3.2.5 PyK cloning and expression

Full length *pyk* (585 amino acids; 1758 bp) from *Listeria monocytogenes* serotype 4b strain F4244 was amplified by PCR using the following primers: LmPyK-F 5'-GCGGCCGCATGAAAAAAACGAAAATT-3' and LmPyK-R 5'-CTCGAGATGTGTTGCTGTTTTTGC -3' with restriction sites *Not*I and *Xho*I, respectively. The gene was first cloned into the cloning vector pGEM-T Easy (Promega) and from that into a *Not*I and *Xho*I digested pET32 (a) expression vector (Novagen). The transformants in both cases were verified by gene sequencing at the Purdue University Genomics Facility. The protein was expressed in *E. coli* BL21 (DE3) expression cells (Novagen) in presence of Ampicillin (50 µg/ml). Recombinant PyK (rPyK) was purified by immobilized Metal Affinity Chromatography (IMAC) using a Nickel column (Thermo Fisher Scientific) and further confirmed by MALDI-TOF/TOF (Applied Biomics, Inc). Protein expression in recombinant strains was subsequently confirmed by Western blot

analysis using MAb EM-7H10 and an anti-His monoclonal antibody (Pierce Antibodies, Thermo Fisher Scientific).

3.2.6 Analysis of enzyme activity of rPyK

Enzymatic activity of rPyK was determined by using the PyK assay kit (Biomedical Research Service Center, University at Buffalo, State University of New York; http://www.bmrservice.com/PyruvateKinaseAssay.html; CAT #: E-117). This assay determines catalytic activity of the enzyme by measuring the difference in the UV absorption spectra between the oxidized and reduced forms of NAD+/NADH at 340 nm; and has a detection limit of ~10 μM Pyruvate. All the steps were performed according to the manufacturers instruction. Enzymatic activity of the rPyK was determined by correlating the ATP production with the corresponding amount of the enzyme units.

E. coli BL21 (DE3), *L. monocytogenes* F4244, *B. cereus* WT and recombinant *E. coli* BL21 (DE3) strain harboring pET-32(a)-PyK were grown in LB broth at 37 °C in a shaker incubator to mid-exponential phase (OD_{600} 0.5) and IPTG (1mM) was added to induce over-expression of PyK. Cell pellets were harvested [266] and 0.3 mg of the crude protein preparation was tested for PyK activity. 1 PyK unit is defined as the amount of enzyme needed to catalyze the generation of 100μM ATP.

3.2.7 Analysis of PyK localization in SecA2 mutants

To investigate the role of SecA2 transport protein on the surface expression and secretion of PyK, *L. monocytogenes* F4244, *ΔsecA2* and *secA2*$^{+}$

strains, were tested for differences in PyK expression in the various cell protein fractions: intracellular (IC), cell wall (CW) and secreted or supernatant (SN) protein fractions. SN was collected from centrifuged culture (7,000×g for 10 min at 4°C) and the pellet was retained for preparation of CW and intracellular proteins. The SN was filtered (0.22-μm filter), precipitated with 10% trichloroacetic acid for 40 min on ice, and centrifuged (14,000×g at 4°C for 10 min). The pellet was resuspended in ice-cold acetone and centrifuged. The remaining acetone was evaporated, and the pellet was resuspended in alkaline rehydration buffer (100 mM Tris-base, 3% SDS, 3 mM dithiothreitol, pH 11), boiled for 5 min, and stored at −20°C.

For the CW protein fraction, the pellet was resuspended in 1 M Tris, pH 7.5, and incubated for 1 h in ice. The suspension was centrifuged (13,000×g at 4°C for 5 min) and the SN was filtered (0.45-μm filter) and stored at −20°C.

The pellet from the CW protein preparation was used for IC protein isolation. It was resuspended in the sample solvent (5% SDS, 0.5% β-mercaptoethanol, 1.5% Tris, pH 7.0) and sonicated on ice for 5–7 cycles of 15 sec each using a Sonifier 150D (Branson, Niantic, CT). The samples were centrifuged and the SN fractions were collected and stored at −20°C. SN and CW protein preparations were also tested with a PepC assay [281] to rule out contamination with intracellular or membrane proteins.

Proteins were quantified using the bicinchoninic acid method (Pierce, Rockford, IL) and equivalent amounts of protein (20 μg of each fraction) were separated using SDS polyacrylamide gel electrophoresis (7.5% acrylamide) gel.

The proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, MA) and immunoprobed with anti-LAP antibody MAb-EM10 (1.0 µg/mL) and horseradish peroxidase-coupled anti-mouse antibody (0.2 µg/mL; Jackson Immuno Research, West Grove, PA). The membranes were developed with an enhanced chemiluminescence kit (Pierce).

3.2.8 Reverse transcriptase PCR (RT-PCR) for *pyk*

To determine the regulatory role of SecA2 on PyK transcription, we performed an RT- PCR reaction for the *pyk* in *L. monocytogenes* F4244, *ΔsecA2* and *secA2*$^{+}$ strains. mRNA was extracted by following the manufacturer's instruction provided with the Qiagen RNeasy Mini Kit (# 74104). RNA was then reverse transcribed into cDNA using the BioRad iScript cDNA Synthesis Kit (# 170-8890) and the resulting cDNA was used as a template for subsequent PCR amplification using Applied Biosciences SYBR® Green PCR Master Mix (# 4309155) and the following primers: PyK-qPCR-F 5'-GCGCTGAAGCAAGTGACGTA-3' and PyK-qPCR-R 5'-TCACCGGACAACATAATTGCA-3' and 16sLF 5'-AGCTTGCTCTTCCAAAGT-3' and 16sLR 5'-AAGCAGTTACTCTTATCCT-3'. Amplification was obtained at 60°C for *pyk* and 54°C for the housekeeping control *16s* gene.

To analyze results, the percentage difference in the ratio of threshold values (C_T) for *pyk* to *16s* was calculated for each strain to assess the relative amounts of *pyk* transcript. Similarly, percentage relative change in *pyk*

expression in the three strains was also observed with *pyk* levels in *L. monocytogenes* F4244 serving as the base line.

3.2.9 Effect of growth media on PyK expression

To observe the differential expression of PyK in the cell wall when *Listeria* is grown in the various growth media, *L. monocytogenes* F4244 was grown in 100 ml volume of six different media: nutrient broths-Luria Bertani (LB), Tryptic Soy Broth (TSB) and Brain Heart Infusion (BHI) and Listeria selective enrichment broths-University of Vermont Media (UVM), Frasier Broth (FB) and Buffered Listeria Enrichment Broth (BLEB) for 18 h. Since the bacteria grow- more slowly in selective media, the amount of bacterial culture used in the downstream experiments was normalized by measuring the absorbance of cells at 595nm ($A_{595\ nm}$ ~ 0.6). The cultures were centrifuged (7,000×g for 10 min at 4°C) and the pellets were resuspended in 1 M Tris, pH 7.5, and incubated for 1 h in ice. The suspension was centrifuged (13,000×g at 4°C for 5 min) and the SN were filtered (0.45-μm filter) and stored at −20°C. This cell wall protein fraction was used for Western blot and ELISA experiments to assess the expression level of PyK in different media.

3.2.10 Fiber optics biosensor

Polystyrene waveguides (fibers) were cleaned and coated with 100 μg/mL of streptavidin (NeutrAvidin; Pierce) for 2 h at 4°C as described previously [329]. Fibers were blocked with SuperBlock blocking buffer (Pierce) for 1 h and incubated overnight at 4°C with each of the biotinylated EM-7H10 (200 μg/mL).

The fibers were rinsed gently with PBST and then reacted with biotinylated-BSA (100 µg/mL; Pierce) for 1 h at RT to block unbound streptavidin sites. Subsequently, the fibers were coated with biotinylated MAbs as detailed above and placed in reaction chambers containing 100 µL of freshly harvested bacterial suspensions at various concentrations (10^2 to 10^8 CFU/mL) and incubated for 2 h at RT. Following gentle washing with PBS, the fibers were exposed to Cy5-labeled anti-p66 antibody for 2 h at 4°C, washed with PBST, and signals were acquired with an Analyte 2000 Fluorometer (Research 23 International Co., Monroe, WA). The fluorescence intensity signals were recorded for each fiber for 30 s [143]. For each treatment, 2 waveguides were used.

3.2.11 Adhesion assay

The adhesion profiles of bacteria (10^6 cfu/well) to Caco-2 cells (10^5 cells/well) with multiplicity of exposure (MOE) of 10:1 were analyzed using adhesion assays [267]. Adhered *Listeria* was enumerated on BHI and MOX agar plates. To verify PyK mediated binding, bacterial cells were also pretreated for 1 hour with anti-PyK, EM-7H10 monoclonal antibody, before use in the adhesion experiment; as a control, LAP and anti-LAP EM10 antibody was used [293].

3.2.12 Statistical analysis

All experiments were repeated at least three times independently, and each set of experiments was performed in duplicate or triplicate. Statistical comparisons were carried out using analysis of variance (SAS 9.2, Cary, NC)

and Tukey's multiple comparisons of means at $P<0.05$ to determine significant differences.

3.3 Results

3.3.1 MALDI-TOF MS/MS revealed MAb EM-7H10 reactive protein to be PyK

MAb EM-7H10 (subclass IgG1) [326] was tested in Western blot and data showed that the antibody reacted strongly with a 60 kDa protein present in all the *Listeria* species, except *L. rocourtiae* (Figure 3-1a). This 60-kDa band was cut from the SDS gel and analyzed by a MALDI/TOF-TOF analysis (Applied Biomics, Inc.) (Figure 3-1b). Sequencing revealed that the band was a glycolytic enzyme, Pyruvate kinase (gi: 46881070). The target sequence was then cloned and expressed in *E. coli* BL21 (DE3) cells. rPyK was further verified by sequencing, MALDI/TOF-TOF analysis and subsequent reaction with MAb EM-7H10 in Western blot assay (Figure 3-1c).

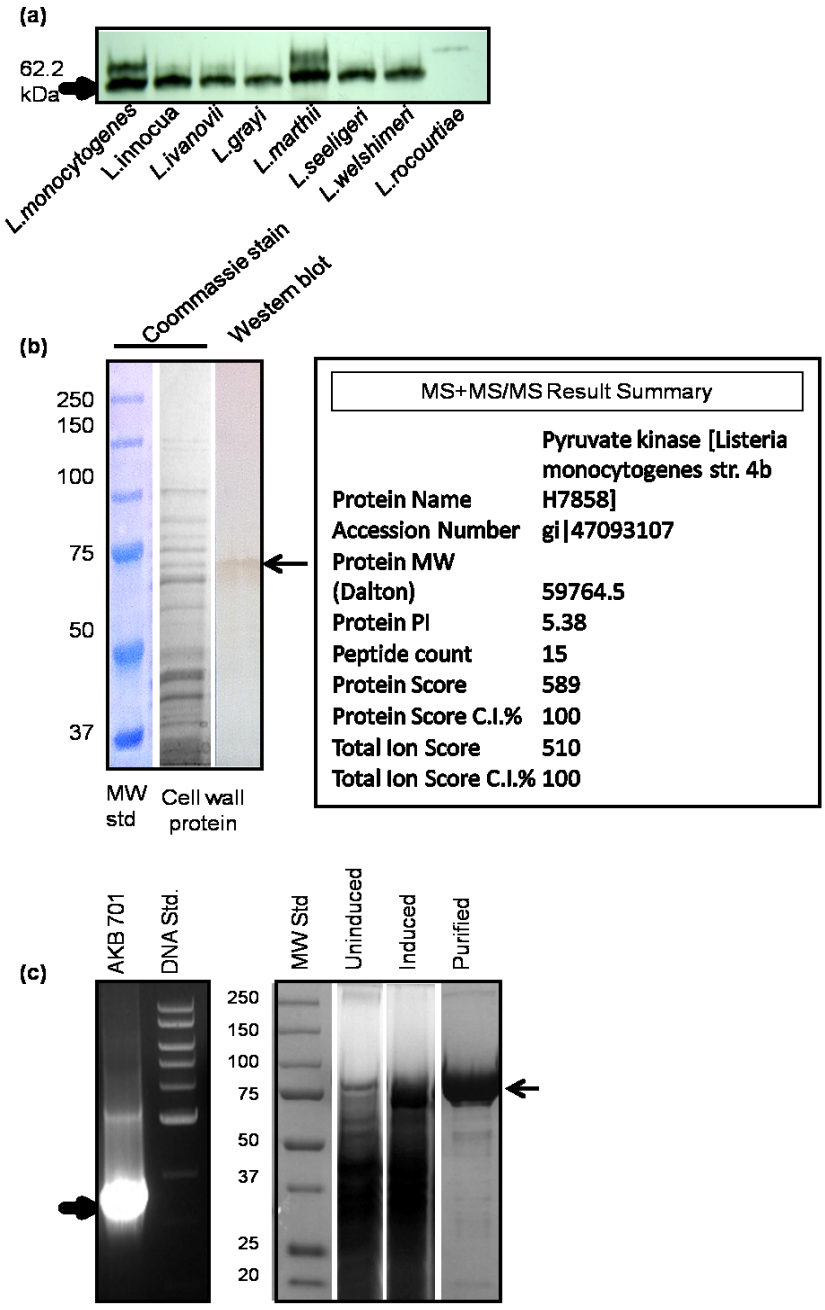

Figure 3-1 (a) Western blot reaction profile of MAb EM7-H10 with *Listeria* spp. (b) Identification of MAb-H7 reactive protein band on SDS-PAGE (7.5% acrylamide) for MALDI-TOF-MS/MS analysis. The 60 kDa band (arrow) was excised from Coommassie stained gel and sent for MALDI analysis. (c). *pyk* was cloned into pGEM T Easy cloning vector in *E. coli* DH10B cells and then into expression vector, pET-32a for production of rPyKin *E. coli* BL21. PyK expression was induced in *E. coli* BL21 cells by growing cells in presence of IPTG (1 mM) and purified by Ni-affinity column. The purified protein showed strong reaction with MAb EM-7H10.

3.3.2 PyK is located on the cell surface of all *Listeria* species

Surface expression of PyK was verified by performing whole cell ELISA experiment and Western blot experiment with the various cellular protein fractions (cell supernatant, cell wall and intracellular fractions) and further demonstrated by immunofluorescence.

3.3.2.1 ELISA

MAb EM-7H10 gave high fluorescence values (~10,000 RFU) when tested against live, whole cell *Listeria* spp., and did not give any cross reaction with other Gram positive or Gram negative bacteria (Figure 3-2a). An ELISA based titration for the MAb showed that the 50 ng of the antibody was sufficient to detect 10^7cfu/ml of the bacteria (Figure 3-2b). Reaction was observed for all *Listeria* spp., however, the intensity was relatively higher in *L. monocytogenes* and *L. marthii* and almost negligible in *L. rocourtiae*.

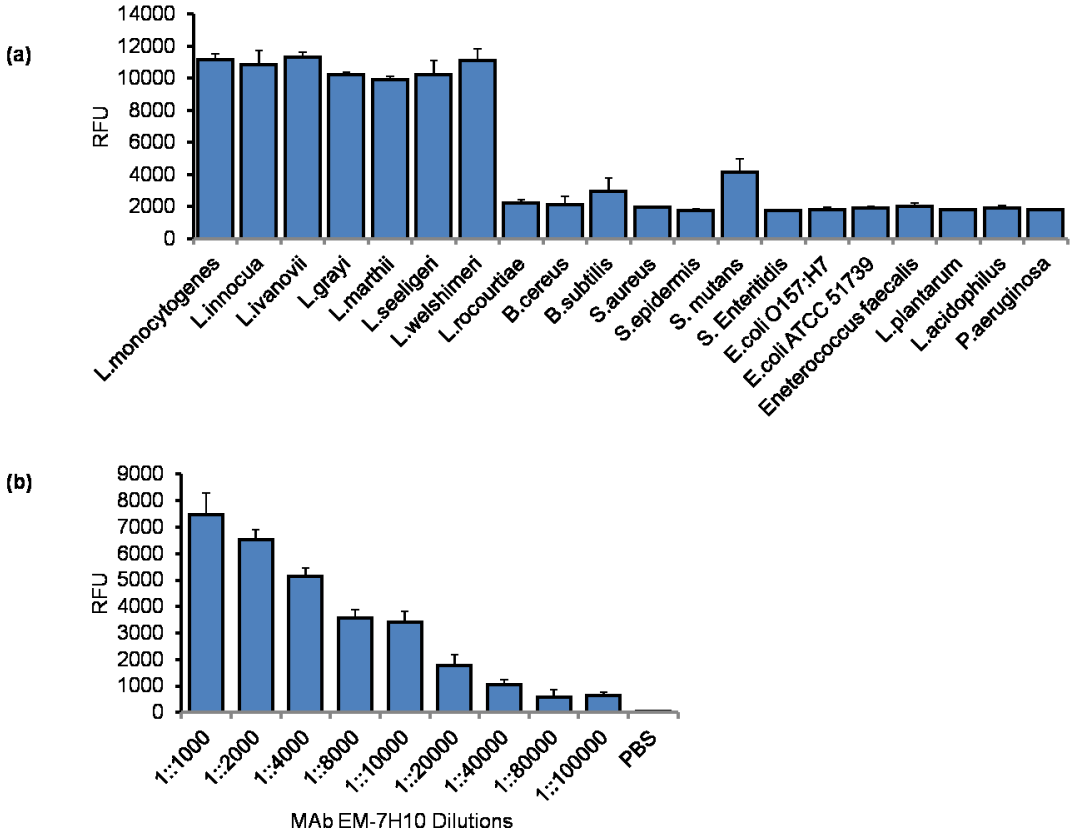

Figure 3-2 Reaction profile of MAb EM-7H10 with various *Listeria* and non-*Listeria* species in (a) ELISA. In ELISA, bacterial cells were adjusted to 10^7 cells/ well (b) Determination of MAb EM-7H10 titer by ELISA using *L. monocytogenes* F4244 cells as (10^7cfu/well) as antigen. In ELISA, data are average of three experiments analyzed in quadruplicate.

3.3.2.2 Immunoflourescence microscopy

Immunofluorescence microscopy confirmed the presence of PyK on the bacterial surface and the specificity of MAb EM-7H10 as it bound with *L. monocytogenes* but not with *B. cereus* and *ΔsecA2* strains (Figure 3-3).

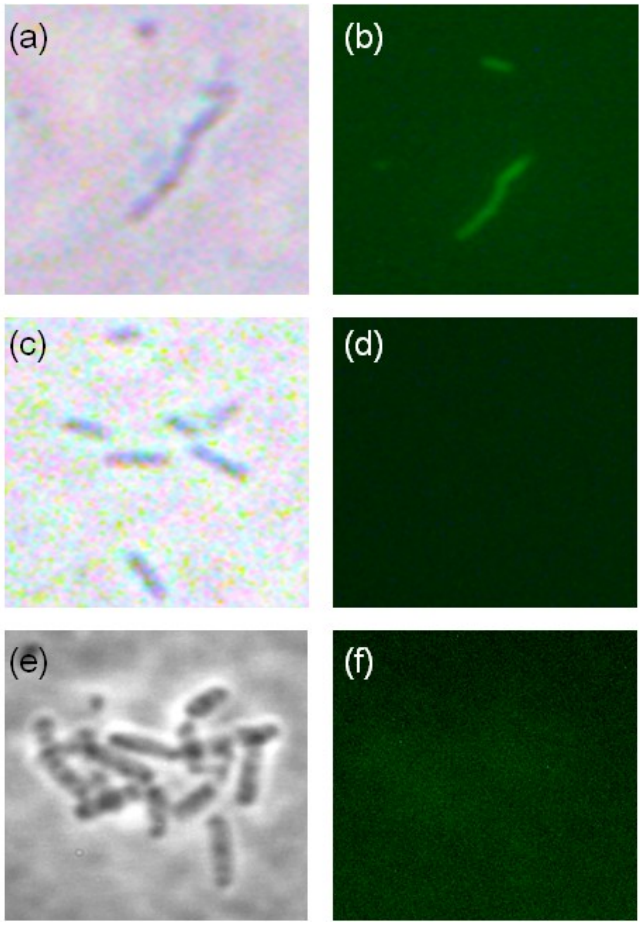

Figure 3-3 Immunofluorescence analysis for the presence of PyK on bacterial surface using anti-PyK MAb EM7-H10. *L.monocytogenes* WT in (a) bright field and (b) FITC exhibited flourescence. *L.mono* SecA2 mutant (c) and (d) did not shown any flourescence, thereby indicating absence of PyK from bacterial surface. *B.cereus* (e) and (f) also did not show any flourescence. All images are taken under 1000X magnification.

3.3.3 PyK translocation to cell wall is SecA2 dependent

To investigate the role of the SecA2 transporter protein on PyK surface expression and secretion, we tested difference in PyK expression in *L. monocytogenes* F4244, Δ*secA2* and *secA2*$^{+}$ strains. An ELISA experiment with whole bacterial cells showed that PyK level was significantly reduced in the Δ*secA2* strain but restored to WT levels in the *secA2*$^{+}$ strain (Figure 3-4a).

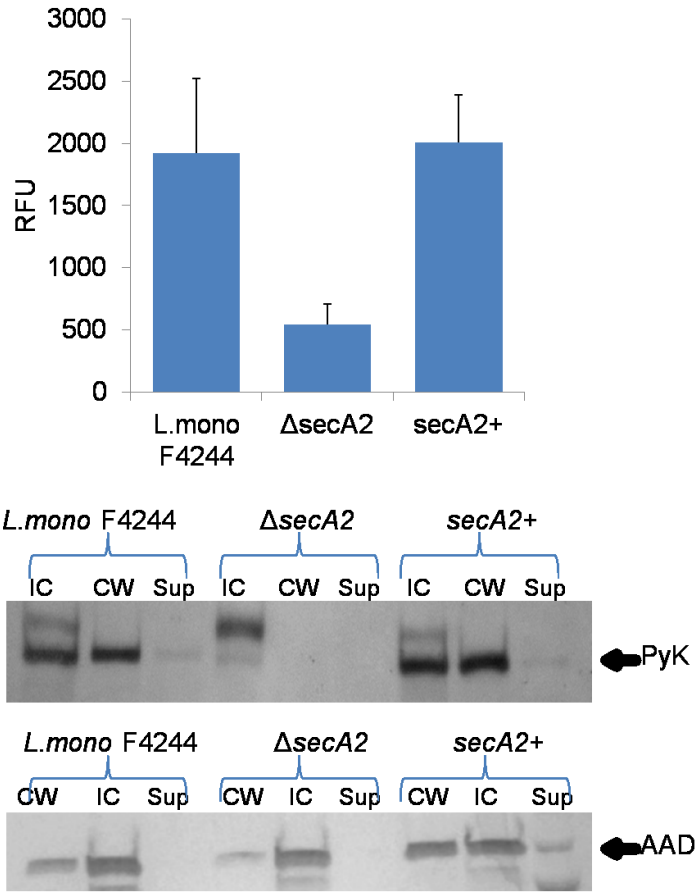

Figure 3-4 Analysis of pyruvate kinase by SecA2 pathway. Effect of SecA2 mutation and complementation on pyruvate kinase surface expression and secretion as shown in the reaction profiles of the MAb EM-7H10 with *L.monocytogenes* whole cells in (a) ELISA and with the intracellular, cell wall and supernatant protein fractions in (b) Western blot. In ELISA, cells were adjusted to 10^8 cfu/ml before sensitizing the wells and in Western blot, bacterial cells were adjusted to an OD_{600} = 1.2 (~10^9 cells/ml) prior to the cell wall protein extraction. In ELISA, data are average of three experiments analyzed in triplicate.

Western blot analysis also exhibited a similar pattern: PyK levels were reduced in all the three protein fractions of the Δ*secA2* strain, with almost none detected in the CW and SN; however, the protein levels were restored to WT amounts in the *secA2*$^{+}$ strain (Figure 3-4b). These results show that SecA2 protein is essential for PyK transport to the bacterial surface and the extracellular mileu. Reduction in the intracellular levels of PyK in the Δ*secA2* strain may also indicate a regulatory influence of SecA2, similar to its effect on MurA expression, which is also transported by SecA2 [299].

3.3.4 Pyk transcript reduced in Δ*secA2* strain

An RT-PCR was performed to determined the influence of SecA2 on *pyk* transcription. Preliminary PCR confirmed the presence of *pyk* in *L. monocytogenes* F4244, Δ*secA2* and *secA2*$^{+}$ (Figure 3-5a). 16S rRNA was used as internal positive control. The percentage ratio difference of the internal positive control to *pyk* clearly shows similar transcript levels in F4244 (91.46%) and *secA2*$^{+}$ (95.41%) with relatively decreased levels in Δ*secA2* (51.18%) (Figure 3-5b). Comparing *pyk* transcript expression between the three strains exhibits significantly elevated amounts in F4244 (79% increase, $p<0.01$) and *secA2*$^{+}$ (86% increase, $p<0.01$) as compared to Δ*secA2* (Figure 3-5c). This supports the hypothesis that SecA2 not only affects PyK transport and secretion, but also gene transcription.

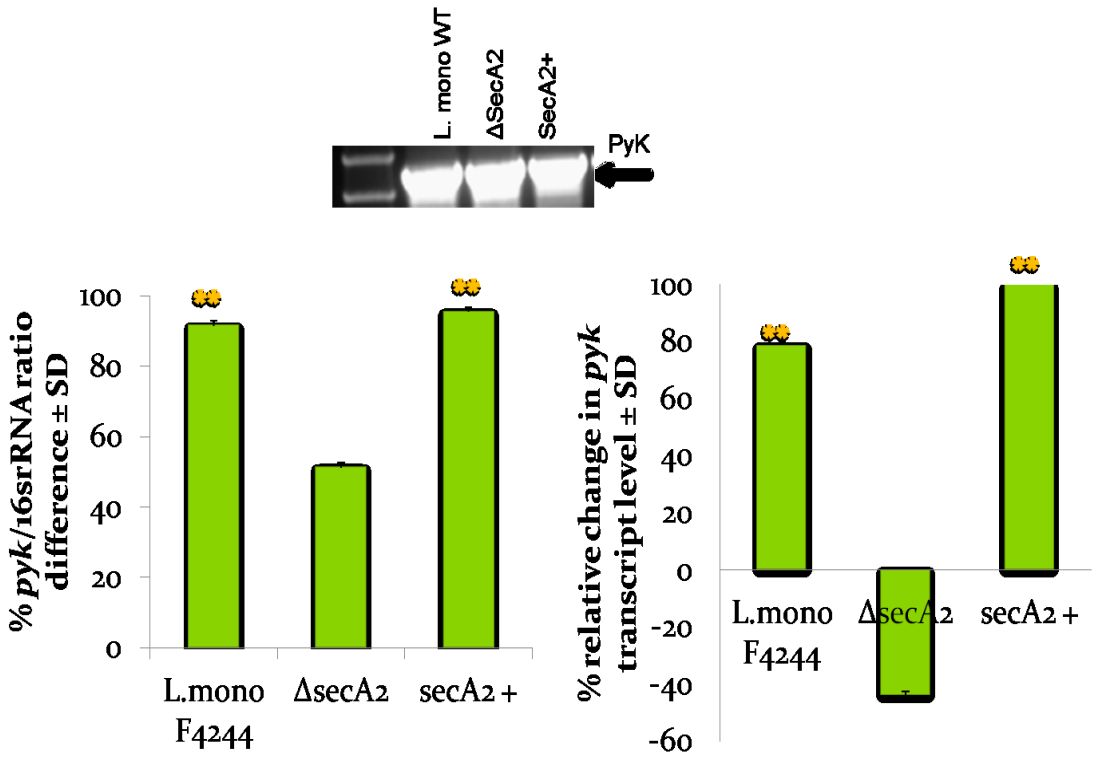

Figure 3-5 (a) PCR for *pyk* in F4244, Δ*secA2* and *secA2*$^{+}$. (b) 16s ribosomal RNA was used as internal positive control and the data is presented here as the % ratio difference of *pyk* expression levels to that of the internal positive control. (c) Relative increase in *pyk* expression levels as compared to expression in Δ*secA2*. The ** indicates statistically significant difference at P<0.01. Values are an average of two experiments run in duplicate.

3.3.5 Enzyme activity of PyK

Enzyme activity of PyK was examined to determine if the recombinant enzyme could still assist in bacterial growth and metabolism. Crude rPyK preparation along with protein extracts from *E. coli* BL21 (DE3), *L. monocytogenes* F4244 and *B. cereus* were tested for PyK activity. rPyK displayed 14.3 mU (mg protein)$^{-1}$ PyK activity (Table 3-3) which was higher, but not significantly different from the enzyme activity of all negative controls. This

indicates that rPyK may not be enzymatically active and it is possible that the renaturation of recombinant protein caused this loss of functionality.

Table 3-3 rPyK enzyme activity

PyK source	PyK activity (mU)
E. coli pET-32(a)-PyK	14.346 ± 1.06E-02^{A}
E. coli BL21 (DE3) Parent	13.938 ± 1.96E-02^{A}
L. monocytogenes F4244	13.97 ± 9.33E-02^{A}
B. cereus	13.766 ± 4.53E-02^{A}

PyK enzyme activity of rPyK and negative controls *E. coli* BL21 (DE3) Parent, *L. monocytogenes* F4244 and *B. cereus*. Means ± SD are shown. Values in a column labeled with A were analyzed by Tukey's test at $P<0.05$. Results are an average of two separate experiments.

3.3.6 Differential PyK expression in selective enrichment broths

To understand the influence of different growth and enrichment media on the expression of surface displayed PyK, *L. monocytogenes* F4244 was grown in six different media and protein levels were examined by ELISA and Western Blot reactions. Results show that PyK expression was lowest in the selective media, UVM and FB and highest in TSB, BHI and BLEB (Figure 3-6a). Nearly10 fold increase in PyK expression was observed between UVM and BLEB, BHI and TSB. Expression in minimal media, LB, was 5 fold higher than that in UVM. These results also correlated with the Western blot results (Figure 3-6b). We hence conclude that enrichment in BLEB is preferred over other selective enrichment broths if MAb EM-7H10 is used for *Listeria* detection.

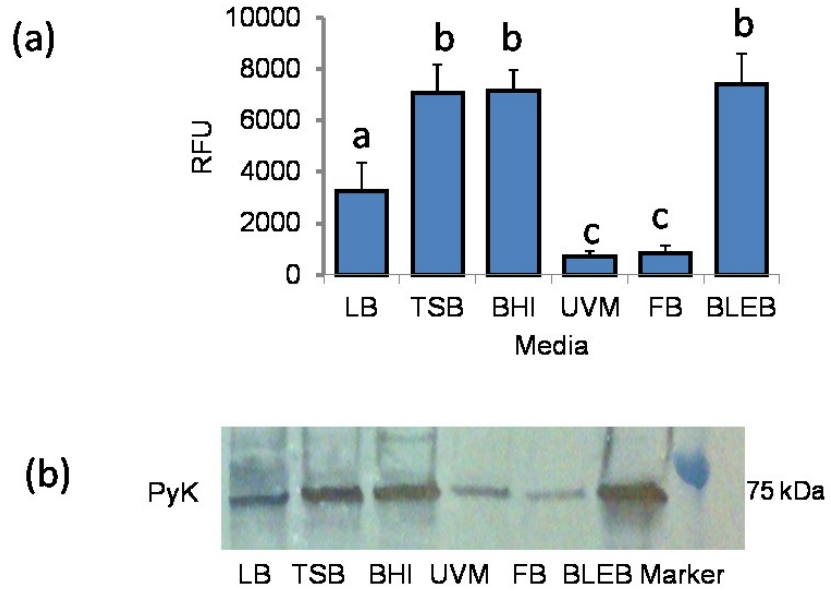

Figure 3-6 Effect of enrichment broth on the expression of pyruvate kinase analyzed by (a) ELISA and (b) Western blot. In ELISA, cells were adjusted to 10^8 cfu/ml before sensitizing the wells and in Western blot, bacterial cells were adjusted to an OD_{600} = 1.2 (~10^9 cells/ml) prior to the cell wall protein extraction. In ELISA, data are average of three experiments analyzed in quadruplicate. Bars labeled with different letters (a, b, c) are significantly different at $P<0.05$.

3.3.7 *Listeria* detection with fiber optics

MAb EM-7H10 gave high fluorescence values (~22,000 RFU) when tested against live, whole cell *Listeria* spp. However, slightly elevated cross reactivity was observed with other Gram positive or Gram negative bacteria (Figure 3-7a,b). The evanescent wave based biosensor can selectively capture the tested *Listeria* spp. and is capable of detecting up to 10^3 cfu/ml of the pathogen (Figure 3-7c,d).

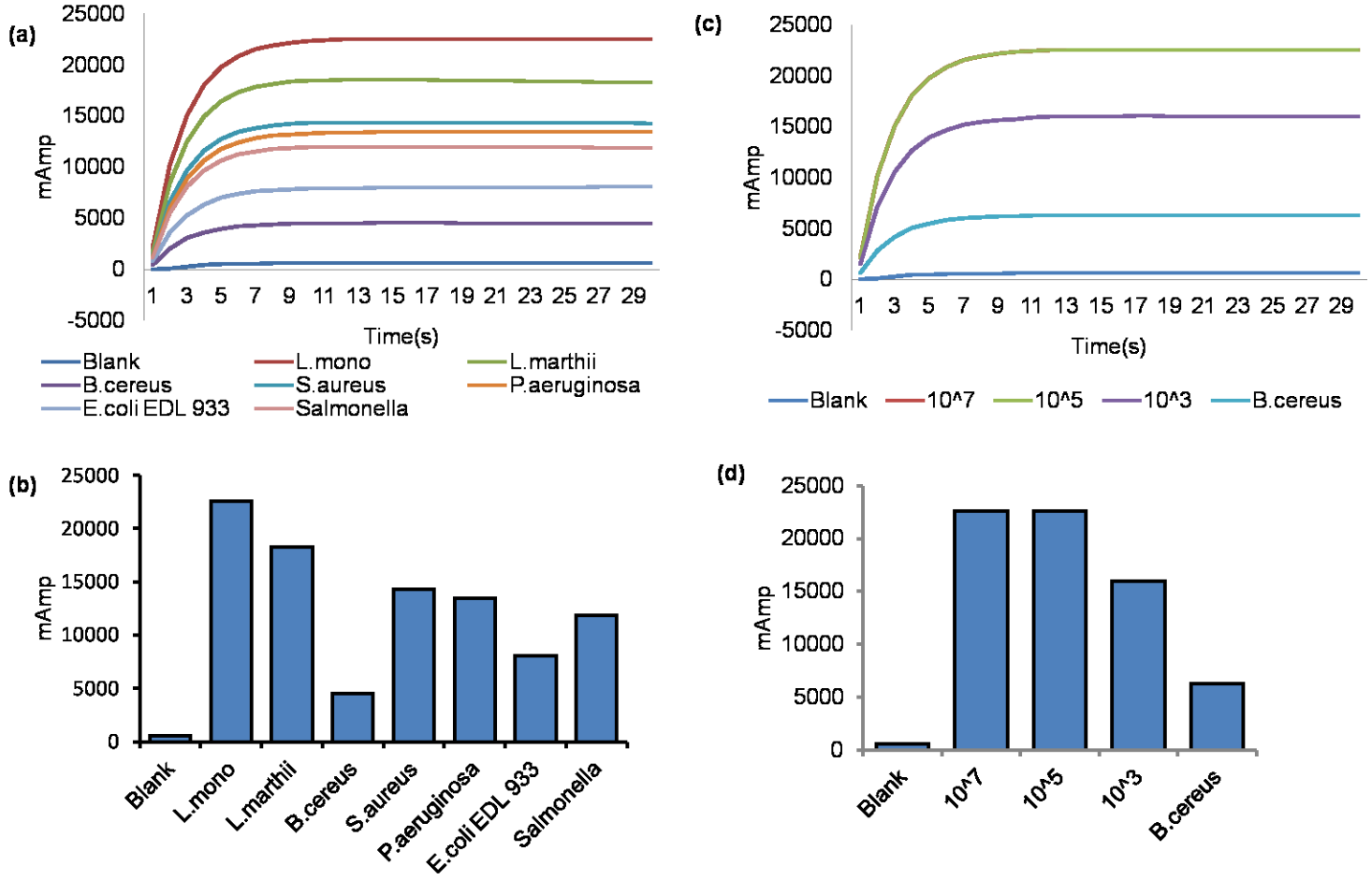

Figure 3-7 Determination of MAb EM-7H10 specificity (a) & (b) and sensitivity (c) & (d), towards *Listeria* species by fiber optics biosensor. Bacterial whole cells were used as the antigen while biotinylated MAb EM-7H10 was used as the capture antibody and Cy5 labeled p66 as the reporter antibody.

3.3.8 Role of PyK as a bacterial adhesion

Adhesion assay to determine the role of PyK as *Listeria* surface adhesin suggested that it may be involved in enhancing bacterial adhesion. Nearly 1 log reduced *L. monocytogenes* binding was observed when Caco-2 cells were exposed to MAb EM7-H10 and the results were comparable with the reduction observed with LAP (Figure 3-8).

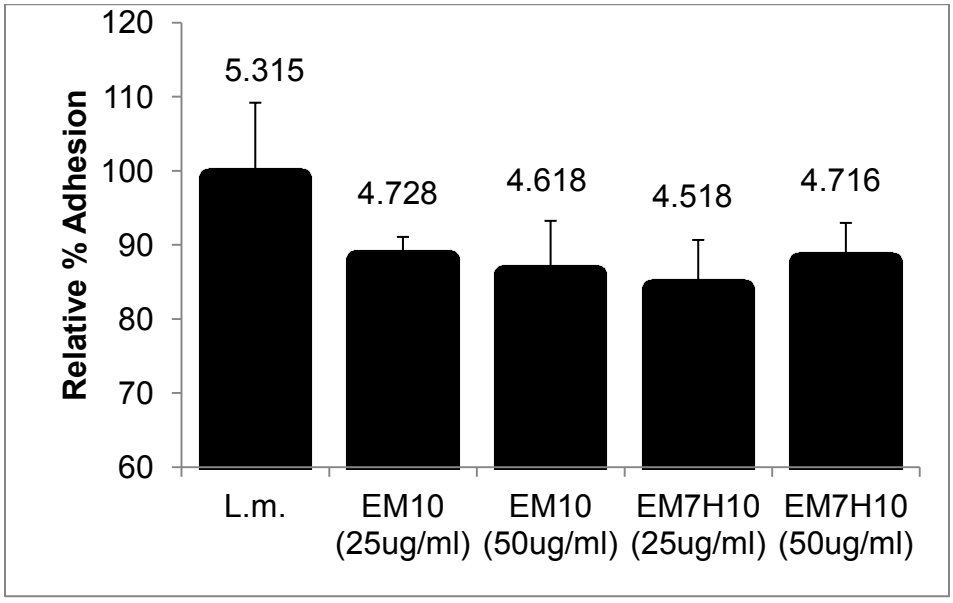

Figure 3-8 Analysis of pyruvate kinase as an adhesin on Caco-2 cell monolayers. Bacterial cells were exposed to different antibodies, anti-LAP EM10 and anti-PyK EM7-H10, before adding to Caco-2 cells monolayers at MOI of 10:1. Data are average of two experiments analyzed in triplicate.

3.4 Discussion

In this study we have identified Pyruvate kinase to be present on the surface of *Listeria* spp (with the exception of *L. rocortiae*) (Figure3-1 and Figure 3-2). Pyruvate kinase [EC: 2.7.1.40] is a key glycolytic enzyme, which catalyzes the rate limiting step of conversion of phosphoenol pyruvate (PEP) to pyruvate

with the production of ATP. The sequence of PyK is highly conserved and does not display any alterations or additional motifs for cell surface anchorage. While uncommon, it is not unusual to find PyK enzyme on the surface of bacteria. *Streptococcus pyogenes* [303, 304], *Streptococcus suis* serotype 9 [305], *Streptococcus iniae* [306] *Clostridium deficille* [307] and certain lactic acid bacteria such as *Lactococcus lactis* IL1403 [308] all exhibit this enzyme on the surface. A multiple sequence alignment of the PyK sequences identified in *Streptococcus pyogenes* M3, *Lactococcus lactis* IL1403 with the PyK sequence from *L. monocytogenes* F4244 showed a highly conserved architecture (Figure 3-9), which is expected (the multiple sequence alignment was done with T-Coffee web server [330, 331]). Additionally, the tertiary structure of PyK consists of homo-tetramers of identical subunits, contributing to the allosteric regulation of the enzyme [309, 332]. The exact reason behind the presence of this enzyme on the bacterial surface remains to be identified; however, studies pertaining to a two-component regulatory system in *Streptococcus iniae*, Siv S/R, indicates possible involvement of the surface displayed Pyruvate kinase in malate metabolism [306]. In *L. lactis*, PyK present on the bacterial surface has been found to bind with the yeast mannin [308, 310].

Another critical finding from this study was that the pyruvate kinase enzyme is transported to the bacterial surface by the auxillary SecA2 transport system (Figure 3-4). Similar to SecA, SecA2 also mediates protein transport across the cell membrane by utilizing structural changes induced by the ATP hydrolysis; however, unlike SecA, SecA2 is not vital to cell viability, and functions

only to further facilitate protein translocation and increase the overall transport efficiency [287, 292]. Some of the virulence proteins secreted by the SecA2 pathway include FbpA, lipoprotein LpeA and LAP [287, 293]. SecA2 systems also transports several non-conventional cytosolic proteins, such as p60, GAPDH, PFK and SodA, which do not possess the conventional signal sequences, such as the LPXTG motif or the NXXTX sorting signal [281, 290, 300, 302]. PyK sequence also lacks a canonical signal sequence or anchoring mechanism. The reduction in PyK transcript levels and the subsequent decrease in PyK expression in the cytoplasm, cell and cell supernatant in the SecA2 mutants clearly indicates that *pyk* expression is SecA2-dependent. Besides transporting PyK, SecA2 also appears to play a regulatory role in PyK production. A reverse-transcriptase PCR for the *pyk* gene in wild-type *L. monocytogenes*, Δ*secA2* and *secA2*$^{+}$ strains showed that pyk mRNA levels were considerably reduced in the Δ*secA2* strain and then restored to wild-type levels in the *secA2*$^{+}$ strain. These results suggest that SecA2 might contribute in *pyk* production at the transcription stage. Similar observations were made for the expression of the MurA protein which displayed reduced protein expression in the cell wall, membrane and cytoplasm in Δ*secA2* strain [299]. Further investigation is required to understand why and how these proteins are targeted for export and the exact regulatory aspect of SecA2 transporter in their expression. The function of this glycolytic enzyme on the bacterial surface is also needs to be elucidated.

Curiously, the decrease in intracellular PyK expression coincided with the accumulation of another 90kDa protein, as indicated in Figure 3-4b. This protein

band was also cut and sent for MALDI-TOF/TOF analysis (figure not shown). Results indicated this protein to be a bifunctional acetaldehyde-CoA/alcohol dehydrogenase, or LAP, from *Listeria monocytogenes* serotype 4b str. F4244. This interdependence of these two proteins, reduction of one leading to accumulation of the other was an interesting observation because LAP and PyK share little functional or sequential similarity.

To assess the enzymatic activity of the rPyK protein, we performed an enzyme assay on protein extracts from the *E. coli* BL21 pET-32(a)-PyK and parent *E. coli* BL21 (DE3), *L. monocytogenes* F4244 and *B. cereus*. A relatively higher enzyme activity for rPyK was observed (Table 3-3), confirming enzyme functionality.

Growth media and environmental conditions, such as temperature, osmotic stress, nutrient availability, carbon source and acidity can influence expression of proteins [251, 333-336], which may affect immunologic pathogen detection [313]. To evaluate this, PyK expression was studied in nutrient-rich media, TSB and BHI; minimal medium, LB and Listeria selective media, UVM, FB and BLEB by ELISA and Western blot reactions. Selective and non-selective media for Listeria growth have been shown to have major impact on the expression of various proteins such as enolase, flavocytochrome C fumarate reductase, glyceraldehydedehydrogenase [337], internalin B, and ActA [338]. It was not surprising, therefore, to observe differential PyK expression in different media (Figure 3-7). PyK expression was highest in nutrient rich media, TSB and BHI, and also Listeria selective medium, BLEB; and least protein expression was

observed in the selective UVM and FB media. The variation in PyK expression can be explained by the differences in the carbon source in these media. BHI, TSB and BLEB contain 2 g/L glucose, 2.5 g/L glucose and 2.5 g/L dextrose respectively; on the other hand,LB, UVM and FB lack readily metabolizable sugars such as glucose or dextrose. To further verify the involvement of glucose in PyK surface expression, this experiment will be repeated following the addition of 2-2.5g/L of glucose to LB, UVM and FB media and then observing the change in protein surface expression, if any. Both UVM [339, 340] and FB [341] are recommended by the USDA-FSIS method to be used as enrichment bases for specific isolation and cultivation of *Listeria*, whereas the BLEB medium is FDA/BAM recommended (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm). Based up on the results, we therefore infer that this PyK-MAb EM7-H10 system works best for bacterial detection when the pre-enrichment is performed according to the FDA method.

Pyruvate kinase has thus emerged as a new and unique target for detection of *Listeria* species (with the exception of *L. rocourtiae* as it lacks PyK on the surface). However, the difference in protein expression in different media must be kept in mind when using MAb EM-7H10 for pathogen detection. Besides the ability to detect low numbers of the bacteria on a fiber optic biosensor, MAb EM-7H10 also holds potential for use on multiple different bacterial detection platforms.

Figure 3-9 Multiple sequence alignment for Pyruvate kinase from *Streptococcus pyogenes* M3, *Lactococcus lactis* IL1403 an *L. monocytogenes* F2365.

CHAPTER 4. TIR (TRANSLOCATED INTIMIN RECEPTOR) FOR CAPTURE AND DETECTION OF STEC

4.1 Introduction

Shiga-toxigenic strains of *E. coli* (STEC), also known as Verocytotoxin (VT)-producing *E. coli* (VTEC) or Enterohaemorrhagic *E. coli* (EHEC), were first identified in 1977 [164], and have since emerged as major foodborne pathogens raising significant public health concern. Various STEC strains have been implicated in human diseases like diarrhea, gastroenteritis, thrombotic thrombocytopenic purpurea (TTP), hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) [342-344]. STEC serovar O157 is listed as one of the top 5 pathogens contributing to domestically acquired foodborne illnesses resulting in hospitalizations by the CDC. Though STEC O157:H7 was the most common cause of these infections, other serovars such as O26, O45, O145, O121, O111 and O104 have also been implicated.

The key virulence factors in EHEC are Shiga toxins (Stxs). Shiga toxins are family of structurally and functionally related proteins secreted by the *Shigella dysenteriae* serotype I and by STEC [174, 175]. Stxs are iron regulated toxins that catalytically inactivate the 60S ribosomal subunits of eukaryotic cells thereby blocking mRNA translation and causing cell death [181-183].

A characteristic step in the pathogenic mechanism of STEC or EHEC is the formation of the attachment/effacement (A/E) lesion. The bacteria colonize the gut and disrupt enterocyte function by forming A/E lesions. An attachment/effacement (A/E) lesion is characterized by destruction (effacement) of the brush border villi followed by intimate attachment of the bacterium to the host cells and cytoskeletal rearrangements for the formation of a pedestal like structure [190-192]. For EPEC, LEE encodes all the genes that are necessary and sufficient for formation of the A/E lesion [193, 194]; however, for EHEC, LEE is necessary but not entirely sufficient [195].

LEE contains genes involved in formation of the type three secretion system (TTSS), intimate attachment *eaeA*/Intimin and *espE*/TIR and several other effector proteins like EspA, EspB, EspD, EspF, EspG and EspH. EaeA or Intimin is a 94 kDa EPEC and EHEC outer membrane adhesion protein, encoded by the *eae* gene in the LEE, which is critical for virulence, as it mediates intimate bacterial attachment and triggers downstream events for actin pedestal formation [193, 197-201]. Intimin binds to the translocated intimin receptor (TIR), a 78 kDa protein, produced in *E. coli* and translocated to the host cell membrane by the T3SS [203, 204]. Within the host cell, TIR undergoes phosphorylation and gets expressed on the cell surface. TIR-Intimin association leads to intimate binding between bacteria and host cell which in turn triggers a chain of reactions that induce actin polymerization, depolymerization, cytoskeletal rearrangement and pedestal formation.

Intimin-TIR interaction has been studied extensively. The C-terminal region of intimin (Int190) has been shown to be the TIR-binding region [208]. Similar structural and biochemical analysis of TIR protein indicates that the C-terminal and N-terminal domains of TIR (called C-TIR and N-TIR) are membrane associated whereas the 55 amino acid long middle extracellular region (M-TIR) contains the intimin binding domain (TIR-IBD) [209-211]. The crystal structure of TIR-intimin complex was analyzed by Luo et al [212] and they determined that the intimin-TIR-IBD binding affinity constant (K_a) was $3.2x10^6$ M^{-1} at 37°C, and it was similar to the binding constant of full length TIR with intimin. More specifically, the binding occurred between the lectin-like D3 domain of intimin and β-hairpin and N-terminal of the helix HB of TIR-IBD [212].

CesT, or chaperone for *E. coli* secreted TIR, is encoded by a 15 kb locus (previously known as OrfU), located between the *TIR* and *eae* (intmin) genes on the LEE [185, 219]. CesT is a cytoplasmic protein which has been shown to be essential for stable TIR production [219]. CesT also plays an important role in guiding TIR to the T3SS for secretion by interacting with a specific T3SS ATPase, EscN [220]. The CesT binding domain (CBD) of TIR is located on the N-terminal end of the protein and is distinct from its IBD [219, 221]. Therefore, CesT binding with TIR does not interfere with TIR-intimin interaction. Apart from TIR, CesT also assists in production and secretion of other T3SS proteins, most notably, Map [222] and NleA [223].

In this study, the goal is to utilize this TIR-intimin interaction for specific STEC detection. Immobilized TIR on a pathogen detection platform, such as a

microtiter plate or an evanescent wave based biosensor, will be used for specific detection of EHEC and EPEC.

4.2 Materials and methods

4.2.1 Bacterial cultures and growth conditions

All cultures, plasmids, and primers used in this study are listed in Table 3-1. All wild-type strains were grown at 37°C under aerobic conditions in the Brain Heart Infusion (BHI) broth. Recombinant strains were grown in Luria Bertani (LB) media with appropriate antibiotics (as listed in Table 3-1). The *E. coli* BL21 Star (DE3) pLysS strains were grown in the presence of Chloramphenicol (Cm^R 10 µg/mL).

Table 4-1 Bacterial cultures, plasmids and primers used for cloning

Bacteria	**Strains**	**Description**	**Source**
E. coli	EDL933	O157:H7; Wild type	Our collection
E. coli BL21	TIR (AKB 801)	*E. coli* BL21 containing, but not expressing TIR from *E. coli* EDL933 (Kan^R 30 µg/mL)	This study
E. coli BL21	CesT (AKB 802)	*E. coli* BL21 expressing CesT from *E. coli* EDL933 (Am^R 50 µg/mL)	This study
E. coli BL21 Star (DE3) pLysS	IBD (AKB 803)	*E. coli* BL21 Star (DE3) pLysS containing, but not expressing TIR-IBD from *E. coli* EDL933 (Am^R 50 µg/mL+ Cm^R 10 µg/mL)	This study
E. coli BL21	TIR-CesT (AKB 804)	*E. coli* BL21 co-expressing TIR and CesT from *E. coli* EDL933 (Kan^R 30 µg/mL+Am^R 50 µg/mL)	This study
Plasmids			
pGEM-T easy		Cloning vector (Am^R 50 µg/mL)	Promega

Table 4-1 continued

pET 28(a)		Expression vector (Kan^R 30 µg/mL)	Promega
pET 32(a)		Expression vector (Am^R 50 µg/mL)	Promega
pGEX-6P-1		Expression vector (Am^R 100 µg/mL)	GELifesciences
pET 28(a)-TIR		pET 28(a) carrying *tir* (Kan^R 30 µg/mL)	This study
pET 32(a)-CesT		pET 32(a) carrying *cest* (Am^R 50 µg/mL)	This study
pET 32(a)-IBD		pET 32(a) carrying *tir-IBD* (Am^R 50 µg/mL)	This study
Primers			
TIR (1677 bp)	Forward (HindIII)	5'CCCAAGCTTATGCCTATTGGTAATCTT3'	This study
	Reverse (XhoI)	5'CCGCTCGAGTTAGACGAAACGATGGG3'	This study
TIR-pgex; 1677 bp)	Forward (BglII)	5'AAAAGATCTATGCCTATTGGTAACCTT3'	This study
	Reverse (SalI)	5'AAAGTCGACGTTCAGATCTTGATGACAT3'	This study
TIR-IBD (345 bp)	Forward (HindIII)	5'CCCAAGCTTATGCCGGAGCCGGATAGC3'	This study
	Reverse (XhoI)	5'CCGCTCGAGACCAAGAATCAATGCGCC3'	This study
CesT (471 bp)	Forward (BamHI)	5'CGCGGATCCATGTCATCAAGATCTGAACTTTTA3'	This study
	Reverse (SalI)	5'CGCGTCGACTTATCTTCCGGCGTAATAATGTTT3'	This study
TIR-qPCR (158 bp)	Forward	5'AACGAAAGAAGCGTTCCAGA3'	This study
	Reverse	5'TTTCAATGGCTTGCTGTTTG3'	This study

ATCC, American Type Culture Collection; NCDO.

4.2.2 Cloning and expression of TIR, TIR-IBD, CesT & TIR-CesT

Gene encoding full length TIR (1677 bp; 558 amino acids) from *E. coli* O157:H7 strain EDL933 (NC_002655.2) was amplified by PCR using the following primers: TIR-F 5'-CCCAAGCTTATGCCTATTGGTAATCTT-3' and TIR-R 5'-CCGCTCGAGTTAGACGAAACGATGGG-3' containing restriction sites for *Hind*III and *Xho*I, respectively. The gene was first cloned into the cloning vector pGEM-T Easy (Promega) and from that into *Hind*III and *Xho*I digested pET-32(a) and pET-28(a) expression vectors (Novagen). The transformants in both cases were verified by gene sequencing at the Purdue University Genomics Facility. The protein was expressed in *E. coli* BL21 (DE3) expression cells (Novagen) in the presence of Ampicillin (50 µg/ml) (for pET-32(a) plasmid) and Kanamycin (30 µg/ml) (for pET 28(a) plasmid); transformants in *E. coli* BL21 Star (DE3) pLysS cells were grown in the presence of Chloramphenicol (10 µg/ml).

Full length TIR gene (1677 bp; 558 amino acids) from *E. coli* O157:H7 strain EDL933 (NC_002655.2) was amplified by PCR using the following primers: TIR-pgex-F 5'- AAAAGATCTATGCCTATTGGTAACCTT-3' and TIR-pgex-R 5'-AAAGTCGACGTTCAGATCTTGATGACAT-3' containing restriction sites *Bgl*II and *Sal*I, respectively. The gene was first cloned into the cloning vector pGEM-T Easy (Promega) and from that into a *Bgl*II and *Sal*I digested pGEX-6P-1 expression vectors (Novagen). The transformants were verified by gene sequencing at the Purdue University Genomics Facility. The protein was expressed in *E. coli* BL21 (DE3) expression cells (Novagen) in the presence of Ampicillin (50 µg/ml).

TIR-IBD gene fragment was (345 bp;115 amino acids) from *E. coli* O157:H7 strain EDL933 (NC_002655.2) was amplified by PCR using the following primers: TIR-IBD-F 5'-CCCAAGCTTATGCCGGAGCCGGATAGC-3' and TIR-IBD-R 5'-CCGCTCGAGACCAAGAATCAATGCGCC-3' with restriction sites *Hind*III and *Xho*I, respectively. The gene was first cloned into the cloning vector pGEM-T Easy (Promega) and from that into a *Hind*III and *Xho*I digested pET-32(a) and pET-28(a) expression vectors (Novagen). The transformants in both cases were verified by gene sequencing at the Purdue University Genomics Facility. The protein was expressed in *E. coli* BL21 Star (DE3) pLysS cells in the presence of Ampicillin (50 µg/ml) (for pET-32(a) plasmid)/ Kanamycin (30 µg/ml) (for pET 28(a) plasmid) and Chloramphenicol (10 µg/ml).

Full length CesT gene (471 bp; 156 amino acids) from *E. coli* O157:H7 strain EDL 933 (NC_002655.2) was amplified by PCR using the following primers: CesT-F 5'- CGCGGATCCATGTCATCAAGATCTGAACTTTTA-3' and CesT-R 5'-CGCGTCGACTTATCTTCCGGCGTAATAATGTTT-3' with restriction sites *Bam*HI and *Sal*I, respectively. The gene was first cloned into the cloning vector pGEM-T Easy (Promega) and from that into a *Bam*HI and *Sal*I digested pET-32(a) expression vector (Novagen). The transformants was verified by gene sequencing at the Purdue University Genomics Facility. The protein was expressed in *E. coli* BL21 (DE3) expression cells (Novagen) in presence of Ampicillin (50 µg/ml).

For TIR-CesT co-expressing strains, recombinant *E. coli* BL21 cells containing CesT expressing pET32(a) plasmid were transformed with TIR-

containing pET-28(a) plasmid. Similarly, *E. coli* BL21 cells with TIR-containing pET-28(a) plasmid were transformed with the CesT expressing pET-32(a) plasmid. The eventual TIR-CesT co-expressing *E. coli* BL21 cells would therefore contain both CesT (in pET-32(a) plasmid) and TIR (pET-28(a) plasmid). The presence of two distinct plasmids enables dual selection in the presence of both Ampicillin (100 µg/ml) for CesT and Kanamycin (30 µg/ml) for TIR.

Recombinant His-tagged TIR-CesT proteins were purified by Immobilized Metal Affinity Chromatography (IMAC) using a Nickel column (Thermo Fisher Scientific). Protein expression in recombinant strains was subsequently confirmed by Western blot analysis using MAb anti-His monoclonal antibody (Pierce Antibodies, Thermo Fisher Scientific).

4.2.3 Recombinant protein expression

Recombinant cell lines were induced with IPTG (1-2 mM) to increase and optimize protein production. Whole cell bacterial proteins were extracted by heat killing the cells at 95°C for 10 minutes and then resuspending the cell pellet in the sample solvent (5% SDS, 0.5% β-mercaptoethanol, 1.5% Tris, pH 7.0) followed by sonication on ice for 5–7 cycles of 15 sec each using a Sonifier 150D (Branson, Niantic, CT). The samples were centrifuged and the supernatant fractions were collected and stored at −20°C.

Proteins were quantified using the bicinchoninic acid method (Pierce, Rockford, IL) and equivalent amounts of protein (20 µg of each fraction) were separated using SDS polyacrylamide gel electrophoresis (7.5% acrylamide) gel.

In case of 15 kDa long TIR-IBD protein, a 4-20% pre-cast gradient SDS gel (Bio-Rad Laboratories) was used to ensure retention of the recombinant protein. The proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, MA) and immunoprobed with anti-TIR antibody PAb-TIR (1.0 µg/mL) and horseradish peroxidase-coupled anti-mouse antibody (0.2 µg/mL; Jackson Immuno Research, West Grove, PA). The membranes were developed with an enhanced chemiluminescence kit (Pierce). The membrane was also immunoprobed with anti-His-tag MAb (0.1 µg/mL; Thermo Fisher Scientific) for detection of His-tagged recombinant proteins.

4.2.4 TIR antibody development

To predict the most antigenic TIR peptide sequence that should be used to develop its antibody, two methods were considered and the consensus sequence from both results was chosen for antibody development. These two methods were (i) Kolaskar and Togaonkar Method [345] and (ii) BCPREDS: B-cell epitope prediction server [346-348]. A BLAST-P search was also performed on the consensus peptide obtained to ensure specificity. The selected peptide sequence showed a 100% similarity only with TIR from *E. coli* strains. This antigenic peptide is: PSGVLKDDVVANI and constitutes a 13 amino acid long (306-318) TIR-IBD region.

This peptide sequence was sent for polyclonal antibody (PAb) development to EZBiolab (Cat # AB203). The antibody was raised in rabbits as

hosts and a partially purified antibody from 30-50 ml antiserum of each rabbit by ammonium sulfate precipitation method was provided by EZBiolab.

The partially purified PAb-TIR was further purified by affinity chromatography using a Protein A column. The purification was performed according to manufacturer's instructions and the final antibody concentration was obtained as 0.3 mg/ml.

4.2.5 Reverse transcriptase (RT) PCR

To inspect the presence of TIR and TIR-IBD RNA in all transformants, we performed a quantitative reverse-transcriptase PCR reaction for the TIR gene. mRNA was extracted by following the manufacturer's instruction provided with the Qiagen RNeasy Mini Kit (# 74104). RNA was then reverse transcribed into cDNA using the BioRad iScript cDNA Synthesis Kit (# 170-8890) and the resulting cDNA was used as a template for subsequent PCR amplification by using the following primers: TIR-qPCR-F 5'- AACGAAAGAAGCGTTCCAGA-3' and TIR-qPCR-R 5'- TTTCAATGGCTTGCTGTTTG-3'. Amplification of the 158 bp TIR fragment was obtained at 60°C and 64°C. RNA from *E. coli* O145 and *E. coli* O157 WT strains was used positive controls whereas RNA from the parent cell lines, *E. coli* BL21 (DE3) and *E. coli* BL21 Star (DE3) pLysS as well as *Listeria innocua* F4248 was used as negative controls.

4.2.6 Effect of growth media on TIR expression

To observe the differential expression of TIR when recombinant strains are grown in the various growth media, the strains were grown in 100 ml volume

of three different media: Luria Bertani (LB), Tryptic Soy Broth (TSB) and Brain Heart Infusion (BHI) for 18 h. The amount of bacterial culture used in the downstream experiments was normalized by measuring and normalizing the absorbance of cells at 595nm (A_{595nm} ~ 0.8). The cultures were centrifuged (7,000×g for 10 min at 4°C) and whole cell protein was extracted and used for Western blot. For whole cell protein extraction, heat-killed (95°C for 10 minutes) bacterial pellets were resuspended in sample solvent (5%SDS, 20% Glycerol, 1.5% Tris base and 10% β-Mercaptoethanol; pH adjusted to 6.8) and lysed by sonicating the suspension on ice in four 30-second bursts. The solution was centrifuged at 12,000 x rpm for 10 min and the supernatant was collected as the whole cell protein fraction.

4.2.7 ELISA

Bacterial cell pellets from freshly grown bacterial cultures ($A_{595\ nm}$ ~ 1.2) were resuspended in equal volume of 0.05 M sodium carbonate coating buffer, pH 9.6, immobilized in 96-well Immulon 4HBX plates (Thermo Scientific, Waltham, MA), and stored at 4°C for 48 h. Following bacterial immobilization, the plate wells were sequentially reacted with PAb TIR 300 ng/ml) and anti-mouse HRP-conjugated antibody (1:4000 dilution; Jackson Immunologicals). For all steps, plates were held at RT for 1 h and washed 3 times with PBST between steps. Finally 100 µL of a fluorescent substrate, either Super Red (10-acetyl-3,7-dihydroxyphenoxazine; Virolabs, Chantilly, VA; Ex: 540 nm, Em: 600 nm) or Quanta Blu (Ex: 320 nm, Em: 460 nm), was added to each well and fluorescence

was measured using a Spectramax fluorescent reader (Gemini, Sunnyvale, CA) every 15 min for 1 h. To determine nonspecific protein binding, control reactions without bacteria and PAb TIR were included. Fluorescent readings obtained from these controls were subtracted from the test results to obtain true values.

To determine any non-specific binding of the CesT protein with pathogenic and other bacteria, an ELISA was done with whole bacterial cells as above. Following bacterial immobilization, the plate wells were sequentially reacted with recombinant CesT protein (1 µg/well) and then exposed to MAb anti-His-tag (250 ng/ml; Jackson Immunologicals).

To determine the STEC detection capability of the TIR-CesT conjugated proteins, an ELISA was performed as above; the plate wells were sequentially reacted with recombinant TIR-CesT proteins (1 µg/well) and then with MAb anti-His-tag (250 ng/ml; Jackson Immunologicals). *E. coli* O157, O145, O121, O111, O103, O45 and O26 serovars were used as positive controls and non-pathogenic *E. coli* BL21, *E. coli* ATCC 51739, *L. monocytogenes* F4244, *S. aureus* subspecies aureus ATCC 25923 and *B. cereus* UW85 were used as negative controls.

4.2.8 Statistical analysis

All experiments were repeated at least three times independently, and each set of experiment was performed in duplicate or triplicate. Statistical comparisons were carried out using analysis of variance (SAS 9.2, Cary, NC)

and Tukey's multiple comparisons of means at $P < 0.05$ to determine significant differences.

4.3 Results and discussion

4.3.1 TIR antibody

The TIR antibody developed was tested for specificity and sensitivity by Western Blot (Figure 1a) and ELISA (Figure 1b) reactions. Results showed that the antibody selectively detected pathogenic *E. coli*, thus indicating that it bound only with the TIR protein expressed on the surface of various pathogenic *E. coli* strains. Anti-TIR PAb gave higher fluorescence values when tested against live, whole cell pathogenic *E. coli* spp., and did not show any cross reaction with other non-pathogenic *E. coli* strains.

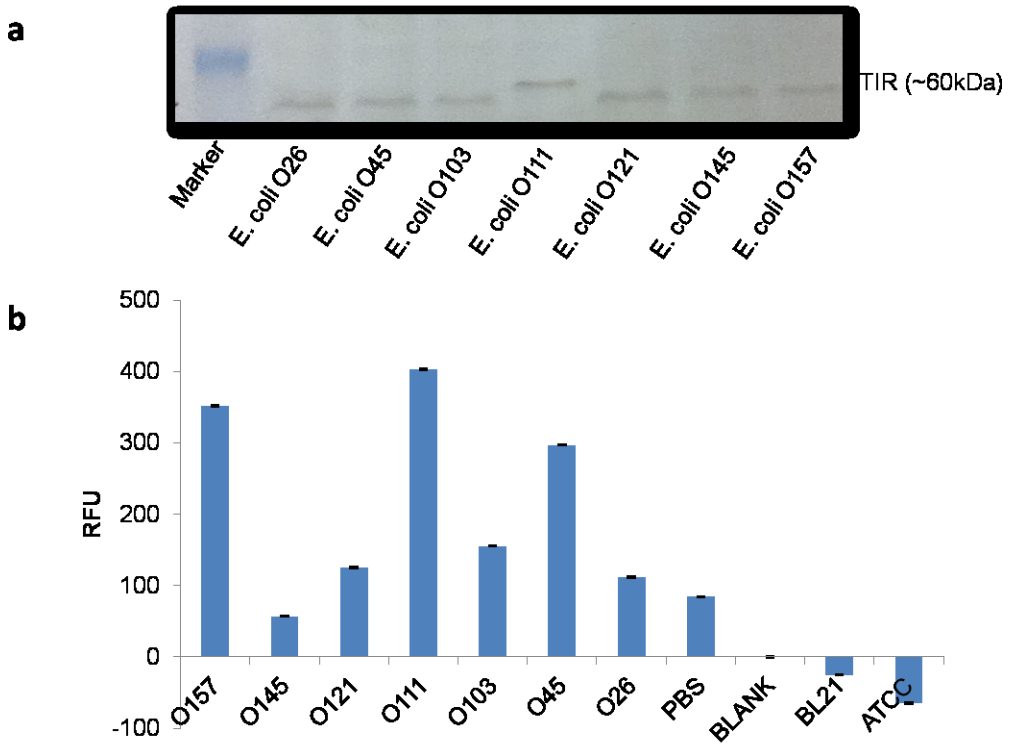

Figure 4-1(a) Western blot and (b) ELISA reaction profile of PAb TIR with *E. coli* spp. In ELISA, bacterial cells were adjusted to 10^8 cells/well and in Western blot, bacterial cells were adjusted to an OD_{600} = 1.5 (~10^9 cells/ml) prior to total protein extraction. In ELISA, data are average of three experiments analyzed in quadruplicate.

4.3.2 TIR protein cloning and expression

The 1677 bp long TIR gene from *E. coli* EDL933 strain was cloned into pET-28(a) and pET-32(a) expression vectors and subsequently transformed into *E. coli* BL21 (DE3) and *E. coli* BL21 Star (DE3) pLysS expression cell lines (Figure 3-2a). The pET-28(a) vector carries an N-terminal His-Tag along with an optional C-terminal His-Tag and contains a Kanamycin resistance marker. pET-32(a) is another commonly used vector that carries a cleavable His-Tag and S-tag for protein detection and purification and contains an Ampicillin resistance

marker [349]. Both *E. coli* BL21 (DE3) and *E. coli* BL21 Star (DE3) pLysS are used for protein expression, however, *E. coli* BL21 Star (DE3) pLysS contains a T7 lysozyme (in the pLysS plasmid) which lowers the background expression level of target genes but does not interfere with the level of expression achieved following induction by IPTG [350, 351]. In addition, the *E. coli* BL21 Star (DE3) pLysS strain also carries a mutated *rne* gene (*rne131*) which encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA, resulting in an increase in mRNA stability and consequently an overall increase in target protein expression.

Expression of recombinant protein was analyzed by a Western blot reaction using an anti-His-tag antibody (Jackson Immunologicals) and an anti-TIR antibody developed above (Figure 3-2b). No TIR expression was observed in any of the transformants. However, some degraded protein product was observed at the bottom of the gel lanes.

Since growth media and environmental conditions also influence expression of proteins, the expression of the recombinant-TIR was studied in nutrient-rich media (Trypticase Soy Broth [TSB] and Brain Heart Infusion [BHI]), minimal medium (Luria-Bertani [LB]) by Western blotting (Figure 3-2c). Once again, no protein expression was observed in any of the recombinant-clones.

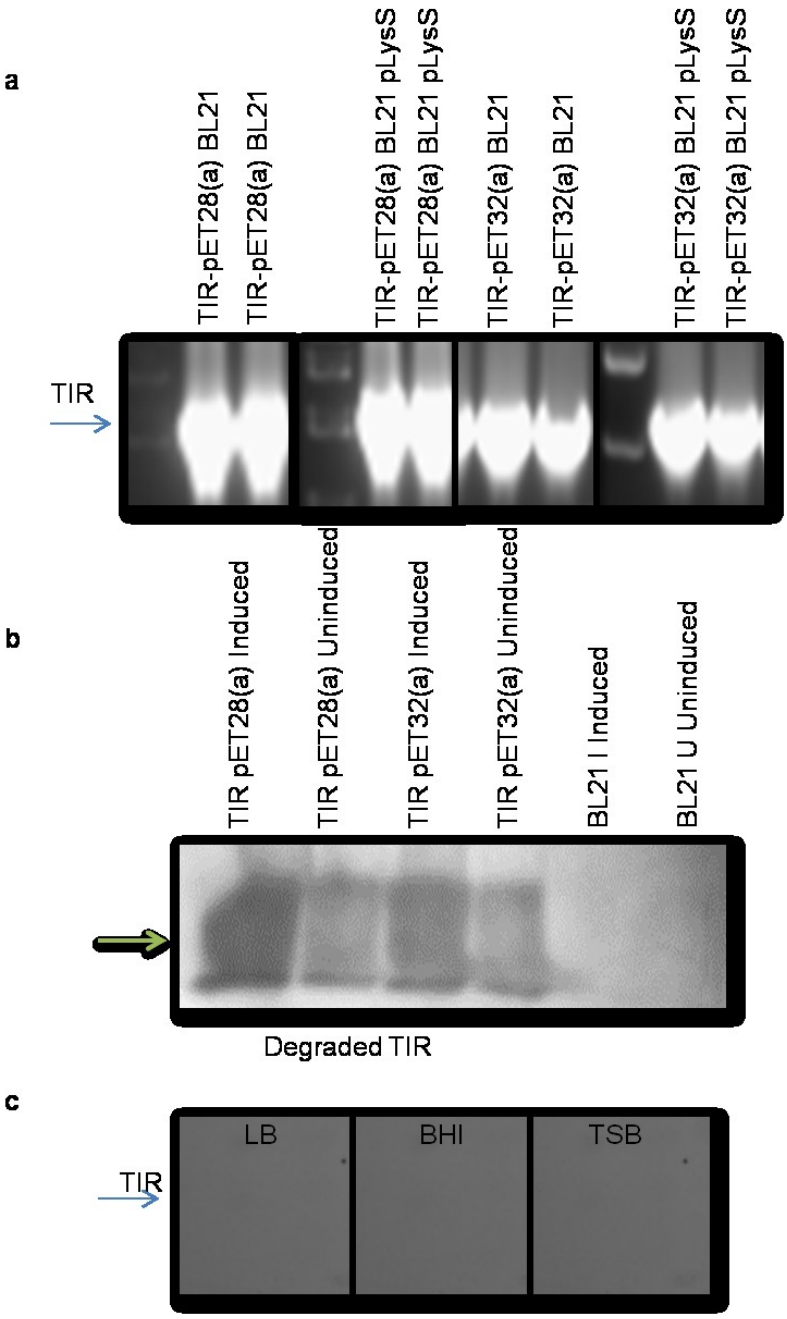

Figure 4-2 (a) *tir* (NC_002655.2) was cloned into pGEM T Easy cloning vector in *E. coli* DH10B cells and then into expression vectors, pET28(a) and pET-32a, for production of TIR in *E. coli* BL21 and *E. coli* BL21 Star (DE3) pLysS. (b)TIR expression was induced in *E. coli* BL21 cells by growing cells in presence of IPTG (1 mM) (c)TIR expression was also induced and observed in different media: LB, BHI and TSB.

4.3.3 TIR-IBD cloning and expression

In the absence of TIR protein expression, we decided to take an alternate approach by focusing on the Intimin Binding Domain (TIR-IBD) of the protein. The 347 bp long TIR-IBD was cloned into pET-28(a) and pET-32(a) expression vectors and then transformed into the *E. coli* BL21 Star (DE3) pLysS expression cell line (Figure 3-3).

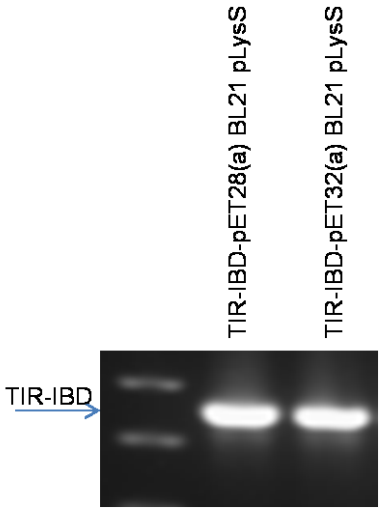

Figure 4-3 TIR-IBD was cloned into pGEM T Easy cloning vector in *E. coli* DH10B cells and then into expression vectors, pET28(a) and pET-32a, for production of TIR-IBD in *E. coli* BL21 Star (DE3) pLysS.

Expression of recombinant TIR-IBD protein was analyzed by a Western Blot reaction using an anti-His-tag antibody (Jackson Immunologicals) and once again, no protein expression was observed in any of the transformants (image not shown). Variation in induction duration (1-8 hours of induction with 1mM IPTG) also failed to result in affirmative protein expression.

4.3.4 TIR and TIR-IBD RT-PCR

To determine the presence of TIR and TIR-IBD RNA transcripts in the recombinant strains, a reverse transcriptase PCR (RT-PCR) was performed. Results showed that all the recombinant strains contained relatively high levels of the TIR or TIR-IBD transcripts (Figure 3-4). We conclude therefore that the target genes are successfully transcribed; however, the RNA is not being translated into a functional protein. It is possible that the over-expression of recombinant TIR renders the protein unstable, resulting in a highly degraded product.

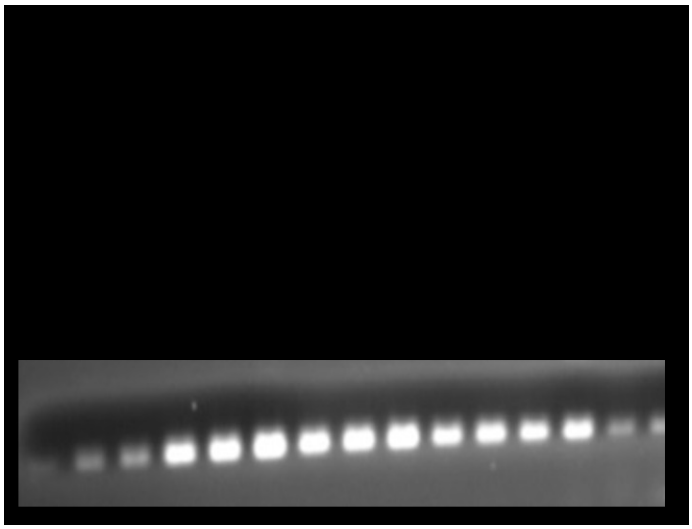

Figure 4-4 Reverse transcriptase PCR of TIR transformants developed along with negative controls.

4.3.5 TIR-CesT coexpression

To assist TIR production, we decided to co-express TIR with its chaperone, CesT. Analysis of whole cell proteins from recombinant cells showed that only the cells which contained CesT and were later transformed with TIR containing pET-28(a) plasmid expressed both the targeted proteins (Figure 3-5a). Cells

containing TIR and subsequently transformed with CesT containing pET32(a) plasmid failed to express both TIR and CesT.

The presence of CesT clearly helped stabilize and increase TIR production; and a further optimization of induction conditions revealed that best TIR expression was observed after an overnight induction with 2 mM IPTG (Figure 3-5b,c).

His-tagged TIR and CesT recombinant conjugate protein complex was purified by IMAC chromatography using a Nickel column (Thermo Fisher Scientific) (Figure 3-5d).

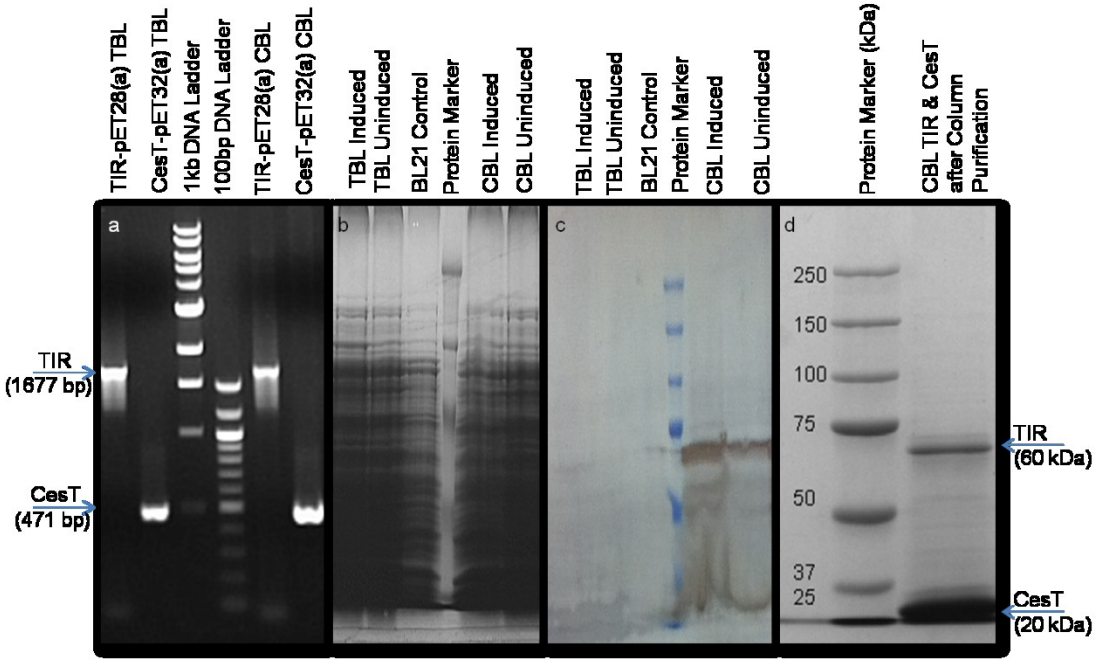

Figure 4-5(a) Transformation of TIR in CesT containing *E. coli* BL21 cells (CBL) and transformation of CesT in TIR contaning *E. coli* BL21 (TBL) (b) SDS-PAGE (7.5% acrylamide) with CBL and TBL strains and parent strain *E. coli* BL21 as control (c) Western blot reaction profile of CBL and TBL strains with MAb His-tag antibody (d) TIR-CesT conjugate was purified by Ni-affinity column. The purified protein showed strong reaction with MAb anti-His-tag.

4.3.6 TIR based STEC detection

Non-specificity of CesT: Recombinant CesT protein was tested for specificity towards the pathogenic *E. coli* strains. It was observed that CesT bound and gave a high fluorescence values for all bacterial strains, including the Gram Positive controls such as *L. monocytogenes*, *S. aureus* and *B. cereus* (Figure 3-6) used in the reaction. However, the values for the pathogenic *E. coli* strains, particularly O157:H7, were relatively lower.

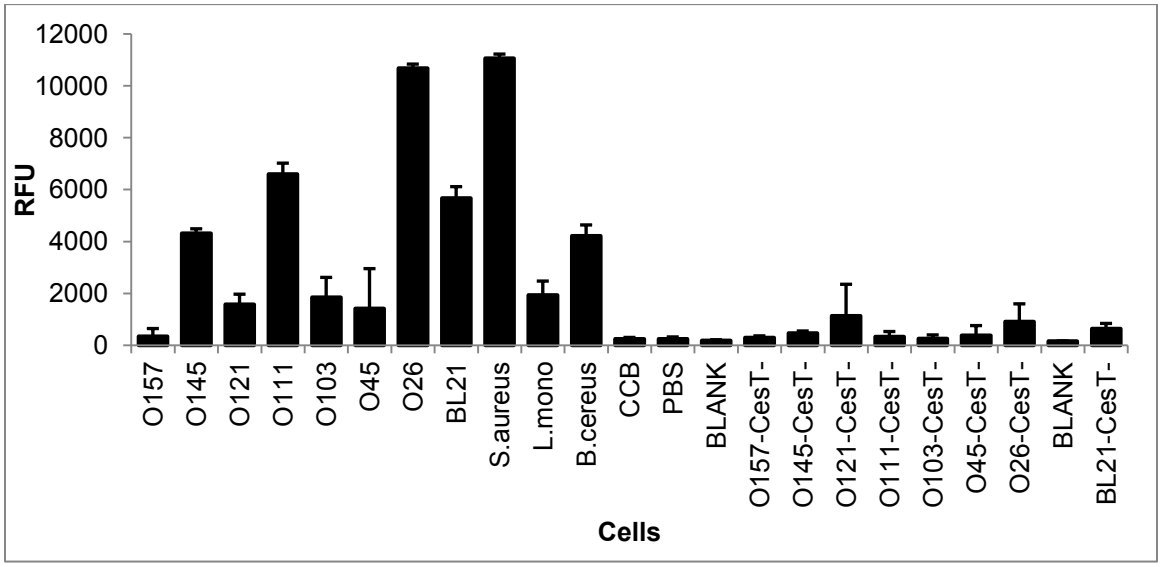

Figure 4-6 ELISA reaction profile of recombinant CesT with *E. coli* spp. Bacterial cells were adjusted to 10^8 cells/well prior to immobilization on the plate. CesT-indiates well to which no CesT protein was added. BLANK indicates emplty wells. Data are an average of three experiments analyzed in quadruplicate.

TIR-CesT conjugate protein-based STEC detection: Recombinant TIR-CesT conjugated proteins were tested for STEC detection. All pathogenic *E. coli* strains gave a high fluorescence values, but unfortunately, all the negative controls used also yielded high values (Figure 3-7). High fluorescence values towards the pathogenic *E. coli* strains indicate that recombinant TIR is binding with the intimin.

However, since the TIR-intimin interaction is highly specific, the non-specificity towards other controls can be attributed to CesT. We therefore conclude that if the TIR-CesT conjugate can be separated, we can utilize the purified TIR for STEC detection and the non-specificity towards other bacterial strains can be minimized.

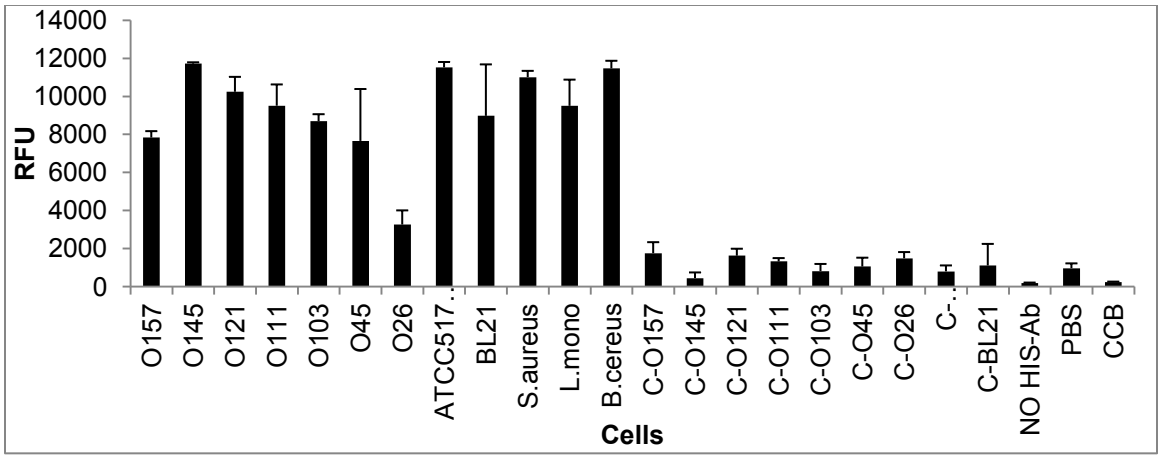

Figure 4-7 ELISA reaction profile of recombinant TIR-CesT conjugate with *E. coli* spp. Bacterial cells were adjusted to 10^8 cells/well prior to immobilization on the plate. C- indicates wells to which the the TIR-CesT conjugate was not added. Data are an average of three experiments analyzed in quadruplicate.

4.3.7 TIR-CesT protein separation

To separate TIR-CesT conjugate, we utilized various chaotropic agents for protein separation. We used 6M and 8M Guanidine hydrochloride (GndHCl) and 4M and 8M Urea for TIR-CesT separation. GndHCl is one of the strongest denaturants used to achieve protein unfolding. 6M GndHCl has been shown to loosen the well ordered structures of most proteins [352]. Similarly, direct interaction of proteins with urea causes weakening of the intermolecular bonds within the protein that results in denaturation and loss of structure. TIR-CesT

complex was exposed to these chaotropic agents for 5 min, 30 min and 1 hour, and the proteins were further separated with a 50 kDa molecular weight cut off membrane and then analyzed in an SDS gel. This treatment still failed to separate these two proteins. It is also speculated that prolonged exposure to harsh reagents may have degraded TIR and reduced overall protein stability (Figure 3-8).

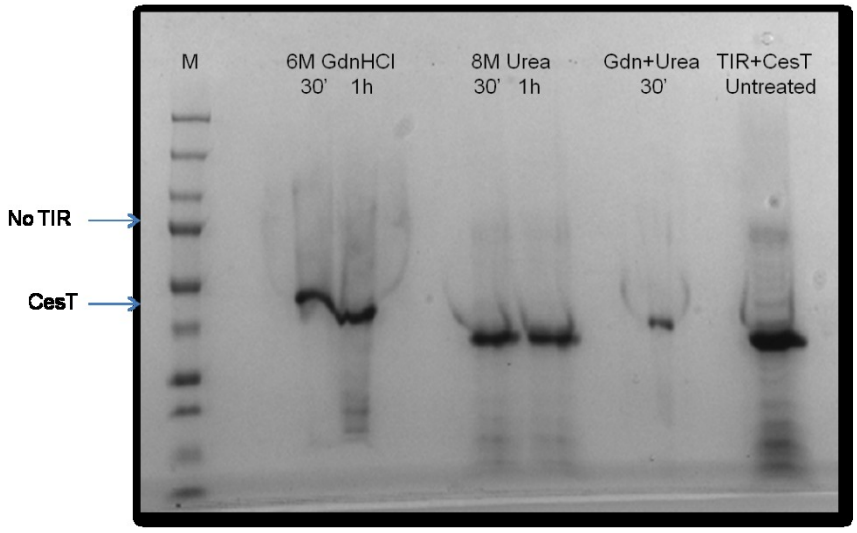

Figure 4-8 TIR-CesT separation using chaotropic reagents Guanidimium hydrochloride and Urea for 30 minutes and 1h.

4.3.8 Next steps

An alternate to having both TIR and CesT as HIS-tagged proteins would be to develop one of the proteins as a GST-tagged protein. This would address both the issues observed in this study: firstly, the co-expression of CesT with TIR would enhance TIR stability and secondly, since both TIR and CesT would be tagged with different amino acids (HIS and GST), separation of the proteins would be easily achieved by using different affinity chromatography columns.

To obtain TIR protein with a GST tag, full length *tir* gene was first cloned into the cloning vector pGEM-T Easy (Promega) and from that into a *Bgl*II and *Sal*I digested pGEX-6P-1 expression vector which contains a GST-tag at the N-terminal. GST is a 26 kDa protein which enables stability and solubility of the recombinant protein. Figure 3-9 shows tir gene in pGEM-T Easy cloning vector.

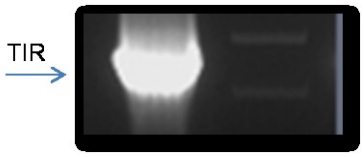

Figure 4-9 *tir* gene in pGEM T Easy vector prior to insertion in *Bgl*II and *Sal*I digested pGEX-6P-1 expression vector.

Next steps involve inserting the gene into pGEX-6P-1 expression vector and then GST-tagged TIR protein expression and purification and utilization of the recombinant TIR for STEC detection.

REFERENCES

REFERENCES

1. Scallan, E., et al., *Foodborne illness acquired in the United States--major pathogens.* Emerg Infect Dis, 2011. **17**(1): p. 7-15.
2. *The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010.* Euro Surveill, 2012. **17**(10).
3. Kuchenmuller, T., et al., *Estimating the global burden of foodborne diseases--a collaborative effort.* Euro Surveill, 2009. **14**(18).
4. Frenzen, P.D., A. Drake, and F.J. Angulo, *Economic cost of illness due to Escherichia coli O157 infections in the United States.* J Food Prot, 2005. **68**(12): p. 2623-30.
5. Buzby, J.C. and T. Roberts, *Economic costs and trade impacts of microbial foodborne illness.* World Health Stat Q, 1997. **50**(1-2): p. 57-66.
6. Buzby, J.C. and T. Roberts, *The economics of enteric infections: human foodborne disease costs.* Gastroenterology, 2009. **136**(6): p. 1851-62.
7. Bhunia, A.K., *Biosensors and bio-based methods for the separation and detection of foodborne pathogens.* Adv Food Nutr Res, 2008. **54**: p. 1-44.
8. Banerjee, P. and A.K. Bhunia, *Mammalian cell-based biosensors for pathogens and toxins.* Trends Biotechnol, 2009. **27**(3): p. 179-88.
9. Allmann, M., et al., *Polymerase Chain-Reaction (Pcr) for Detection of Pathogenic Microorganisms in Bacteriological Monitoring of Dairy-Products.* Research in Microbiology, 1995. **146**(1): p. 85-97.
10. Scheu, P.M., K. Berghof, and U. Stahl, *Detection of pathogenic and spoilage micro-organisms in food with the polymerase chain reaction.* Food Microbiology, 1998. **15**(1): p. 13-31.
11. Wang, R.F., W.W. Cao, and C.E. Cerniglia, *A universal protocol for PCR detection of 13 species of foodborne pathogens in foods.* Journal of Applied Microbiology, 1997. **83**(6): p. 727-736.
12. Bansal, N.S., *Development of a polymerase chain reaction assay for the detection of Listeria monocytogenes in foods.* Lett Appl Microbiol, 1996. **22**(5): p. 353-6.
13. Bansal, N.S., et al., *Multiplex PCR assay for the routine detection of Listeria in food.* Int J Food Microbiol, 1996. **33**(2-3): p. 293-300.
14. Olsen, J.E., *DNA-based methods for detection of food-borne bacterial pathogens.* Food Research International, 2000. **33**(3-4): p. 257-266.

15. Almeida, C., et al., *Fluorescence in situ hybridization method using a peptide nucleic acid probe for identification of Salmonella spp. in a broad spectrum of samples.* Appl Environ Microbiol, 2010. **76**(13): p. 4476-85.
16. McLoughlin, K.S., *Microarrays for pathogen detection and analysis.* Brief Funct Genomics, 2011. **10**(6): p. 342-53.
17. Rasooly, A. and K.E. Herold, *Food microbial pathogen detection and analysis using DNA microarray technologies.* Foodborne Pathog Dis, 2008. **5**(4): p. 531-50.
18. Fang, H., et al., *An FDA bioinformatics tool for microbial genomics research on molecular characterization of bacterial foodborne pathogens using microarrays.* BMC Bioinformatics, 2010. **11 Suppl 6**: p. S4.
19. Lazcka, O., F.J. Del Campo, and F.X. Munoz, *Pathogen detection: a perspective of traditional methods and biosensors.* Biosens Bioelectron, 2007. **22**(7): p. 1205-17.
20. Zordan, M.D., et al., *Detection of pathogenic E. coli O157:H7 by a hybrid microfluidic SPR and molecular imaging cytometry device.* Cytometry A, 2009. **75**(2): p. 155-62.
21. Valadez, A.M., et al., *Evanescent wave fiber optic biosensor for salmonella detection in food.* Sensors (Basel), 2009. **9**(7): p. 5810-24.
22. Leach, K.M., J.M. Stroot, and D.V. Lim, *Same-day detection of Escherichia coli O157:H7 from spinach by using electrochemiluminescent and cytometric bead array biosensors.* Appl Environ Microbiol, 2010. **76**(24): p. 8044-52.
23. Pividori, M.I., A. Merkoci, and S. Alegret, *Electrochemical genosensor design: immobilisation of oligonucleotides onto transducer surfaces and detection methods.* Biosens Bioelectron, 2000. **15**(5-6): p. 291-303.
24. Koo, O.K., et al., *Targeted capture of pathogenic bacteria using a mammalian cell receptor coupled with dielectrophoresis on a biochip.* Anal Chem, 2009. **81**(8): p. 3094-101.
25. Kastritis, P.L. and A.M. Bonvin, *On the binding affinity of macromolecular interactions: daring to ask why proteins interact.* J R Soc Interface, 2013. **10**(79): p. 20120835.
26. Ruhlmann, A., et al., *Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. Crystal structure determination and stereochemistry of the contact region.* J Mol Biol, 1973. **77**(3): p. 417-36.
27. Lim, Y.C., A.Z. Kouzani, and W. Duan, *Aptasensors: a review.* J Biomed Nanotechnol, 2010. **6**(2): p. 93-105.
28. Tombelli, S., M. Minunni, and M. Mascini, *Biosensors for RNA aptamers-protein interaction.* Methods Mol Biol, 2008. **419**: p. 109-19.
29. Minunni, M., et al., *Development of biosensors with aptamers as bio-recognition element: the case of HIV-1 Tat protein.* Biosens Bioelectron, 2004. **20**(6): p. 1149-56.
30. Wang, R., et al., *Immobilisation of DNA probes for the development of SPR-based sensing.* Biosens Bioelectron, 2004. **20**(5): p. 967-74.

31. Schultz, J.S., *Sensitivity and dynamics of bioreceptor-based biosensors.* Ann N Y Acad Sci, 1987. **506**: p. 406-14.
32. Richman, S.A., D.M. Kranz, and J.D. Stone, *Biosensor detection systems: engineering stable, high-affinity bioreceptors by yeast surface display.* Methods Mol Biol, 2009. **504**: p. 323-50.
33. Saitakis, M., A. Tsortos, and E. Gizeli, *Probing the interaction of a membrane receptor with a surface-attached ligand using whole cells on acoustic biosensors.* Biosens Bioelectron, 2010. **25**(7): p. 1688-93.
34. Sassolas, A., L.J. Blum, and B.D. Leca-Bouvier, *Immobilization strategies to develop enzymatic biosensors.* Biotechnol Adv, 2012. **30**(3): p. 489-511.
35. Yang, H., *Enzyme-based ultrasensitive electrochemical biosensors.* Curr Opin Chem Biol, 2012. **16**(3-4): p. 422-8.
36. Bar-Haim, E., et al., *[Laboratory methods for detection and identification of biological pathogens].* Harefuah, 2005. **144**(5): p. 341-6, 382.
37. Iqbal, S.S., et al., *A review of molecular recognition technologies for detection of biological threat agents.* Biosens Bioelectron, 2000. **15**(11-12): p. 549-78.
38. Banada, P.P., et al., *Optical forward-scattering for detection of Listeria monocytogenes and other Listeria species.* Biosens Bioelectron, 2007. **22**(8): p. 1664-71.
39. Bae, E., et al., *Biophysical modeling of forward scattering from bacterial colonies using scalar diffraction theory.* Appl Opt, 2007. **46**(17): p. 3639-48.
40. Banada, P.P., et al., *Label-free detection of multiple bacterial pathogens using light-scattering sensor.* Biosens Bioelectron, 2009. **24**(6): p. 1685-92.
41. Rajwa, B., et al., *Discovering the Unknown: Detection of Emerging Pathogens Using a Label-Free Light-Scattering System.* Cytometry Part A, 2010. **77A**(12): p. 1103-1112.
42. Kaittanis, C., S. Santra, and J.M. Perez, *Emerging nanotechnology-based strategies for the identification of microbial pathogenesis.* Adv Drug Deliv Rev, 2010. **62**(4-5): p. 408-23.
43. Lucore, L.A., M.A. Cullison, and L.A. Jaykus, *Immobilization with metal hydroxides as a means to concentrate food-borne bacteria for detection by cultural and molecular methods.* Appl Environ Microbiol, 2000. **66**(5): p. 1769-76.
44. Mead, P.S., et al., *Food-related illness and death in the United States.* Emerg Infect Dis, 1999. **5**(5): p. 607-25.
45. Kingsley, R.A., et al., *Epidemic multiple drug resistant Salmonella Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype.* Genome Research, 2009. **19**(12): p. 2279-2287.

46. Sheikh, A., R. C. Charles, S. Rollins, J. B. Harris, M. S. Bhuiyan, F. Khanam, A. Bukka, A. Kalsy, S. Porwollik, A. Brooks, R. C. LaRocque, M. McClelland, T. Logvinenko, A. Cravioto, S. B. Calderwood, J. E. Graham, F. Qadri, and E. T. Ryan. 2, *High throughput gene expression profiling of Salmonella enterica serovar Paratyphi A in the blood of bacteremic patients in Bangladesh.* Salmonella: Biology, Pathogenesis and Prevention. American Society for Microbiology, Washington, DC., 2009.
47. Andrews-Polymenis, H.L., et al., *Taming the elephant: Salmonella biology, pathogenesis, and prevention.* Infect Immun, 2010. **78**(6): p. 2356-69.
48. Frenzen P, R.T., Buzby J, Breuer T, Roberts T, Voetsch D, *Salmonella cost estimate update using FoodNet data.* Food Reviews, 1999. **22**(10): p. 5.
49. Fatica, M.K. and K.R. Schneider, *Salmonella and produce: survival in the plant environment and implications in food safety.* Virulence, 2011. **2**(6): p. 573-9.
50. Werber, D., et al., *Outbreaks of virulent diarrheagenic Escherichia coli--are we in control?* BMC Med, 2012. **10**: p. 11.
51. Farfan, M.J. and A.G. Torres, *Molecular mechanisms that mediate colonization of Shiga toxin-producing Escherichia coli strains.* Infect Immun, 2012. **80**(3): p. 903-13.
52. Lim, J.Y., J. Yoon, and C.J. Hovde, *A brief overview of Escherichia coli O157:H7 and its plasmid O157.* J Microbiol Biotechnol, 2010. **20**(1): p. 5-14.
53. Ferens, W.A. and C.J. Hovde, *Escherichia coli O157:H7: animal reservoir and sources of human infection.* Foodborne Pathog Dis, 2011. **8**(4): p. 465-87.
54. Rebagliati, V., et al., *Prevention of foodborne listeriosis.* Indian J Pathol Microbiol, 2009. **52**(2): p. 145-9.
55. Camejo, A., et al., *The arsenal of virulence factors deployed by Listeria monocytogenes to promote its cell infection cycle.* Virulence, 2011. **2**(5): p. 379-94.
56. Le Loir, Y., F. Baron, and M. Gautier, *Staphylococcus aureus and food poisoning.* Genet Mol Res, 2003. **2**(1): p. 63-76.
57. Pinchuk, I.V., E.J. Beswick, and V.E. Reyes, *Staphylococcal enterotoxins.* Toxins (Basel), 2010. **2**(8): p. 2177-97.
58. Schelin, J., et al., *The formation of Staphylococcus aureus enterotoxin in food environments and advances in risk assessment.* Virulence, 2011. **2**(6): p. 580-92.
59. Adams M.R., M.M.O., *Bacterial agents of foodborne illness—Staphylococcus aureus.* Food Microbiology. Cambridge: Royal Society of Chemistry, 2008.
60. Poropatich, K.O., C.L. Walker, and R.E. Black, *Quantifying the association between Campylobacter infection and Guillain-Barre syndrome: a systematic review.* J Health Popul Nutr, 2010. **28**(6): p. 545-52.

61. Smith, J.L., *Campylobacter jejuni infection during pregnancy: long-term consequences of associated bacteremia, Guillain-Barre syndrome, and reactive arthritist.* J Food Prot, 2002. **65**(4): p. 696-708.
62. van Putten, J.P., et al., *Molecular mechanisms of campylobacter infection.* Curr Top Microbiol Immunol, 2009. **337**: p. 197-229.
63. Dasti, J.I., et al., *Campylobacter jejuni: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms.* Int J Med Microbiol, 2010. **300**(4): p. 205-11.
64. Sahin, O., T.Y. Morishita, and Q. Zhang, *Campylobacter colonization in poultry: sources of infection and modes of transmission.* Anim Health Res Rev, 2002. **3**(2): p. 95-105.
65. Lin, J., *Novel approaches for Campylobacter control in poultry.* Foodborne Pathog Dis, 2009. **6**(7): p. 755-65.
66. McClane, B.A., *An overview of Clostridium perfringens enterotoxin.* Toxicon, 1996. **34**(11-12): p. 1335-43.
67. Brynestad, S. and P.E. Granum, *Clostridium perfringens and foodborne infections.* Int J Food Microbiol, 2002. **74**(3): p. 195-202.
68. Smith, L.D., *Virulence factors of Clostridium perfringens.* Rev Infect Dis, 1979. **1**(2): p. 254-62.
69. Andersson, A., U. Ronner, and P.E. Granum, *What problems does the food industry have with the spore-forming pathogens Bacillus cereus and Clostridium perfringens?* Int J Food Microbiol, 1995. **28**(2): p. 145-55.
70. Narayan, K.G., *Food borne infection with Clostridium perfringens type A.* Int J Zoonoses, 1982. **9**(1): p. 12-32.
71. Bartlett, J.M. and D. Stirling, *A short history of the polymerase chain reaction.* Methods Mol Biol, 2003. **226**: p. 3-6.
72. Chamberlain, J.S., et al., *Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification.* Nucleic Acids Res, 1988. **16**(23): p. 11141-56.
73. Hayden, M.J., et al., *Multiplex-ready PCR: a new method for multiplexed SSR and SNP genotyping.* BMC Genomics, 2008. **9**: p. 80.
74. Purcell, M.K., et al., *Quantitative polymerase chain reaction (PCR) for detection of aquatic animal pathogens in a diagnostic laboratory setting.* J Aquat Anim Health, 2011. **23**(3): p. 148-61.
75. Paton, A.W. and J.C. Paton, *Detection and characterization of STEC in stool samples using PCR.* Methods Mol Med, 2003. **73**: p. 45-54.
76. Yu, W.L. and K. Nielsen, *Review of detection of Brucella spp. by polymerase chain reaction.* Croat Med J, 2010. **51**(4): p. 306-13.
77. Ospino, D.U., G. Young, and O.A. Navarro, *Viral gastroenteritis and diversity of Rotavirus strains in Colombian children: a systematic review.* J Infect Dev Ctries, 2008. **2**(2): p. 99-105.
78. Abubakar, I., et al., *A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food.* Health Technol Assess, 2007. **11**(36): p. 1-216.

79. Vasconcelos, N.G., et al., *Molecular detection of enterotoxins E, G, H and I in Staphylococcus aureus and coagulase-negative staphylococci isolated from clinical samples of newborns in Brazil.* J Appl Microbiol, 2011. **111**(3): p. 749-62.
80. Gunaydin, B., O. Aslantas, and C. Demir, *Detection of superantigenic toxin genes in Staphylococcus aureus strains from subclinical bovine mastitis.* Trop Anim Health Prod, 2011. **43**(8): p. 1633-7.
81. Nakamura, Y., et al., *[Food-borne botulism].* Brain Nerve, 2011. **63**(9): p. 979-85.
82. Park, C.E., M. Akhtar, and M.K. Rayman, *Evaluation of a commercial enzyme immunoassay kit (RIDASCREEN) for detection of staphylococcal enterotoxins A, B, C, D, and E in foods.* Appl Environ Microbiol, 1994. **60**(2): p. 677-81.
83. Kim, J.S., et al., *Multiplexed magnetic microsphere immunoassays for detection of pathogens in foods.* Sens Instrum Food Qual Saf, 2010. **4**(2): p. 73-81.
84. Trainer, V.L. and D.G. Baden, *An enzyme immunoassay for the detection of Florida red tide brevetoxins.* Toxicon, 1991. **29**(11): p. 1387-94.
85. Nagahama, M., et al., *Enzyme-linked immunosorbent assay for rapid detection of toxins from Clostridium perfringens.* FEMS Microbiol Lett, 1991. **68**(1): p. 41-4.
86. Ekong, T.A., K. McLellan, and D. Sesardic, *Immunological detection of Clostridium botulinum toxin type A in therapeutic preparations.* J Immunol Methods, 1995. **180**(2): p. 181-91.
87. Dezfulian, M. and J.G. Bartlett, *Detection of Clostridium botulinum type A toxin by enzyme-linked immunosorbent assay with antibodies produced in immunologically tolerant animals.* J Clin Microbiol, 1984. **19**(5): p. 645-8.
88. Doellgast, G.J., et al., *Sensitive enzyme-linked immunosorbent assay for detection of Clostridium botulinum neurotoxins A, B, and E using signal amplification via enzyme-linked coagulation assay.* J Clin Microbiol, 1993. **31**(9): p. 2402-9.
89. Scotcher, M.C., L.W. Cheng, and L.H. Stanker, *Detection of botulinum neurotoxin serotype B at sub mouse LD(50) levels by a sandwich immunoassay and its application to toxin detection in milk.* PLoS ONE, 2010. **5**(6): p. e11047.
90. Clotilde, L.M., et al., *Microbead-based immunoassay for simultaneous detection of Shiga toxins and isolation of Escherichia coli O157 in foods.* J Food Prot, 2011. **74**(3): p. 373-9.
91. Sano, T., C.L. Smith, and C.R. Cantor, *Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates.* Science, 1992. **258**(5079): p. 120-2.
92. Malou, N. and D. Raoult, *Immuno-PCR: a promising ultrasensitive diagnostic method to detect antigens and antibodies.* Trends Microbiol, 2011. **19**(6): p. 295-302.

93. He, X., et al., *Sensitive detection of Shiga Toxin 2 and some of its variants in environmental samples by a novel immuno-PCR assay.* Appl Environ Microbiol, 2011. **77**(11): p. 3558-64.
94. Zhang, W., et al., *New immuno-PCR assay for detection of low concentrations of shiga toxin 2 and its variants.* J Clin Microbiol, 2008. **46**(4): p. 1292-7.
95. Chao, H.Y., et al., *A highly sensitive immuno-polymerase chain reaction assay for Clostridium botulinum neurotoxin type A.* Toxicon, 2004. **43**(1): p. 27-34.
96. Fischer, A., et al., *A quantitative real-time immuno-PCR approach for detection of staphylococcal enterotoxins.* J Mol Med (Berl), 2007. **85**(5): p. 461-9.
97. Allen, R.C., et al., *An immuno-PCR method for detecting Bacillus thuringiensis Cry1Ac toxin.* J Immunol Methods, 2006. **308**(1-2): p. 109-15.
98. Uyttendaele, M., I. Van Hoorde, and J. Debevere, *The use of immuno-magnetic separation (IMS) as a tool in a sample preparation method for direct detection of L-monocytogenes in cheese.* International Journal of Food Microbiology, 2000. **54**(3): p. 205-212.
99. Su, X.L. and Y. Li, *Quantum dot biolabeling coupled with immunomagnetic separation for detection of Escherichia coli O157:H7.* Anal Chem, 2004. **76**(16): p. 4806-10.
100. Koujitani, E., et al., *Immuno-magnetic separation and agar layer methods for the isolation of freeze-injured Yersinia enterocolitica O:8 from water.* J Vet Med Sci, 2006. **68**(3): p. 195-9.
101. Wang, H., et al., *Rapid, Sensitive, and Simultaneous Detection of Three Foodborne Pathogens Using Magnetic Nanobead-Based Immunoseparation and Quantum Dot-Based Multiplex Immunoassay.* Journal of Food Protection, 2011. **74**(12): p. 2039-2047.
102. Docherty, L., et al., *The magnetic immuno-polymerase chain reaction assay for the detection of Campylobacter in milk and poultry.* Lett Appl Microbiol, 1996. **22**(4): p. 288-92.
103. Widjojoatmodjo, M.N., et al., *The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples.* J Clin Microbiol, 1992. **30**(12): p. 3195-9.
104. Yazdankhah, S.P., et al., *Development and evaluation of an immunomagnetic separation-ELISA for the detection of Staphylococcus aureus thermostable nuclease in composite milk.* Vet Microbiol, 1999. **67**(2): p. 113-25.
105. Massey, S., B. Quinones, and K. Teter, *A cell-based fluorescent assay to detect the activity of Shiga toxin and other toxins that inhibit protein synthesis.* Methods Mol Biol, 2011. **739**: p. 49-59.
106. Lee, D.H., et al., *In vitro bioassay of endotoxin using fluorescein as a pH indicator in a macrophage cell culture system.* Yonsei Med J, 2005. **46**(2): p. 268-74.

107. Manger, R.L., et al., *Detection of sodium channel toxins: directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts.* J AOAC Int, 1995. **78**(2): p. 521-7.
108. Pancrazio, J.J., et al., *Development and application of cell-based biosensors.* Ann Biomed Eng, 1999. **27**(6): p. 697-711.
109. Rider, T.H., et al., *A B cell-based sensor for rapid identification of pathogens.* Science, 2003. **301**(5630): p. 213-5.
110. Wampler, J.L., et al., *Heat shock protein 60 acts as a receptor for the Listeria adhesion protein in Caco-2 cells.* Infect Immun, 2004. **72**(2): p. 931-6.
111. Asphahani, F. and M. Zhang, *Cellular impedance biosensors for drug screening and toxin detection.* Analyst, 2007. **132**(9): p. 835-41.
112. Toh, Y.C., et al., *A novel 3D mammalian cell perfusion-culture system in microfluidic channels.* Lab Chip, 2007. **7**(3): p. 302-9.
113. Banerjee, P., et al., *A novel and simple cell-based detection system with a collagen-encapsulated B-lymphocyte cell line as a biosensor for rapid detection of pathogens and toxins.* Lab Invest, 2008. **88**(2): p. 196-206.
114. Hwang, B.H., et al., *Specific multiplex analysis of pathogens using a direct 16S rRNA hybridization in microarray system.* Anal Chem, 2012. **84**(11): p. 4873-9.
115. Lee, J.Y., et al., *A subtractively optimized DNA microarray using non-sequenced genomic probes for the detection of food-borne pathogens.* Appl Biochem Biotechnol, 2011. **164**(2): p. 183-93.
116. Kostrzynska, M. and A. Bachand, *Application of DNA microarray technology for detection, identification, and characterization of food-borne pathogens.* Can J Microbiol, 2006. **52**(1): p. 1-8.
117. Suo, B., et al., *Development of an oligonucleotide-based microarray to detect multiple foodborne pathogens.* Mol Cell Probes, 2010. **24**(2): p. 77-86.
118. Quinones, B., et al., *Identification of Escherichia coli O157 by using a novel colorimetric detection method with DNA microarrays.* Foodborne Pathog Dis, 2011. **8**(6): p. 705-11.
119. Wang, Q., et al., *Development of a DNA microarray for detection and serotyping of enterotoxigenic Escherichia coli.* J Clin Microbiol, 2010. **48**(6): p. 2066-74.
120. Gui, J. and I.R. Patel, *Recent advances in molecular technologies and their application in pathogen detection in foods with particular reference to yersinia.* J Pathog, 2011. **2011**: p. 310135.
121. Bahl, A., et al., *A novel multifunctional oligonucleotide microarray for Toxoplasma gondii.* BMC Genomics, 2010. **11**: p. 603.
122. Lakey, J.H. and E.M. Raggett, *Measuring protein-protein interactions.* Curr Opin Struct Biol, 1998. **8**(1): p. 119-23.
123. Rich, R.L. and D.G. Myszka, *Survey of the year 2004 commercial optical biosensor literature.* J Mol Recognit, 2005. **18**(6): p. 431-78.

124. Soelberg, S.D., et al., *A portable surface plasmon resonance sensor system for real-time monitoring of small to large analytes.* J Ind Microbiol Biotechnol, 2005. **32**(11-12): p. 669-74.
125. Bergwerff, A.A. and F. van Knapen, *Surface plasmon resonance biosensors for detection of pathogenic microorganisms: strategies to secure food and environmental safety.* J AOAC Int, 2006. **89**(3): p. 826-31.
126. Kausaite, A., et al., *[Surface plasmon resonance and its application to biomedical research].* Medicina (Kaunas), 2007. **43**(5): p. 355-65.
127. Nanduri, V., et al., *SPR biosensor for the detection of L. monocytogenes using phage-displayed antibody.* Biosens Bioelectron, 2007. **23**(2): p. 248-52.
128. Baccar, H., et al., *Surface plasmon resonance immunosensor for bacteria detection.* Talanta, 2010. **82**(2): p. 810-4.
129. Dudak, F.C. and I.H. Boyaci, *Rapid and label-free bacteria detection by surface plasmon resonance (SPR) biosensors.* Biotechnol J, 2009. **4**(7): p. 1003-11.
130. Jyoung, J.Y., et al., *Immunosensor for the detection of Vibrio cholerae O1 using surface plasmon resonance.* Biosens Bioelectron, 2006. **21**(12): p. 2315-9.
131. Swain, M.D., et al., *Llama-derived single-domain antibodies for the detection of botulinum A neurotoxin.* Anal Bioanal Chem, 2010. **398**(1): p. 339-48.
132. Spangler, B.D., et al., *Comparison of the Spreeta (R) surface plasmon resonance sensor and a quartz crystal microbalance for detection of Escherichia coli heat-labile enterotoxin.* Analytica Chimica Acta, 2001. **444**(1): p. 149-161.
133. Li, J.Q., Q.L. Kong, and Z. Fan, *[SPR detection of affinity of antibacterial peptides in bactericidal/permeability-increasing protein domain for endotoxin].* Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi, 2010. **26**(11): p. 1143-5.
134. Marazuela, D. and M.C. Moreno-Bondi, *Fiber-optic biosensors--an overview.* Anal Bioanal Chem, 2002. **372**(5-6): p. 664-82.
135. Bosch, M.E., et al., *Recent development in optical fiber biosensors.* Sensors, 2007. **7**(6): p. 797-859.
136. Ogert, R.A., et al., *Detection of Clostridium-Botulinum Toxin-a Using a Fiber Optic-Based Biosensor.* Analytical Biochemistry, 1992. **205**(2): p. 306-312.
137. Kramer, M.F. and D.V. Lim, *A rapid and automated fiber optic-based biosensor assay for the detection of Salmonella in spent irrigation water used in the sprouting of sprout seeds.* J Food Prot, 2004. **67**(1): p. 46-52.
138. Cao, L.K., et al., *Detection of Yersinia pestis fraction 1 antigen with a fiber optic biosensor.* J Clin Microbiol, 1995. **33**(2): p. 336-41.
139. DeMarco, D.R. and D.V. Lim, *Direct detection of Escherichia coli O157 : H7 in unpasteurized apple juice with an evanescent wave biosensor.* Journal of Rapid Methods and Automation in Microbiology, 2001. **9**(4): p. 241-257.

140. Demarco, D.R. and D.V. Lim, *Detection of Escherichia coli O157:H7 in 10- and 25-gram ground beef samples with an evanescent-wave biosensor with silica and polystyrene waveguides.* J Food Prot, 2002. **65**(4): p. 596-602.
141. Tempelman, L.A., et al., *Quantitating staphylococcal enterotoxin B in diverse media using a portable fiber-optic biosensor.* Analytical Biochemistry, 1996. **233**(1): p. 50-57.
142. Tims, T.B., et al., *Detection of low levels of Listeria monocytogenes within 20 hours using an evanescent wave biosensor.* Am Clin Lab, 2001. **20**(8): p. 28-9.
143. Geng, T., M.T. Morgan, and A.K. Bhunia, *Detection of low levels of Listeria monocytogenes cells by using a fiber-optic immunosensor.* Applied and Environmental Microbiology, 2004. **70**(10): p. 6138-6146.
144. Bayraktar, B., et al., *Feature extraction from light-scatter patterns of Listeria colonies for identification and classification.* J Biomed Opt, 2006. **11**(3): p. 34006.
145. Huff, K., et al., *Light-scattering sensor for real-time identification of Vibrio parahaemolyticus, Vibrio vulnificus and Vibrio cholerae colonies on solid agar plate.* Microb Biotechnol, 2012. **5**(5): p. 607-20.
146. Aguirre, S.D., et al., *Detection of bacteria using fluorogenic DNAzymes.* J Vis Exp, 2012(63).
147. Temur, E., et al., *A highly sensitive detection platform based on surface-enhanced Raman scattering for Escherichia coli enumeration.* Anal Bioanal Chem, 2010. **397**(4): p. 1595-604.
148. Smolina, I., N.S. Miller, and M. Frank-Kamenetskii, *PNA-based microbial pathogen identification and resistance marker detection: an accurate, isothermal rapid assay based on genome-specific features.* Artif DNA PNA XNA, 2010. **1**(2): p. 1-7.
149. Shin, G.W., et al., *A two-step quantitative pathogen detection system based on capillary electrophoresis.* Analytical Biochemistry, 2008. **383**(1): p. 31-7.
150. Chakravorty, S., et al., *Rapid Universal Identification of Bacterial Pathogens from Clinical Cultures by Using a Novel Sloppy Molecular Beacon Melting Temperature Signature Technique.* Journal of Clinical Microbiology, 2010. **48**(1): p. 258-267.
151. Mori, Y. and T. Notomi, *Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases.* Journal of Infection and Chemotherapy, 2009. **15**(2): p. 62-69.
152. DebRoy, C., E. Roberts, and P.M. Fratamico, *Detection of O antigens in Escherichia coli.* Anim Health Res Rev, 2011. **12**(2): p. 169-85.
153. Kaper, J.B., J.P. Nataro, and H.L. Mobley, *Pathogenic Escherichia coli.* Nat Rev Microbiol, 2004. **2**(2): p. 123-40.

154. Rodas, C., et al., *Enterotoxins, colonization factors, serotypes and antimicrobial resistance of enterotoxigenic Escherichia coli (ETEC) strains isolated from hospitalized children with diarrhea in Bolivia.* Brazilian Journal of Infectious Diseases, 2011. **15**(2): p. 132-7.
155. Robins-Browne, R.M. and E.L. Hartland, *Escherichia coli as a cause of diarrhea.* Journal of Gastroenterology and Hepatology, 2002. **17**(4): p. 467-475.
156. Ochoa, T.J. and C.A. Contreras, *Enteropathogenic escherichia coli infection in children.* Curr Opin Infect Dis, 2011. **24**(5): p. 478-83.
157. Humphries, R.M. and G.D. Armstrong, *Sticky situation: localized adherence of enteropathogenic Escherichia coli to the small intestine epithelium.* Future Microbiol, 2010. **5**(11): p. 1645-61.
158. Ochoa, T.J., et al., *New insights into the epidemiology of enteropathogenic Escherichia coli infection.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 2008. **102**(9): p. 852-856.
159. Kenny, B., et al., *Enteropathogenic Escherichia coli protein secretion is induced in response to conditions similar to those in the gastrointestinal tract.* Infection and Immunity, 1997. **65**(7): p. 2606-2612.
160. Nataro, J.P., *Enteroaggregative Escherichia coli pathogenesis.* Current Opinion in Gastroenterology, 2005. **21**(1): p. 4-8.
161. Nataro, J.P., et al., *Patterns of adherence of diarrheagenic Escherichia coli to HEp-2 cells.* Pediatr Infect Dis J, 1987. **6**(9): p. 829-31.
162. Scaletsky, I.C.A., et al., *Diffusely adherent Escherichia coli as a cause of acute diarrhea in young children in northeast Brazil: a case-control study.* Journal of Clinical Microbiology, 2002. **40**(2): p. 645-648.
163. Kenny, B., et al., *Enteropathogenic Escherichia coli protein secretion is induced in response to conditions similar to those in the gastrointestinal tract.* Infect Immun, 1997. **65**(7): p. 2606-12.
164. Konowalchuk, J., J.I. Speirs, and S. Stavric, *Vero response to a cytotoxin of Escherichia coli.* Infect Immun, 1977. **18**(3): p. 775-9.
165. Edelman, R., M.A. Karmali, and P.A. Fleming, *From the National Institutes of Health. Summary of the International Symposium and Workshop on Infections due to Verocytotoxin (Shiga-like toxin)-producing Escherichia coli.* J Infect Dis, 1988. **157**(5): p. 1102-4.
166. Karmali, M.A., et al., *The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing Escherichia coli.* J Infect Dis, 1985. **151**(5): p. 775-82.
167. Scotland, S.M., et al., *Vero cytotoxin-producing strains of Escherichia coli from children with haemolytic uraemic syndrome and their detection by specific DNA probes.* J Med Microbiol, 1988. **25**(4): p. 237-43.
168. Smith, H.R. and S.M. Scotland, *Vero cytotoxin-producing strains of Escherichia coli.* J Med Microbiol, 1988. **26**(2): p. 77-85.
169. Lipp, M.J., et al., *[EHEC-associated colon stenosis after ulcerous-chronic haemorrhagic colitis and consecutive resulting ileus].* Z Gastroenterol, 2012. **50**(5): p. 453-6.

170. Bielaszewska, M. and H. Karch, *Consequences of enterohaemorrhagic Escherichia coli infection for the vascular endothelium.* Thromb Haemost, 2005. **94**(2): p. 312-8.
171. Suri, R.S., et al., *Relationship between Escherichia coli O157:H7 and diabetes mellitus.* Kidney International, 2009. **75**: p. S44-S46.
172. Obata, F., *Influence of Escherichia coli shiga toxin on the mammalian central nervous system.* Adv Appl Microbiol, 2010. **71**: p. 1-19.
173. Weissenborn, K., et al., *Neurologic manifestations of E coli infection-induced hemolytic-uremic syndrome in adults.* Neurology, 2012. **79**(14): p. 1466-73.
174. Johannes, L. and W. Romer, *Shiga toxins - from cell biology to biomedical applications.* Nature Reviews Microbiology, 2010. **8**(2): p. 105-116.
175. Engedal, N., et al., *Shiga toxin and its use in targeted cancer therapy and imaging.* Microbial Biotechnology, 2011. **4**(1): p. 32-46.
176. Pina, D.G., L. Johannes, and M.A.R.B. Castanho, *Shiga toxin B-subunit sequential binding to its natural receptor in lipid membranes.* Biochimica Et Biophysica Acta-Biomembranes, 2007. **1768**(3): p. 628-636.
177. Stein, P.E., et al., *Crystal structure of the cell-binding B oligomer of verotoxin-1 from E. coli.* Nature, 1992. **355**(6362): p. 748-50.
178. Fraser, M.E., et al., *Structure of shiga toxin type 2 (Stx2) from Escherichia coli O157:H7.* J Biol Chem, 2004. **279**(26): p. 27511-7.
179. Donohuerolfe, A., M. Jacewicz, and G.T. Keusch, *Isolation and Characterization of Functional Shiga Toxin Subunits and Renatured Holotoxin.* Molecular Microbiology, 1989. **3**(9): p. 1231-1236.
180. Yu, M. and D.B. Haslam, *Shiga toxin is transported from the endoplasmic reticulum following interaction with the luminal chaperone HEDJ/ERdj3.* Infection and Immunity, 2005. **73**(4): p. 2524-2532.
181. Smith, W.E., et al., *Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells.* Infection and Immunity, 2003. **71**(3): p. 1497-1504.
182. Lee, S.Y., et al., *Shiga toxin 1 induces apoptosis through the endoplasmic reticulum stress response in human monocytic cells.* Cellular Microbiology, 2008. **10**(3): p. 770-780.
183. Reisbig, R., S. Olsnes, and K. Eiklid, *The cytotoxic activity of Shigella toxin. Evidence for catalytic inactivation of the 60 S ribosomal subunit.* J Biol Chem, 1981. **256**(16): p. 8739-44.
184. Deng, W., et al., *Dissecting virulence: systematic and functional analyses of a pathogenicity island.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3597-602.
185. Elliott, S.J., et al., *Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic Escherichia coli.* Mol Microbiol, 1999. **33**(6): p. 1176-89.

186. Mellies, J.L., et al., *The Per regulon of enteropathogenic Escherichia coli : identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler).* Mol Microbiol, 1999. **33**(2): p. 296-306.
187. Sperandio, V., et al., *Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic Escherichia coli.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15196-201.
188. Elliott, S.J., et al., *The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic Escherichia coli E2348/69.* Mol Microbiol, 1998. **28**(1): p. 1-4.
189. Wong, A.R., et al., *Enteropathogenic and enterohaemorrhagic Escherichia coli: even more subversive elements.* Mol Microbiol, 2011. **80**(6): p. 1420-38.
190. Knutton, S., et al., *Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic Escherichia coli.* Infect Immun, 1989. **57**(4): p. 1290-8.
191. Knutton, S., D.R. Lloyd, and A.S. McNeish, *Adhesion of enteropathogenic Escherichia coli to human intestinal enterocytes and cultured human intestinal mucosa.* Infect Immun, 1987. **55**(1): p. 69-77.
192. Knutton, S., et al., *A novel EspA-associated surface organelle of enteropathogenic Escherichia coli involved in protein translocation into epithelial cells.* EMBO J, 1998. **17**(8): p. 2166-76.
193. Donnenberg, M.S., J.B. Kaper, and B.B. Finlay, *Interactions between enteropathogenic Escherichia coli and host epithelial cells.* Trends Microbiol, 1997. **5**(3): p. 109-14.
194. McDaniel, T.K. and J.B. Kaper, *A cloned pathogenicity island from enteropathogenic Escherichia coli confers the attaching and effacing phenotype on E. coli K-12.* Mol Microbiol, 1997. **23**(2): p. 399-407.
195. Elliott, S.J., J. Yu, and J.B. Kaper, *The cloned locus of enterocyte effacement from enterohemorrhagic Escherichia coli O157:H7 is unable to confer the attaching and effacing phenotype upon E. coli K-12.* Infect Immun, 1999. **67**(8): p. 4260-3.
196. Phillips, A.D., *The medium is the messenger.* Gut, 1998. **43**(4): p. 456-457.
197. Frankel, G., et al., *Characterization of the C-terminal domains of intimin-like proteins of enteropathogenic and enterohemorrhagic Escherichia coli, Citrobacter freundii, and Hafnia alvei.* Infect Immun, 1994. **62**(5): p. 1835-42.
198. Jerse, A.E., et al., *A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells.* Proc Natl Acad Sci U S A, 1990. **87**(20): p. 7839-43.
199. Donnenberg, M.S. and J.B. Kaper, *Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector.* Infect Immun, 1991. **59**(12): p. 4310-7.

200. Donnenberg, M.S., et al., *The role of the eae gene of enterohemorrhagic Escherichia coli in intimate attachment in vitro and in a porcine model.* J Clin Invest, 1993. **92**(3): p. 1418-24.
201. Dean-Nystrom, E.A., et al., *Escherichia coli O157:H7 requires intimin for enteropathogenicity in calves.* Infect Immun, 1998. **66**(9): p. 4560-3.
202. Ito, K., et al., *Intimin types determined by heteroduplex mobility assay of intimin gene (eae)-positive Escherichia coli strains.* J Clin Microbiol, 2007. **45**(3): p. 1038-41.
203. Kenny, B., et al., *Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells.* Cell, 1997. **91**(4): p. 511-20.
204. Rosenshine, I., et al., *A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation.* Embo Journal, 1996. **15**(11): p. 2613-2624.
205. DeVinney, R., et al., *Enterohemorrhagic Escherichia coli O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated.* Infect Immun, 1999. **67**(5): p. 2389-98.
206. Campellone, K.G. and J.M. Leong, *Tails of two Tirs: actin pedestal formation by enteropathogenic E-coli and enterohemorrhagic E-coli O157 : H7.* Current Opinion in Microbiology, 2003. **6**(1): p. 82-90.
207. Frankel, G., et al., *The cell-binding domain of intimin from enteropathogenic Escherichia coli binds to beta1 integrins.* J Biol Chem, 1996. **271**(34): p. 20359-64.
208. Batchelor, M., et al., *Structural basis for recognition of the translocated intimin receptor (Tir) by intimin from enteropathogenic Escherichia coli.* Embo Journal, 2000. **19**(11): p. 2452-2464.
209. Jenkins, C., et al., *Antibody response of patients infected with verocytotoxin-producing Escherichia coli to protein antigens encoded on the LEE locus.* Journal of Medical Microbiology, 2000. **49**(1): p. 97-101.
210. de Grado, M., et al., *Identification of the intimin-binding domain of Tir of enteropathogenic Escherichia coli.* Cellular Microbiology, 1999. **1**(1): p. 7-17.
211. Hartland, E.L., et al., *Binding of intimin from enteropathogenic Escherichia coli to Tir and to host cells.* Mol Microbiol, 1999. **32**(1): p. 151-8.
212. Luo, Y., et al., *Crystal structure of enteropathogenic Escherichia coli intimin-receptor complex.* Nature, 2000. **405**(6790): p. 1073-7.
213. Phillips, N., R.D. Hayward, and V. Koronakis, *Phosphorylation of the enteropathogenic E. coli receptor by the Src-family kinase c-Fyn triggers actin pedestal formation.* Nat Cell Biol, 2004. **6**(7): p. 618-25.
214. Hawrani, A., et al., *Effect of protein kinase A-mediated phosphorylation on the structure and association properties of the enteropathogenic Escherichia coli Tir virulence protein.* Journal of Biological Chemistry, 2003. **278**(28): p. 25839-25846.
215. Vingadassalom, D., et al., *Insulin receptor tyrosine kinase substrate links the E. coli O157:H7 actin assembly effectors Tir and EspF(U) during pedestal formation.* Proc Natl Acad Sci U S A, 2009. **106**(16): p. 6754-9.

216. Brady, M.J., et al., *Enterohaemorrhagic and enteropathogenic Escherichia coli Tir proteins trigger a common Nck-independent actin assembly pathway.* Cellular Microbiology, 2007. **9**(9): p. 2242-53.
217. Garmendia, J., et al., *TccP is an enterohaemorrhagic Escherichia coli O157 : H7 type III effector protein that couples Tir to the actin-cytoskeleton.* Cellular Microbiology, 2004. **6**(12): p. 1167-1183.
218. Campellone, K.G., D. Robbins, and J.M. Leong, *EspF(U) is a translocated EHEC effector that interacts with Tir and N-WASP and promotes nck-independent actin assembly.* Developmental Cell, 2004. **7**(2): p. 217-228.
219. Abe, A., et al., *Enteropathogenic Escherichia coli translocated intimin receptor, Tir, requires a specific chaperone for stable secretion.* Mol Microbiol, 1999. **33**(6): p. 1162-75.
220. Gauthier, A. and B.B. Finlay, *Translocated intimin receptor and its chaperone interact with ATPase of the type III secretion apparatus of enteropathogenic Escherichia coli.* J Bacteriol, 2003. **185**(23): p. 6747-55.
221. Crawford, J.A. and J.B. Kaper, *The N-terminus of enteropathogenic Escherichia coli (EPEC) Tir mediates transport across bacterial and eukaryotic cell membranes.* Mol Microbiol, 2002. **46**(3): p. 855-68.
222. Creasey, E.A., et al., *CesT is a bivalent enteropathogenic Escherichia coli chaperone required for translocation of both Tir and Map.* Molecular Microbiology, 2003. **47**(1): p. 209-221.
223. Thomas, N.A., et al., *CesT is a multi-effector chaperone and recruitment factor required for the efficient type III secretion of both LEE- and non-LEE-encoded effectors of enteropathogenic Escherichia coli.* Molecular Microbiology, 2005. **57**(6): p. 1762-1779.
224. Riley, L.W., et al., *Hemorrhagic colitis associated with a rare Escherichia coli serotype.* N Engl J Med, 1983. **308**(12): p. 681-5.
225. Rangel, J.M., et al., *Epidemiology of Escherichia coli O157:H7 outbreaks, United States, 1982-2002.* Emerg Infect Dis, 2005. **11**(4): p. 603-9.
226. Vazquez-Boland, J.A., et al., *Listeria pathogenesis and molecular virulence determinants.* Clin Microbiol Rev, 2001. **14**(3): p. 584-640.
227. Graves, L.M., et al., *Listeria marthii sp. nov., isolated from the natural environment, Finger Lakes National Forest.* Int J Syst Evol Microbiol, 2010. **60**(Pt 6): p. 1280-8.
228. Leclercq, A., et al., *Listeria rocourtiae sp. nov.* Int J Syst Evol Microbiol, 2010. **60**(Pt 9): p. 2210-4.
229. Lang Halter, E., K. Neuhaus, and S. Scherer, *Listeria weihenstephanensis sp. nov., isolated from the water plant Lemna trisulca of a German fresh water pond.* Int J Syst Evol Microbiol, 2012.
230. Guillet, C., et al., *Human listeriosis caused by Listeria ivanovii.* Emerg Infect Dis, 2010. **16**(1): p. 136-8.
231. Swaminathan, B. and P. Gerner-Smidt, *The epidemiology of human listeriosis.* Microbes and Infection, 2007. **9**(10): p. 1236-1243.
232. Ramaswamy, V., et al., *Listeria--review of epidemiology and pathogenesis.* J Microbiol Immunol Infect, 2007. **40**(1): p. 4-13.

233. Jackson, K.A., M. Iwamoto, and D. Swerdlow, *Pregnancy-associated listeriosis.* Epidemiol Infect, 2010. **138**(10): p. 1503-9.
234. Lamont, R.F., et al., *Listeriosis in human pregnancy: a systematic review.* J Perinat Med, 2011. **39**(3): p. 227-36.
235. Laksanalamai, P., et al., *Genomic characterization of Listeria monocytogenes strains involved in a multistate listeriosis outbreak associated with cantaloupe in US.* PLoS One, 2012. **7**(7): p. e42448.
236. Ivanek, R., et al., *The cost and benefit of Listeria monocytogenes food safety measures.* Crit Rev Food Sci Nutr, 2004. **44**(7-8): p. 513-23.
237. FAO/WHO, *Food and Agriculture Organization/World Health Organization: Risk assessment of Listeria monocytogenes in ready to eat foods- Technical report.* In. , 2004. **5**: p. 1-267.
238. Evans, J.R., et al., *Follow-up study of survivors of fetal and early onset neonatal listeriosis.* Clin Invest Med, 1984. **7**(4): p. 329-34.
239. Schuchat, A., et al., *Bacterial meningitis in the United States in 1995.* New England Journal of Medicine, 1997. **337**(14): p. 970-976.
240. Siebers, A. and B.B. Finlay, *M cells and the pathogenesis of mucosal and systemic infections.* Trends in Microbiology, 1996. **4**(1): p. 22-29.
241. Jensen, V.B., J.T. Harty, and B.D. Jones, *Interactions of the invasive pathogens Salmonella typhimurium, Listeria monocytogenes, and Shigella flexneri with M cells and murine Peyer's patches.* Infect Immun, 1998. **66**(8): p. 3758-66.
242. Wampler, J.L., et al., *Heat shock protein 60 acts as a receptor for the Listeria adhesion protein in Caco-2 cells.* Infection and Immunity, 2004. **72**(2): p. 931-936.
243. Mengaud, J., et al., *E-cadherin is the receptor for internalin, a surface protein required for entry of L-monocytogenes into epithelial cells.* Cell, 1996. **84**(6): p. 923-932.
244. Shen, Y., et al., *InlB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase.* Cell, 2000. **103**(3): p. 501-510.
245. Braun, L., B. Ghebrehiwet, and P. Cossart, *gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of Listeria monocytogenes.* Embo Journal, 2000. **19**(7): p. 1458-1466.
246. Alvarez-Dominguez, C., et al., *Host cell heparan sulfate proteoglycans mediate attachment and entry of Listeria monocytogenes, and the listerial surface protein ActA is involved in heparan sulfate receptor recognition.* Infect Immun, 1997. **65**(1): p. 78-88.
247. Gaillard, J.L., et al., *Invitro Model of Penetration and Intracellular Growth of Listeria-Monocytogenes in the Human Enterocyte-Like Cell-Line Caco-2.* Infection and Immunity, 1987. **55**(11): p. 2822-2829.
248. Alvarez-Dominguez, C., R. Roberts, and P.D. Stahl, *Internalized Listeria monocytogenes modulates intracellular trafficking and delays maturation of the phagosome.* J Cell Sci, 1997. **110 (Pt 6)**: p. 731-43.

249. Mengaud, J., et al., *Identification of the structural gene encoding the SH-activated hemolysin of Listeria monocytogenes: listeriolysin O is homologous to streptolysin O and pneumolysin.* Infect Immun, 1987. **55**(12): p. 3225-7.
250. Geoffroy, C., et al., *Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from Listeria monocytogenes.* Infect Immun, 1987. **55**(7): p. 1641-6.
251. Dussurget, O., J. Pizarro-Cerda, and P. Cossart, *Molecular determinants of Listeria monocytogenes virulence.* Annual Review of Microbiology, 2004. **58**: p. 587-610.
252. Kayal, S., et al., *Listeriolysin O-dependent activation of endothelial cells during infection with Listeria monocytogenes: activation of NF-kappa B and upregulation of adhesion molecules and chemokines.* Mol Microbiol, 1999. **31**(6): p. 1709-22.
253. Tang, P., et al., *Listeriolysin O activates mitogen-activated protein kinase in eucaryotic cells.* Infect Immun, 1996. **64**(6): p. 2359-61.
254. Tang, P., I. Rosenshine, and B.B. Finlay, *Listeria monocytogenes, an invasive bacterium, stimulates MAP kinase upon attachment to epithelial cells.* Mol Biol Cell, 1994. **5**(4): p. 455-64.
255. Wadsworth, S.J. and H. Goldfine, *Listeria monocytogenes phospholipase C-dependent calcium signaling modulates bacterial entry into J774 macrophage-like cells.* Infection and Immunity, 1999. **67**(4): p. 1770-1778.
256. Nishibori, T., et al., *Induction of cytokine gene expression by listeriolysin O and roles of macrophages and NK cells.* Infect Immun, 1996. **64**(8): p. 3188-95.
257. Guzman, C.A., et al., *Apoptosis of mouse dendritic cells is triggered by listeriolysin, the major virulence determinant of Listeria monocytogenes.* Molecular Microbiology, 1996. **20**(1): p. 119-126.
258. Gaillard, J.L., et al., *In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2.* Infect Immun, 1987. **55**(11): p. 2822-9.
259. Beckerle, M.C., *Spatial control of actin filament assembly: lessons from Listeria.* Cell, 1998. **95**(6): p. 741-8.
260. Chakraborty, T., *The molecular mechanisms of actin-based intracellular motility by Listeria monocytogenes.* Microbiologia, 1996. **12**(2): p. 237-44.
261. Cossart, P., *Actin-based motility of pathogens: the Arp2/3 complex is a central player.* Cellular Microbiology, 2000. **2**(3): p. 195-205.
262. Smith, G.A. and D.A. Portnoy, *How the Listeria monocytogenes ActA protein converts actin polymerization into a motile force.* Trends Microbiol, 1997. **5**(7): p. 272-6.
263. Cossart, P., J. Pizarro-Cerda, and M. Lecuit, *Invasion of mammalian cells by Listeria monocytogenes: functional mimicry to subvert cellular functions.* Trends Cell Biol, 2003. **13**(1): p. 23-31.

264. Pentecost, M., et al., *Listeria monocytogenes internalin B activates junctional endocytosis to accelerate intestinal invasion.* PLoS Pathog, 2010. **6**(5): p. e1000900.
265. Pandiripally, V.K., et al., *Surface protein p104 is involved in adhesion of Listeria monocytogenes to human intestinal cell line, Caco-2.* J Med Microbiol, 1999. **48**(2): p. 117-24.
266. Jagadeesan, B., et al., *LAP, an alcohol acetaldehyde dehydrogenase enzyme in Listeria, promotes bacterial adhesion to enterocyte-like Caco-2 cells only in pathogenic species.* Microbiology, 2010. **156**(Pt 9): p. 2782-95.
267. Kim, K.P., et al., *Adhesion characteristics of Listeria adhesion protein (LAP)-expressing Escherichia coli to Caco-2 cells and of recombinant LAP to eukaryotic receptor Hsp60 as examined in a surface plasmon resonance sensor.* FEMS Microbiol Lett, 2006. **256**(2): p. 324-32.
268. Jaradat, Z.W., J.W. Wampler, and A.W. Bhunia, *A Listeria adhesion protein-deficient Listeria monocytogenes strain shows reduced adhesion primarily to intestinal cell lines.* Med Microbiol Immunol, 2003. **192**(2): p. 85-91.
269. Kim, H. and A.K. Bhunia, *Secreted Listeria adhesion protein (Lap) influences Lap-mediated Listeria monocytogenes paracellular translocation through epithelial barrier.* Gut Pathog, 2013. **5**(1): p. 16.
270. Burkholder, K.M. and A.K. Bhunia, *Listeria monocytogenes uses Listeria adhesion protein (LAP) to promote bacterial transepithelial translocation and induces expression of LAP receptor Hsp60.* Infect Immun, 2010. **78**(12): p. 5062-73.
271. Vazquez-Boland, J.A., et al., *Pathogenicity islands and virulence evolution in Listeria.* Microbes and Infection, 2001. **3**(7): p. 571-584.
272. de las Heras, A., et al., *Regulation of Listeria virulence: PrfA master and commander.* Current Opinion in Microbiology, 2011. **14**(2): p. 118-27.
273. Scortti, M., et al., *The PrfA virulence regulon.* Microbes and Infection, 2007. **9**(10): p. 1196-1207.
274. Hamon, M., H. Bierne, and P. Cossart, *Listeria monocytogenes: a multifaceted model.* Nature Reviews Microbiology, 2006. **4**(6): p. 423-434.
275. Banada, P.P., Bhunia, A. K., *Antibodies and immunoassays for detection of bacterial pathogens.* In: Principles of Bacterial DetectionL Biosensors, Recognition Receptors and Microsystems. , 2008. **Edited by- Zourob M, Elwary S., Turner A. Manchester: Cambridge University**: p. 567-602.
276. Roberts, A.J. and M. Wiedmann, *Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis.* Cell Mol Life Sci, 2003. **60**(5): p. 904-18.
277. Trost, M., et al., *Comparative proteome analysis of secretory proteins from pathogenic and nonpathogenic Listeria species.* Proteomics, 2005. **5**(6): p. 1544-57.
278. Cabanes, D., et al., *Surface proteins and the pathogenic potential of Listeria monocytogenes.* Trends Microbiol, 2002. **10**(5): p. 238-45.

279. Bierne, H. and P. Cossart, *Listeria monocytogenes surface proteins: from genome predictions to function.* Microbiol Mol Biol Rev, 2007. **71**(2): p. 377-97.
280. Popowska, M. and Z. Markiewicz, *Classes and functions of Listeria monocytogenes surface proteins.* Pol J Microbiol, 2004. **53**(2): p. 75-88.
281. Schaumburg, J., et al., *The cell wall subproteome of Listeria monocytogenes.* Proteomics, 2004. **4**(10): p. 2991-3006.
282. Glaser, P., et al., *Comparative genomics of Listeria species.* Science, 2001. **294**(5543): p. 849-52.
283. Bonazzi, M., M. Lecuit, and P. Cossart, *Listeria monocytogenes internalin and E-cadherin: from bench to bedside.* Cold Spring Harb Perspect Biol, 2009. **1**(4): p. a003087.
284. Garcia-del Portillo, F., et al., *Association of ActA to peptidoglycan revealed by cell wall proteomics of intracellular Listeria monocytogenes.* Journal of Biological Chemistry, 2011. **286**(40): p. 34675-89.
285. Koo, O.K., A. Aroonnual, and A.K. Bhunia, *Human heat-shock protein 60 receptor-coated paramagnetic beads show improved capture of Listeria monocytogenes in the presence of other Listeria in food.* J Appl Microbiol, 2011. **111**(1): p. 93-104.
286. Mendonca, M., et al., *Highly specific fiber optic immunosensor coupled with immunomagnetic separation for detection of low levels of Listeria monocytogenes and L. ivanovii.* BMC Microbiol, 2012. **12**: p. 275.
287. Desvaux, M. and M. Hebraud, *The protein secretion systems in Listeria: inside out bacterial virulence.* FEMS Microbiol Rev, 2006. **30**(5): p. 774-805.
288. Desvaux, M., et al., *Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue.* Trends Microbiol, 2009. **17**(4): p. 139-45.
289. Lenz, L.L. and D.A. Portnoy, *Identification of a second Listeria secA gene associated with protein secretion and the rough phenotype.* Mol Microbiol, 2002. **45**(4): p. 1043-56.
290. Rigel, N.W. and M. Braunstein, *A new twist on an old pathway--accessory Sec [corrected] systems.* Mol Microbiol, 2008. **69**(2): p. 291-302.
291. Mishra, K.K., et al., *Genetic organization and molecular characterization of secA2 locus in Listeria species.* Gene, 2011. **489**(2): p. 76-85.
292. Braunstein, M., et al., *Two nonredundant SecA homologues function in mycobacteria.* J Bacteriol, 2001. **183**(24): p. 6979-90.
293. Burkholder, K.M., et al., *Expression of LAP, a SecA2-dependent secretory protein, is induced under anaerobic environment.* Microbes Infect, 2009. **11**(10-11): p. 859-67.
294. Economou, A. and W. Wickner, *SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion.* Cell, 1994. **78**(5): p. 835-43.

295. Hartl, F.U., et al., *The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the E. coli plasma membrane.* Cell, 1990. **63**(2): p. 269-79.
296. Braunstein, M., et al., *SecA2 functions in the secretion of superoxide dismutase A and in the virulence of Mycobacterium tuberculosis.* Mol Microbiol, 2003. **48**(2): p. 453-64.
297. Chen, Q., H. Wu, and P.M. Fives-Taylor, *Investigating the role of secA2 in secretion and glycosylation of a fimbrial adhesin in Streptococcus parasanguis FW213.* Mol Microbiol, 2004. **53**(3): p. 843-56.
298. Bensing, B.A. and P.M. Sullam, *An accessory sec locus of Streptococcus gordonii is required for export of the surface protein GspB and for normal levels of binding to human platelets.* Mol Microbiol, 2002. **44**(4): p. 1081-94.
299. Machata, S., et al., *Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in Listeria monocytogenes.* J Bacteriol, 2005. **187**(24): p. 8385-94.
300. Lenz, L.L., et al., *SecA2-dependent secretion of autolytic enzymes promotes Listeria monocytogenes pathogenesis.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12432-7.
301. Dramsi, S., et al., *FbpA, a novel multifunctional Listeria monocytogenes virulence factor.* Mol Microbiol, 2004. **53**(2): p. 639-49.
302. Renier, S., et al., *Exoproteomic analysis of the SecA2-dependent secretion in Listeria monocytogenes EGD-e.* J Proteomics, 2013. **80C**: p. 183-195.
303. Severin, A., et al., *Proteomic analysis and identification of Streptococcus pyogenes surface-associated proteins.* J Bacteriol, 2007. **189**(5): p. 1514-22.
304. Cole, J.N., et al., *Surface analyses and immune reactivities of major cell wall-associated proteins of group a streptococcus.* Infect Immun, 2005. **73**(5): p. 3137-46.
305. Wu, Z., W. Zhang, and C. Lu, *Immunoproteomic assay of surface proteins of Streptococcus suis serotype 9.* FEMS Immunol Med Microbiol, 2008. **53**(1): p. 52-9.
306. Bolotin, S., et al., *The two-component system sivS/R regulates virulence in Streptococcus iniae.* FEMS Immunol Med Microbiol, 2007. **51**(3): p. 547-54.
307. Wright, A., et al., *Proteomic analysis of cell surface proteins from Clostridium difficile.* Proteomics, 2005. **5**(9): p. 2443-52.
308. Katakura, Y., et al., *Lactic acid bacteria display on the cell surface cytosolic proteins that recognize yeast mannan.* Appl Microbiol Biotechnol, 2010. **86**(1): p. 319-26.
309. Zoraghi, R., et al., *Methicillin-resistant Staphylococcus aureus (MRSA) pyruvate kinase as a target for bis-indole alkaloids with antibacterial activities.* Journal of Biological Chemistry, 2011. **286**(52): p. 44716-25.

310. Henderson, B. and A. Martin, *Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease.* Infect Immun, 2011. **79**(9): p. 3476-91.
311. Dwivedi, H.P. and L.A. Jaykus, *Detection of pathogens in foods: the current state-of-the-art and future directions.* Crit Rev Microbiol, 2011. **37**(1): p. 40-63.
312. Byrne, B., et al., *Antibody-based sensors: principles, problems and potential for detection of pathogens and associated toxins.* Sensors (Basel), 2009. **9**(6): p. 4407-45.
313. Hahm, B.K. and A.K. Bhunia, *Effect of environmental stresses on antibody-based detection of Escherichia coli O157:H7, Salmonella enterica serotype Enteritidis and Listeria monocytogenes.* J Appl Microbiol, 2006. **100**(5): p. 1017-27.
314. Hearty, S., et al., *Production, characterisation and potential application of a novel monoclonal antibody for rapid identification of virulent Listeria monocytogenes.* J Microbiol Methods, 2006. **66**(2): p. 294-312.
315. Leonard, P., et al., *A generic approach for the detection of whole Listeria monocytogenes cells in contaminated samples using surface plasmon resonance.* Biosens Bioelectron, 2004. **19**(10): p. 1331-5.
316. Heo, S.A., et al., *Characterization of new hybridoma clones producing monoclonal antibodies reactive against both live and heat-killed Listeria monocytogenes.* J Food Sci, 2007. **72**(1): p. M008-15.
317. Lin, M., et al., *Screening and characterization of monoclonal antibodies to the surface antigens of Listeria monocytogenes serotype 4b.* J Appl Microbiol, 2009. **106**(5): p. 1705-14.
318. Freitag, N.E., G.C. Port, and M.D. Miner, *Listeria monocytogenes from saprophyte to intracellular pathogen.* Nat Rev Microbiol, 2009. **7**(9): p. 623-628.
319. Copley, S.D., *Moonlighting is mainstream: Paradigm adjustment required.* Bioessays, 2012. **34**(7): p. 578-588.
320. Jeffery, C.J., *Moonlighting proteins.* Trends Biochem Sci, 1999. **24**(1): p. 8-11.
321. Henderson, B. and A. Martin, *Bacterial moonlighting proteins and bacterial virulence.* Curr Top Microbiol Immunol, 2013. **358**: p. 155-213.
322. Pancholi, V. and G.S. Chhatwal, *Housekeeping enzymes as virulence factors for pathogens.* International Journal of Medical Microbiology, 2003. **293**(6): p. 391-401.
323. Alvarez-Dominguez, C., et al., *Phagocytosed live Listeria monocytogenes influences Rab5-regulated in vitro phagosome-endosome fusion.* Journal of Biological Chemistry, 1996. **271**(23): p. 13834-43.
324. Alvarez-Dominguez, C., et al., *Characterization of a Listeria monocytogenes protein interfering with Rab5a.* Traffic, 2008. **9**(3): p. 325-37.

325. Burkholder, K.M. and A.K. Bhunia, *Listeria monocytogenes and Host Hsp60 – An invasive pairing*, in *Moonlighting Cell Stress Proteins in Microbial Infections, Heat Shok Proteins*, B. Henderson, Editor. 2013, Springer Science+Business Media: Dordrecht, Germany. p. 267-282.
326. Bhunia, A.K. and M.G. Johnson, *Monoclonal antibody specific for Listeria monocytogenes associated with a 66-kilodalton cell surface antigen.* Appl Environ Microbiol, 1992. **58**(6): p. 1924-9.
327. Harlow, E.a.L., D., *Antibodies, a Laboratory Manual*. 1988, Cold Spring, NY: Cold Spring Harbor Laboratory.

328. Geng, T., et al., *Expression of cellular antigens of Listeria monocytogenes that react with monoclonal antibodies C11E9 and EM-7G1 under acid-, salt- or temperature-induced stress environments.* J Appl Microbiol, 2003. **95**(4): p. 762-72.
329. Ohk, S.H., et al., *Antibody-aptamer functionalized fibre-optic biosensor for specific detection of Listeria monocytogenes from food.* J Appl Microbiol, 2010. **109**(3): p. 808-17.
330. Tommaso, P., et al., *T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension.* Nucleic Acids Research, 2011. **39**: p. W13-W17.
331. Notredame, C., D.G. Higgins, and J. Heringa, *T-Coffee: A novel method for fast and accurate multiple sequence alignment.* Journal of Molecular Biology, 2000. **302**(1): p. 205-217.
332. Zoraghi, R., et al., *Identification of pyruvate kinase in methicillin-resistant Staphylococcus aureus as a novel antimicrobial drug target.* Antimicrob Agents Chemother, 2011. **55**(5): p. 2042-53.
333. Jaradat, Z.W. and A.K. Bhunia, *Glucose and nutrient concentrations affect the expression of a 104-kilodalton Listeria adhesion protein in Listeria monocytogenes.* Appl Environ Microbiol, 2002. **68**(10): p. 4876-83.
334. Datta, A.R. and M.H. Kothary, *Effects of glucose, growth temperature, and pH on listeriolysin O production in Listeria monocytogenes.* Appl Environ Microbiol, 1993. **59**(10): p. 3495-7.
335. Ripio, M.T., et al., *Transcriptional activation of virulence genes in wild-type strains of Listeria monocytogenes in response to a change in the extracellular medium composition.* Res Microbiol, 1996. **147**(5): p. 371-84.
336. Lemes-Marques, E.G. and T. Yano, *Influence of environmental conditions on the expression of virulence factors by Listeria monocytogenes and their use in species identification.* FEMS Microbiol Lett, 2004. **239**(1): p. 63-70.
337. Geng, T., B.K. Hahm, and A.K. Bhunia, *Selective enrichment media affect the antibody-based detection of stress-exposed Listeria monocytogenes due to differential expression of antibody-reactive antigens identified by protein sequencing.* J Food Prot, 2006. **69**(8): p. 1879-86.

338. Lathrop, A.A., P.P. Banada, and A.K. Bhunia, *Differential expression of InlB and ActA in Listeria monocytogenes in selective and nonselective enrichment broths.* Journal of Applied Microbiology, 2008. **104**(3): p. 627-39.
339. Donnelly, C.W. and G.J. Baigent, *Method for Flow Cytometric Detection of Listeria-Monocytogenes in Milk.* Applied and Environmental Microbiology, 1986. **52**(4): p. 689-695.
340. Bailey, J.S., D.L. Fletcher, and N.A. Cox, *Efficacy of Enrichment Media for Recovery of Heat-Injured Listeria-Monocytogenes.* Journal of Food Protection, 1990. **53**(6): p. 473-477.
341. Fraser, J.A. and W.H. Sperber, *Rapid Detection of Listeria Spp in Food and Environmental-Samples by Esculin Hydrolysis.* Journal of Food Protection, 1988. **51**(10): p. 762-765.
342. Karmali, M.A., *Infection by verocytotoxin-producing Escherichia coli.* Clin Microbiol Rev, 1989. **2**(1): p. 15-38.
343. Nataro, J.P. and J.B. Kaper, *Diarrheagenic Escherichia coli.* Clin Microbiol Rev, 1998. **11**(1): p. 142-201.
344. Karmali, M.A., V. Gannon, and J.M. Sargeant, *Verocytotoxin-producing Escherichia coli (VTEC).* Vet Microbiol, 2010. **140**(3-4): p. 360-70.
345. Kolaskar, A.S. and P.C. Tongaonkar, *A semi-empirical method for prediction of antigenic determinants on protein antigens.* FEBS Lett, 1990. **276**(1-2): p. 172-4.
346. Chen, J., et al., *Prediction of linear B-cell epitopes using amino acid pair antigenicity scale.* Amino Acids, 2007. **33**(3): p. 423-8.
347. El-Manzalawy, Y., D. Dobbs, and V. Honavar, *Predicting linear B-cell epitopes using string kernels.* J Mol Recognit, 2008. **21**(4): p. 243-55.
348. El-Manzalawy, Y., D. Dobbs, and V. Honavar, *Predicting flexible length linear B-cell epitopes.* Comput Syst Bioinformatics Conf, 2008. **7**: p. 121-32.
349. LaVallie, E.R., et al., *A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm.* Biotechnology (N Y), 1993. **11**(2): p. 187-93.
350. Davanloo, P., et al., *Cloning and expression of the gene for bacteriophage T7 RNA polymerase.* Proc Natl Acad Sci U S A, 1984. **81**(7): p. 2035-9.
351. Studier, F.W. and B.A. Moffatt, *Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes.* J Mol Biol, 1986. **189**(1): p. 113-30.
352. Ness, F., et al., *Guanidine hydrochloride inhibits the generation of prion "seeds" but not prion protein aggregation in yeast.* Mol Cell Biol, 2002. **22**(15): p. 5593-605.
353. Jagadeesan, B., et al., *N-terminal Gly(224)-Gly(411) domain in Listeria adhesion protein interacts with host receptor Hsp60.* PLoS One, 2011. **6**(6): p. e20694.

APPENDICES

Appendix A : Identifying specific amino-acids involved in LAP-Hsp60 interaction

Listeria adhesion protein (LAP) is a 104kDa bifunctional housekeeping alcohol-acetaldehyde dehydrogenase enzyme. It has been identified to play a critical role in mediating bacterial adhesion to the host cells during the intestinal phase of *L. monocytogenes* infection [265, 268].

Hsp60 is a ubiquitous heat shock protein or a chaperonin and is found in almost all living organisms. Hsp60 plays crucial roles in protein folding, production of cytokines, innate and adaptive immunity, acting as ligand and specific receptors for bacterial toxins, autoimmune diseases and inflammatory responses, reproduction, cardiovascular problems and providing tumor immunity among others. Its involvement in such a vast range of biological processes is fascinating. What particularly makes Hsp60 unique is that all these functions are carried out via different mechanisms, many of which are distinct from the shared mechanisms of other heat shock proteins.

Hsp60 was identified to act as the receptor for LAP from Caco-2 cell line [110]. It has been shown that a low level of infection with *L. monocytogenes* is capable of elevating host Hsp60 expression, which further aggrandizes LAP mediated adhesion to host cells and aids in its translocation across intestinal

epithelial monolayers [270]. Previous studies have shown that it is the 21kDa N2 domain (Gly_{224}–Gly_{411}) of LAP which is involved with binding with Hsp60 [353]. The objective of study was to identify the exact amino acids involved in LAP-Hsp60 protein interaction.

Materials and methods

Identification of surface residues on N2

In order to understand the binding of LAP with its receptor Hsp60, we began by *in-silico* modeling of the LAP protein using ModBase, a database of protein homology models. An aldehyde dehydrogenase (PDB ID:3K9D) was chosen as the most suitable template based on sequence similarity and alignment.

The N2 domain of LAP (Figure A) was subjected to a computational analysis that identified the surface residues of the protein which could be a part of the ligand-receptor interaction. The domain was further analyzed by protein-protein interaction site prediction methods, BindML (developed by David La in Dr. Daisuke Kihara's lab, Computer Science, Purdue University) and Meta-PPISP. Also, as it is a known fact that lysine (K) residues are often involved in adhesion and binding, the helices were also ranked according to the number of lysine residues in their sequences.

ELISA to determine specifiv peptide binding with Hsp60

The peptides were synthesized (EZBiolabs) with yellow helix (TDKEVQKAFGIRMKACR) serving as test and the blue

(KTAKIKRSVNDIILSKSFDQGMICA) and red (DKEVAKEVKAEMEANKCY) helices as negative controls. Purified LAP and N2 proteins were used as positive controls. Peptide and to perform experiments that measure the effect these helices have on LAP adhesion to Caco-2 cells.

$3x10^{14}$ molecules of each peptide as well as LAP and N2 proteins were resuspended in 0.05 M sodium carbonate coating buffer, pH 9.6, immobilized in 96-well Immulon 4HBX plates (Thermo Scientific, Waltham, MA), and stored at 4°C for 48 h. Following peptide/protein immobilization, the plate wells were sequentially reacted with Hsp60 protein (250ng/well), followed by anti-Hsp60 PAb (100ng/well) and finally anti-rabbit HRP-conjugated antibody (1:4000 dilution; Jackson Immunologicals). For all steps, plates were held at RT for 1 h and washed 3 times with PBST between steps. Finally 100 µL of a fluorescent substrate, either Super Red (10-acetyl-3,7-dihydroxyphenoxazine; Virolabs, Chantilly, VA; Ex: 540 nm, Em: 600 nm) or Quanta Blu (Ex: 320 nm, Em: 460 nm), was added to each well and fluorescence was measured using a Spectramax fluorescent reader (Gemini, Sunnyvale, CA) every 15 min for 1 h. To determine nonspecific protein binding, control reactions without peptide/protein and Hsp60 were included. Fluorescent readings obtained from these controls were subtracted from the test results to obtain true values.

LAP cloning for X-ray crystallization

Full length *lap* (*lmo1634*; 2601bp)from *Listeria monocytogenes* serotype 4b strain F4244 was amplified by PCR using the following primers: LAP-F 5'-

CGGTCCCCGGGTACCATGCAATTAAGAAAATGCGGCC -3' and LAP-R 5'-CTCGAGAACACCTTTGTAAGCTTCAAGG -3' with restriction sites *Bam*HI and *Xho*I, respectively. The gene was first cloned into the cloning vector pGEM-T Easy (Promega) and from that into a *Bam*HI and *Xho*I digested pGEX-6P-1 expression vector (Novagen). The transformants in both cases were verified by gene sequencing at the Purdue University Genomics Facility. The protein was expressed in *E. coli* BL21 (DE3) expression cells (Novagen) in presence of Ampicillin (50 µg/ml). Recombinant Gst-tagged protein was purified by immobilized Metal Affinity Chromatography (IMAC) using a Gst column (Thermo Fisher Scientific). Protein expression in recombinant strains was subsequently confirmed by Western blot analysis using MAb-EM10 and an anti-Gst monoclonal antibody (Pierce Antibodies, Thermo Fisher Scientific).

Recombinant LAP protein expression

Recombinant cell lines were induced with IPTG (1 mM) to increase and optimize protein production. Whole cell bacterial proteins were extracted by heat killing the cells at 95°C for 10 minutes and then resuspending the cell pellet in the sample solvent (5% SDS, 0.5% β-mercaptoethanol, 1.5% Tris, pH 7.0) followed by sonication on ice for 5–7 cycles of 15 sec each using a Sonifier 150D (Branson, Niantic, CT). The samples were centrifuged and the supernatant fractions were collected and stored at −20°C.

Proteins were quantified using the bicinchoninic acid method (Pierce, Rockford, IL) and equivalent amounts of protein (20 µg of each fraction) were

separated using SDS polyacrylamide gel electrophoresis (7.5% acrylamide) gel. The proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, MA) and immunoprobed with anti-LAP antibody MAb-EM10 (1.0 µg/mL) and horseradish peroxidase-coupled anti-mouse antibody (0.2 µg/mL; Jackson Immuno Research, West Grove, PA). The membranes were developed with an enhanced chemiluminescence kit (Pierce). The membrane was also immunoprobed with anti-Gst-tag MAb (0.1 µg/mL; Thermo Fisher Scientific) for detection of Gst-tagged recombinant protein.

Results & Discussion

Identification of surface residues in N2 domain

Analysis of homology modeled N2 domain (Figure A-1) showed that the various helices (color coded for convenience) in the N2 structure formed the external surface, while the beta-sheets took part in the LAP inter-domain binding.

Figure A 1 LAP N2 domain structure as predicted by ModBase

To determine the helix with highest probability of interacting with the receptor, they were analyzed and ranked by BindML, Meta-PPISP and lysine count (Table A 1).

Table A 1 Ranking of various N2 helices using multiple selection algorithms

Method/Rank	**BindML**	**Meta-PPISP**	**Lysine (K) count**
1	Green Helix	Blue Helix	Green Helix (6K)
2	Blue Helix	Yellow Helix	Red, Blue and Yellow Helices (3K)
3	Yellow Helix	Orange Helix	Orange Helix (1K)

Although 2 out of 3 methods agree on the green helix (FVKGAEFKKLESYVINPEKGTLNPDVVGKSPAWIANQAGFKVPED), this helix is quite large, possesses a significantly long random coil structure and is much different from the modeling template used earlier (PDB ID:3K9D), and therefore, its actual involvement and structural accuracy is questionable. Hence we now focus on the blue and the yellow helices. The blue helix has a very high scoring

cysteine residue, which makes it energetically unfavorable to be exposed freely on the surface and is believed to be most likely involved in stabilizing the N1-N2 interaction. The yellow helix finally appears to be the consensus among all the methods.

Peptide interaction with Hsp60

If the yellow helix is involved in binding with Hsp60, a relatively elevated fluorescence value will be observed when that peptide is exposed to Hsp60 protein. The results of the ELISA experiment are shown in Figure A-2 whereasTable A-2 lists the expected and observed results of peptide/protein binding with Hsp60.

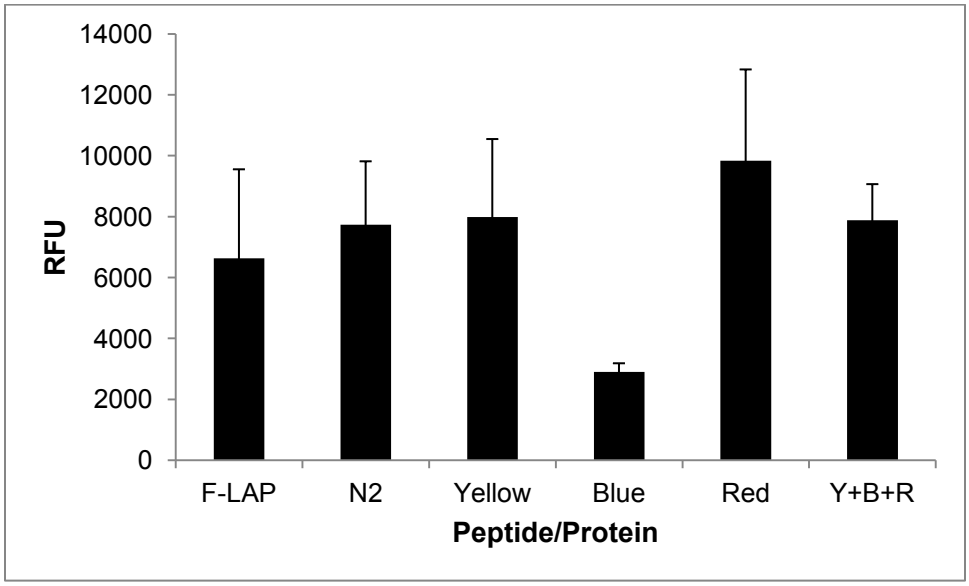

Figure A 2 ELISA binding of synthesized peptides and full length LAP and N2 domain with Hsp60. Data are an average of three experiments analyzed in quadruplicate.

Table A 2 Expected and observed results following exposure of peptides/proteins to Hsp60 protein

Peptides exposed to Hsp60	Expected RFU	Observed RFU
F-LAP	High	High
N2	High	High
Yellow	High	High
Blue	Low	Low
Red	Low	High
Yellow+Red+Blue (Y+B+R)	High	High

As predicted, the yellow helix peptide displays a relatively higher binding with Hsp60. Values for this binding are comparable with full length LAP (F-LAP) and the N2 domain binding with Hsp60 protein. A surprising finding was high binding of the red helix with Hsp60 which was marked as a negative control for this reaction. A possible explanation for this could be that while this helix is not located on N2 domain surface under normal conditions, when the peptide is artificially synthesized and intentionally exposed to Hsp60, it might display affinity towards the protein.

The results therefore indicate that the yellow helix may be involved in LAP-Hsp60 binding. To identify the exact residues participating in the interaction, the helix residues can be sequentially modified and a similar ELISA experiment can be performed to determine which amino acid is critical for N2-Hsp60 interaction.

To deduce the role of lysine residues in binding and adhesion, the lysine residues on the N2 domain could be chemically modified and the subsequent effect on LAP adhesion can be observed. If lysine is shown to have a significant

effect, the lysine residues on the yellow helix could be modified preferentially to determine its role.

LAP cloning for X-ray crystallization

Full length LAP was cloned into GST-tagged pGEX-6P-1 expression vector (Figure A-3a). LAP protein expression was indicated by anti-LAP MAb EM10, however, anti-Gst tag MAb did not show the expected 104 kDa LAP band (Figure A-3b). Further analysis of the recombinants has to be done to confirm protein expression and subsequent purification.

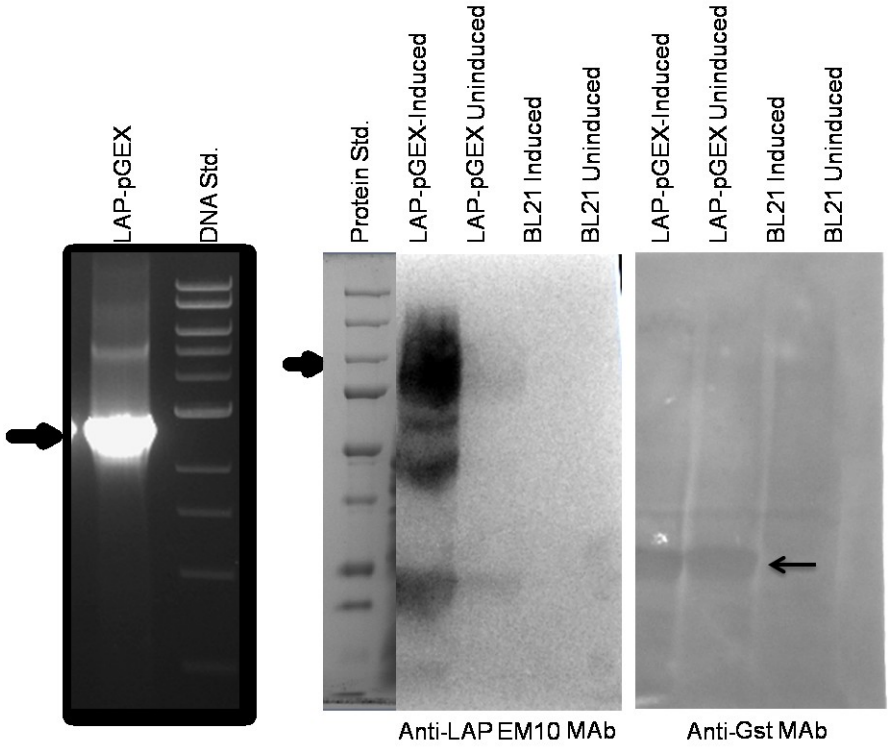

Figure A 3 LAP cloning and purification on pGEX-6P-1 vector.

Appendix B: Identifying natural *Lactobacillus* spp. isolates from cow rumen

This appendix is a continuation of chapter 3 (TIR (Translocated Intimin Receptor) for capture and detection of STEC), with the aim being prevention of *E. coli* O157:H7 gut colonization using recombinant *Lactobacilli* containing TIR. The hypothesis is that natural *Lactobacillus* isolates can be engineered to express TIR. These recombinant *Lactobacilli* can then target and selectively bind with EHEC and EPEC, thereby sequestering the bacteria and preventing their colonization on the host epithelial cells. This specifically designed *Lactobacillus* probiotic strain strains carrying TIR receptor can be fed orally to the will have the ability initiate TIR-intimin binding when exposed to STEC.

Materials and methods

Obtaining cattle rumen samples

Rumen samples (150ml) were isolated from three different fistulated cows at the Purdue Dairy Farm. The samples were pooled together in a thermos. The thermos was maintained at 37°C and anaerobic conditions by using a CO_2 blanket to maintain bacterial viability. The samples were then filtered and the filtrate was diluted in 1% peptone broth for enrichment. The diluted samples were grown on de Man, Rogosa and Sharpe (MRS) agar plates and incubated under anaerobic conditions at 37°C for 24-48 hours. The colonies thus obtained were screened for *Lactobacilli*.

Morphology and phenotype

Colonies obtained were grown in MRS broth under anaerobic conditions at 37°C for 24 h and the cultures were pre-screened for *Lactobacillus* spp. by checking the basic morphology by wet mount and Gram staining. Most *Lactobacillus* spp. are rod-shaped and Gram positive.

Catalase Test

Lactobacillus spp. generally lack the catalase enzyme which oxidizes hydrogen peroxide (H_2O_2) to water and oxygen. To check the production of this enzyme, a catalase test was performed. The bacterial colony was smeared on a clean microscopic slide and a drop of 3% H_2O_2 was added aseptically. Production of bubbles on addition of H_2O_2 indicates oxygen presence due to catalase enzyme activity.

Additional tests

Additional tests for confirming *Lactobacillus* spp. include: (i) Ammonia production from arginine; (ii) Gas production from glucose; (iii) API CHL Medium for *Lactobacillus* identification (bioMérieux, Inc.); (iv) PCR and (vi) Ribotyping with *Eco*RI and *Hind*III restriction enzymes.

Bacterial strains and growth conditions

E. coli strain EDL 933 was grown in Brain – Heart Infusion (BHI) media overnight at 37°C. *E. coli* DH10B was grown at 37° C overnight in Luria-Bertani (LB) broth supplemented with Ampicillin (100µg/ml). *plp*401-*t* expression vector containing *Lactobacillus paracasei* was grown at 37° C overnight in MRS broth

supplemented with Erythromycin (2µg/ml). Plasmid vectors pGEM-T Easy (Promega) and *plp*401-*t* were used as the cloning vector and the expression vector respectively.

TIR cloning in *Lactobacillus* spp.

Full length *tir* gene (1677bp; 558 amino acids) from *E.coli* O157:H7 strain EDL933 was amplified by PCR using primers homologous to TIR regions 5'-CCCGCGGCCGCATGCCTATTGGTAATCTT-3' and 5'-GACGAAACGATGGGATCC-3' and containing restriction sites *Not*I and *Xho*I. The gene was first cloned into the cloning vector pGEM-T Easy. From that it will be inserted into a *Not*I and *Xho*I digested *plp*401-*t* expression vector. The transformants in both cases will be verified by gene sequencing.

Results and discussion

Sixteen possible *Lactobacillus* colonies were isolated based on the phenotype, morphology and catalase test. These colonies are stored at -80°C until additional tests can be performed to confirm *Lactobacillus* species.

TIR was successfully inserted into pGEM-T Easy cloning vector but remains to be ligated into *plp*401-*t* expression vector and then transformed into natural *Lactobacillus* isolates from above as well as lab *Lactobacillus* cultures.

Following recombinant *Lactobacillus* TIR expression, the interaction between the designer probiotic and STEC can be observed by ELISA and other biosensors such as the SPR (surface Plasmon resonance) device. Since this study is intended for use in animals, cell-culture based assays such as adhesion

assay (on Caco-2 cell line) will also be performed to determine the binding efficacy between recombinant *Lactobacillus* and STEC.

Appendix C: Developing polyclonal antibodies for LAP domains (N1, N2, C1 & C2)

To predict the most antigenic peptide sequence that should be used to develop the antibody, two methods were considered and the consensus sequence from both results was chosen for antibody development. These two methods were (i) Kolaskar and Togaonkar Method [345] and (ii) BCPREDS: B-cell epitope prediction server [346-348]. A BLAST-P search was also performed on the consensus peptide obtained to ensure specificity. The selected peptide sequence showed a 100% similarity with *Listeria* strains only. The LAP-domain sequences are listed below and the corresponding antigenic peptides predicted are highlighted.

N1

MAIKENAAQEVLEVQKVIDRLADNGQKALKAFESYNQEQVDNIVHAMALAGLD
QHMPLAKLAVEETGRGLYEDKCIKNIFATEYIWNNIKNNKTVGVINEDVQTGVIEI
AEPVGVVAGVTPVTNPTSTTLFKAIIAIKTRNPIIFAFHPSAQRCSSAAAKVVYDA
AIAAGAPEHCIQWVEKPSLEATKQLMNHDKVALVLATGGAGMVKSAYSTGKPA
LGVGP

N2

GNVPAYIDKTAKIKRSVNDIILSKSFDQGMICASEQAVIVDKEVAKEVKAEMEAN
KCYFVKGAEFKKLESYVINPEKGTLNPDVVGKSPAWIANQAGFKVPEDTKILVA
EIKGVGDKYPLSHEKLSPVLAFIEAANQAEAFDRCEEMLVYGGLGHSAVIHSTD
KEVQKAFGIRMKACRIIVNAPSAQG

C1

GIGDIYNGFIPSLTLGCGSYGKNSVSQNVSATNLLNVKRIADRRNNMQWFKLPP
KIFFEKYSTQYLQKMEGVERVFIVTDPGMGSFKYVDVVIEHLKKRGNDVAYQVF
ADVEPDPSDVTVYKGAELMKDFKPDTIIALGGGSAMDAAKGMWLFYEHPEASF
FGLKQKFLDIRKRTFKYPKLGGKAKFVAIPTTSGTGSEVTPFAVITDKENNIKYPL
ADYELTPDVAIVDAQYVTTV

C2

PAHITADTGMDVLTHAIESYVSVMASDYTRGLSIRAIELVFENLRESVLTGDPDA
REKMHNASALAGMAFANAFLGINHSLAHKIGPEFHIPHGRANAILMPHVIRYNAL
KPKKHALFPRYESFRADEDYARISRIIGFPAATTEEGVKSLVDEIIKLGKDVGIDM
SLKGQNVAKKDLDAVVDTLADRAFMDQCTTANPKQPLVSELKEIYLEAYKGV

Figure C-1 shows anti-C1 PAb reaction with LAP domains (C1, C2, N1 and N2) and full length LAP protein (F-LAP).

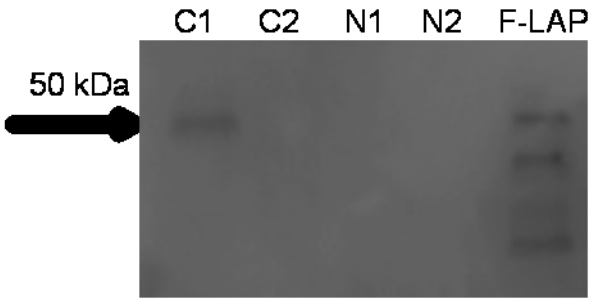

Figure C 1 Reaction profile of anti-C1 PAb with various LAP domains and full lenth LAP (F-LAP)

VITA

VITA

Titiksha Dikshit
Graduate School, Purdue University

EDUCATION

Purdue University - Master of Science, Food Science (August 2010-December 2013)

GPA: 3.87/4

Honors: Golden Key International Honor Society, Phi Tau Sigma Honorary Society

Professional Membership: Center for Food Safety Engineering (CFSE, Purdue University),
American Society for Microbiology (ASM), Institute of Food Technologists Student Association (IFT)

Relevant Coursework:

- **Food Science**: *Food Analysis, Food Chemistry, Product Development, Food Microbiology, Food Processing & Packaging, Food Nutrition, Plant Pathology, Sensory Evaluation Techniques*
- **Molecular Biology**: *Bacterial Physiology, Biochemistry, Advance Techniques for Pathogen Detection*
- **Statistics**: *Regression Analysis, Design of Experiments*

Indian Institute of Technology Guwahati (IIT-G) - Bachelor of Technology, Biotechnology (2006-2010)

GPA: 3.60/4

Honors: Silver Medal, Biotechnology, Class of 2010

SKILLS

Technical: HACCP, GMP and Food Safety standards; Allergen identification & validation; Bacterial growth and pathogen detection techniques; Gene cloning and protein expression; Immunoassays; FACS; Nucleic acid based assays (PCR; qPCR); Cell culture-, protein- and antibody-based assays; Electron Microscopy (TEM and SEM)

Computer: Microsoft Office Suite, C++, PERL, Python, Bioinformatics software and toolssa (SWISS-MODEL, Protein and DNA alignment tools, Homology modeling, Primer design)

Certifications:

- Aseptic Processing and Packaging
- Equipment & Plant Design Workshop for Allergen/Pathogen Control in Low Moisture Foods

EXPERIENCE

Kellogg Company, Food Safety Intern (January-June 2013*)*

- *Evaluated of air quality in food manufacturing plants*
- *Worked with food plants to apply a new, rapid method for allergen detection & validation*
- *Developed a Sanitation Lexicon aimed at harmonizing the use and meaning of terms pertaining to sanitation used across the food industry (The lexicon will be available on www.gmaonline.org)*
- *Approved ingredients used and updated vitamin & mineral specifications*

Purdue University, Research Asst. to Dr. A. K. Bhunia, Molecular Biology and Microbiology Lab. *(*Aug. 2010-Present)

- *Optimized biosensor platforms for capture and detection of pathogenic E. coli*
- *Developed a novel antibody for selective Listeria spp. capture and detection*
- *Developed potential probiotic therapies for preventing Listeria spp. and E. coli infection*

Indian Institute of Technology-Guwahati, Undergrad. Researcher, Molecular Microbiology Lab.(Aug.2009- May 2010)

- *Performed multiple-template homology based 3-D protein modeling of S. aureus global virulence regulator*

National Institute of Immunology, Stem Cell Biology Lab.(May-July 2009)

- *Developed of stem-cell constructs using novel collagen matrices for tissue regeneration*

PUBLICATIONS UNDER PREPARATION

- **Dikshit T** and Bhunia AK. Pyruvate Kinase, a SecA2-Dependent Surface Associated Protein, in *Listeria* Species. **Applied and Environmental Microbiology** 2013
- **Dikshit T** and Bhunia AK. Biosensors: Applications in Detection of Foodorne Toxins. **Trends in Microbiology** 2014

- **Dikshit T** and Bhunia AK. Translocated Intimin Receptor (TIR) based capture and detection of STEC. 2014
- Amalaradjou MA, Singh AK, **Dikshit T**, Bhunia AK. Bio-engineered probiotic expressing adhesion protein from non-pathogenic bacteria protects mice against *Listeria monocytogenes* infection. 2014

LEADERSHIP & ACTIVITIES

- **Languages:** Fluent English and Hindi; Elementary French
- **Member, China Study Abroad Program, Dept. of Food Science, Purdue University**
- **Initiated Zero Illiteracy Zone (Z.I.Z.)**: A non-profit organization for educating young children; established currently as the Brigosha Foundation (NGO)
- **Undergraduate Program Committee Student Representative, Dept. of Biotechnology, IIT-G**
- **Student Representative, Hostel Affairs Board (HAB), IIT-G**
- **Active member of Literary Society, organized debates and other events in *Alcheringa* (**IIT-G Cultural Festival**)**
- **Other interests:** Dancing (Indian classical dance, *Kathak*) and playing *Tabla* (Indian percussion instrument**)**