ABSTRACT

Selection of Aptamers Against Live *E.coli* Cells using Cell SELEX

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Aptamers are oligonucleotides that bind with great specificity and affinity to a target molecule. These oligonucleotides are produced through the course of Systemic Evolution of Ligands by Exponential Enrichment (SELEX). SELEX is a combinatorial chemistry technique used to generate a random DNA or RNA library, which is then incubated with a target molecule. The binding aptamers are divided from the nonbinding random pool DNA/aptamers, and then amplified via polymerase chain reaction (PCR). Double stranded DNA molecules have been used to select against purified target molecules; in this study we have developed a selection technique using live *Escherichia coli* cells as a target and using *Bacillus subtilis* and *Enterobacter aerogenes* as negative controls. Aptamer pools obtained from approximately 8 rounds of Cell SELEX demonstrated an affinity for *E .coli* cells when tested via fluorescence detection.

Selection of Aptamers Against Live *E.coli* Cells using Cell SELEX

by

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LIST OF ABBREVIATIONS

ATP-Adenosine-5'-triphosphate
B.subtilis-Bacillus subtilis
C.jejuni-Campylobacter jejuni
DNA-Deoxyribonucleic acid
dsDNA-Double stranded deoxyribonucleic acid
E. coli-Escherichia coli
E. aerogenes-Enterobacter aerogenes
E. cloacaea-Enterobacter cloacae
FAM-Carboxyfluorescin
FDA- Food and Drug Administration
FITC-Fluorescein isothiocyanate
FRET-Förster resonance energy transfer
GTP-Guanosine-5'-triphosphate
HIV-1-Human immunodeficiency virus 1
HNE-Human nuetrophil elastase
HUS-Hemolytic Uremic Syndrome
IgE-Immunoglobine E
LEE-Locus of enterocyte effacement
NIPH-Norwegian Institute of Public Health
Omp-Outer membrane protein
PCR-Polymerase chain reaction
PDGF-Platelet-derived growth factor
RNA-Ribonucleic acid
TEMED-Tetramethylethylenediamine
S.aureus-Staphylococcus aureus
SELEX-Systematic Evolution of Ligands by Exponential Enrichment
ssDNA-Single stranded deoxyribonucleic acid
STEC-*Shiga* Toxin-Producing Escherichia coli
Stx-*Shiga* toxin
VEGF-Vascular endothelial growth factor

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DEDICATION

To Daddy Ed and Mamma Gayle

CHAPTER ONE

BACKGROUND ON SELEX

Introduction

Combinatorial chemistry is a process that allows for the generation of libraries by producing a large number of unique molecules with similar structures. Nucleic acids are often used in combinatorial chemistry because of their ability to fold into defined secondary and tertiary structures and are easily amplified by polymerase chain reaction (PCR). Exceptionally complex libraries of random sequence oligonucleotides contain about 10^{15} different molecules produced by chemical synthesis. These libraries can then be screened for several different characteristics; such as high affinity ligand binding (Strehlitz *et al.* 2009).

Aptamers or chemical antibodies are oligonucleotides that bind with great specificity and affinity to a target molecule. The molecules primarily function in molecular recognition and can be easily modified for cytometry and *in vivo* applications (Tang *et al.* 2009, Ulrich *et al*. 2009). The phrase aptamer was coined from the Latin phrase *aptus*, which means "to fit." Aptamers are used for three main reasons; stable three-dimensional structures, which are dependent on their primary sequence, length of the nucleic acid molecule, and environmental conditions. This methodology is based on the premise that the distinctive configuration of small oligonucleotides can be applied to identify specific targets with high affinity. Aptamers normally form typical structural motifs based on the number of strands. Double stranded DNA (dsDNA) aptamers form

α-helices, whereas single stranded DNA (ssDNA) aptamers may form stems, internal loops, bulges, hairpin structures, tetra loops, pseudoknots, kissing complexes or G-quadruplex structures which can be seen in Table 1. When aptamers are incubated with target molecules the aptamers tend to undergo adaptive conformational changes, which creates a three-dimensional structure that fits in a specific binding site of the target in question (Strehlitz *et al.* 2009 and Hamula *et al.* 2008).

Table 1. Structural Motifs of Aptamers. Structural motifs aide in the affinity and specificity of the aptamer for the target molecule.

Structure of Aptamers	Type of Aptamer	Reference
Stem Loops	RNA	Davis *et al.*
Internal Loops/Bulge	RNA	Jiang *et al.*
Hairpin	ssDNA	Hamula *et al.*
Tetra Loops	RNA	Sakamoto *et al.*
Psuedoknots	RNA	Kim *et al.*
Kissing Complexes	RNA	Lebars *et al.*
G-quardruplex	ssDNA	Montessarchio *et al.*

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodologies have been employed extensively to assess both single targets as well as complex target structures and mixtures via aptamers (Strehlitz *et al.* 2009). Some of these approaches require previous knowledge of the composition or molecular structure of the target compound for generating successful aptamer pools. Furthermore, if a single target is being used to generate an aptamer pool, the target molecule must exist in high concentration and purity (Mayer 2009).

Intermolecular interactions between aptamer and target molecules are characterized by a combination of complementarily shapes, stacking interactions between aromatic compounds, electrostatic interactions between charged groups, and hydrogen

bonding (Strehiltz *et al.* 2009 and Herman 2003). In many cases, the aptamer binds to its target protein and inhibits biological activities either by interfering with the catalytic site of an enzyme or interfering with sites involved in ligand-receptor recognition. In some cases, it is possible that aptamer-target protein complex binding can induce allosteric effects, such as changes in conformational states that can result in loss of biological activity in the target protein (Ulrich 2005).

Aptamers and their Generation via SELEX

The use of aptamers produced through the course of SELEX was first described twenty years ago by Tuerk and Gold (1990) as an *in vitro* selection and amplification method. This method still remains a prevalent biochemistry technique. As mentioned earlier, SELEX is a combinatorial chemistry technique used to generate a random DNA or RNA library, which is then incubated with a target molecule. SELEX has been applied to various classes of targets, such as inorganic and small organic molecules, peptides, proteins, carbohydrates, antibiotics, whole cells, organisms, or mixtures of several targets (Stoltenburg *et al.* 2007). Aptamers are often developed for molecules connected with nucleotides, cofactors, enzymes, regulatory proteins, growth factors, or organic dyes (Strehlitz *et al.* 2009). Recently, a variety of targets have been successfully identified using SELEX which are depicted in Table 2. Many conventional SELEX methodologies use purified target molecules attached to a solid support like a column; however, approaches such as these may affect that purification of the target molecule and change the conformation of a protein from its native state (Ellington *et al*. 1992).

Table 2. Summary of various aptamers and their targets. In the past twenty years SELEX has been used to produce aptamers with high affinity and specificity for various target molecules; such as, organic dyes, small molecules, antibiotics, cytokines/growth factors, Nucleic acid-binding proteins, serine proteases, antibodies, complement proteins, prion proteins, and peptides.

Target	Type of Aptamer	Reference
Cibacron Blue 3GA	RNA	Ellington & Szostak, 1990
Reactive Brown 10	DNA	Ellington & Szostak, 1992
Reactive Green 19	DNA	Ellington & Szostak, 1992
Sulforhodamine B	DNA	Wilson & Szosrack, 1998
Streptomycin	RNA	Bachler, 1999
Neomycin	RNA	Wallis, 1995
Vascular Endothelial Growth Factor	RNA	Ruckman *et al.* 1998, Tucker *et al.* 1999, Huang *et al.* 2001, Eyetech Study 2002, 2003, Kim *et al.* 2002
Platelet-Derived Growth Factor	DNA	Green *et al.* 1996, Floege *et al.* 1999, Pietras *et al.* 2001
HIV-1 TAT	RNA	Yamamoto *et al.* 2000
α-Thrombin	DNA	Bock *et al.* 1992, Griffin *et al.* 1993, DeAnda *et al.* 1994
Immunoglobulin E	RNA	Wiegand *et al.* 1996
Lipoproteins	RNA	Ishizaki *et al.* 1996
Prion Protein PrP	RNA	Proske *et al.* 2002
Gonadotropin-Releasing Hormone	DNA	Wlotzka *et al.* 2002

Variations of SELEX Methodology

The SELEX process is characterized by iterative cycles of *in vitro* selection and amplification, which seems to mimic a Darwinian process to produce the selection of a small amount of structurally optimized motifs that bind to a specific target (Goringer *et al.* 2006). SELEX starts with the synthesis of a random DNA oligonucleotide library that can be later used for selection of DNA aptamers. This library often contains 10^{13}-10^{15} different sequence motifs and a random region that is approximately 20-80 nucleotides

combined with two primer binding sites, which are approximately 18-20 nucleotides in length on either side (Strehlitz *et al.* 2009). These primer binding sites aid in amplification via PCR. If an RNA aptamer is desired, the DNA oligonucleotide library has to be transferred into an RNA library (Strehlitz *et al.* 2009, Geiger *et al.* 1996 and Goringer *et al.* 2006).

The SELEX methodology is characterized by the reiteration of consecutive steps of binding, partition, elution, and amplification.. The partitioning step is essential to the selection process and often determines the binding feature of the aptamer pool. Various methods of partitioning are used, including affinity chromatography, magnetic separation, filtration, or centrifugation. Irreguardless, all approaches are based on target immobilization to create affinity matrices, selective capturing of the binding complexes, or size separation (Geiger *et al.* 1996). If this step is not executed with great caution, non-binding sequences may remain that could be subsequently amplified during PCR. Therefore, it is imperative that these sequences be removed before the next round of SELEX; and several techniques have been found effective for removing the unbound sequences, including nitrocellulose filtration, affinity chromatography, immunoprecipitation, magnetic beads which are bound to the aptamer, gel electrophoresis and capillary electrophoresis (Strehlitz *et al*. 2009).

The iterative selection and amplification cycles of SELEX allows the original DNA pool ($\sim 10^{15}$) to be narrowed down to only a few aptamers that bind with high affinity and specificity to the target molecule (Strehlitz *et al*. 2009). In most cases, approximately six to twenty SELEX rounds are needed to generate a pool of highly specific aptamers. The number of rounds needed in creating a highly specific aptamer

pool is determined by the target features, concentrations, design of the original DNA pool, the ratio of target molecules and DNA, and the efficiency of the partioning method. As soon as the aptamer pool reaches its maximum specificity and affinity, the sequences are identified by a sequencing procedure; thereafter they are again screened to confirm their binding affinity. The entire process is illustrated in Figure 1 (Chen *et al*. 2007, Strehlitz *et al.* 2009, and Geiger *et al.* 1996).

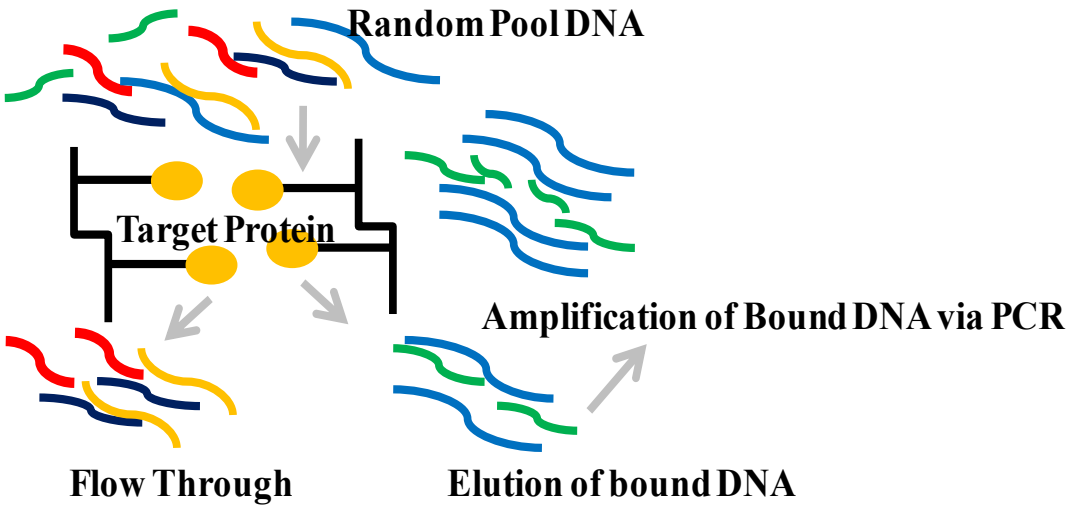

Figure 1. SELEX process in detail. The SELEX methodology is normally characterized by a series of consecutive steps. The random DNA pool is incubated with the target protein, which is generally in the recombinant form and attached to a support. The non-binding DNA is separated from the bound DNA, which is subsequently amplified via PCR. This DNA pool is purified and used in the next round of SELEX. The figure is based on a figure in Okada *et al*. (2008).

The SELEX methodology for the generation of target-specific aptamers is a universal process that is distinguished by the reiteration of five key steps: binding via incubation, partition, elution, amplification, and conditioning. The methodology used during the selection processes must be specified for each specific aptamer; consequently,

some adaptations of SELEX methodology involve changes in the five key steps (Strehlitz *et al.* 2009 and Stoltenburg *et al.* 2007). For instance, supplementary selection steps can be added during each SELEX round to diminish the co-selection of nonspecifically binding oligonucleotides. This modification allows target molecules with similar structures to be differentiated (Goringer *et al*. 1996).. Examples of this approach include negative SELEX, counter SELEX (subtractive SELEX), deconvolution SELEX, subtractive SELEX, Cell SELEX, EMSA SELEX and Spiegelmer technology which generate aptamers in this manner. An outline that demonstrates details of these procedures is shown in Table 3 (Klussman 2006).

Throughout the last decade an increasing number of SELEX experiments have focused on complex targets, such as cells. Cell SELEX, a SELEX modification that is often used to differentiate between normal and disease cell signatures can also be used to differentiate between bacterial cell lines (Kim *et al.* 2009). However, prior knowledge of the number and types of proteins on the target cell membrane surface is not a factor in Cell SELEX. Although in some cases, receptor proteins are known to exist on the cell membrane surfaces, therefore an aptamer pool can be selected to profile the molecular characteristics of a particular cell line or particular receptors (Tang *et al.* 2009).
Cell SELEX methodology requires target cells and the negative cells are selected before any actual lab work is done. The negative cell line generally has different proteins

Table 3. Examples of modifications of the SELEX methodology. The SELEX methodology is a universal process that consists of five general steps; however, the methodology can be modified depending on the aptamer pool that is required. This table provides a synopsis of several modified methods described in Klussman (2006).

Designation	Description	Reference
Negative SELEX	-Decreases the co-selection of unnecessary acid ligands.	Gieger *et al.* 1996, Haller & Sarnow 1997, Blank *et al.* 2001, Vater *et al.* 2003
Counter SELEX	-Creates aptamers that can discriminate between closely related structures.	Jenison *et al.* 1994, Gieger *et al.* 1996, Haller & Sarnow *et al.* 1997, Wang *et al.* 2003, White *et. al.* 2003, Lee & Lee *et al.* 2006
Deconvolution SELEX	-Creates aptamers that can bind to a specific target structure within a complex mixture.	Morris *et al.* 1998, Blank *et al.* 2001
Cell SELEX	-Cell based selection paired with counter selection techniques to gather aptamers that bind to target cells.	Shangguan *et al.* 2006, Hamula *et al.* 2008
EMSA-SELEX	-Electrophoretic mobility shift assay (EMSA) for separateion between each round.	Tasi & Reed 1998

expressed on the cell surface (Kim *et al.* 2009). The use of Cell SELEX does not require prior knowledge of cell surface proteins. An oligonucleotide library that includes a random sequence of 30-40 nucleotides bordered by primer binding sites on either side, which is called the template strand, should be created. The forward primer is labeled with a fluorophore so the sense strand of the PCR that is produced for each round is fluorescently labeled. This fluorescent library can then be monitored for specificity by via microplate reader or flow cytometry (Stoltenburg *et al.* 2007, Chu *et al*. 2006, Hamula *et al.* 2008, and Strehlitz *et al.* 2009).

Cell SELEX is performed after all materials are acquired and prepared. The target cells are prepared for incubation by removing culture media via centrifugation or

other separation methods. Then the aptamer pool is incubated with the target cells for the desired amount of time and temperature. Then the bound aptamers are collected via heat denaturation after the unbound olgionucleotides have been carefully washed away. Next, the surviving bound aptamers are amplified using PCR and sent through another Cell SELEX or counter SELEX round. In counter SELEX, the unbound olgionucleotides are collected, and the bound aptamers are discarded (Strehlitz *et al.* 2009 and Shamah *et al.* 2008). Application of the step allows for the productions of nucleic acid sequences that bind with high affinity and specificity to the target cell line. This process is generally continued for 10-25 rounds. An illustration of this process can be seen in Figure 2. The potentially viable aptamers remaining in the pool are cloned and sequenced. To verify specificity with binding on target cells these sequences can be synthesized with labeled fluorophores and subsequently evaluated via microplate reader, flow cytometry, or fluorescent microscopy, in order to produce a binding affinity (K_D) value (Kim *et al.* 2009, Hamula *et al*. 2008, and Stoltenburg *et al.* 2007).

Cell SELEX methodologies have been especially effective in generating aptamers that bind to molecular markers associated with various cell lines ranging from U251 glioblastoma cells, transformed YPEN-1 endothelial cells, leukemia cells CCRF-CEM, differentiated PC12 cells, *Mycobacterium tuberculosis* cells*, Lactobacillus acidophilus* cells, *Campulobacter jejuni* cells, and *Staphylococcus aureus* cells (Stoltenburg *et al.* 2007, Strehlitz *et al*. 2009, Kim *et al.* 2009, Hamula *et al*. 2008, Chen *et al.* 2007, Dwivedi *et al.* 2010, and Cao *et al*. 2009). This methodology has been particularly helpful in generating aptamer pools that specifically target a wide variety of cancer cells

containing known molecular markers. Table 4 gives more details on these experiments (Kim *et al*. 2009).

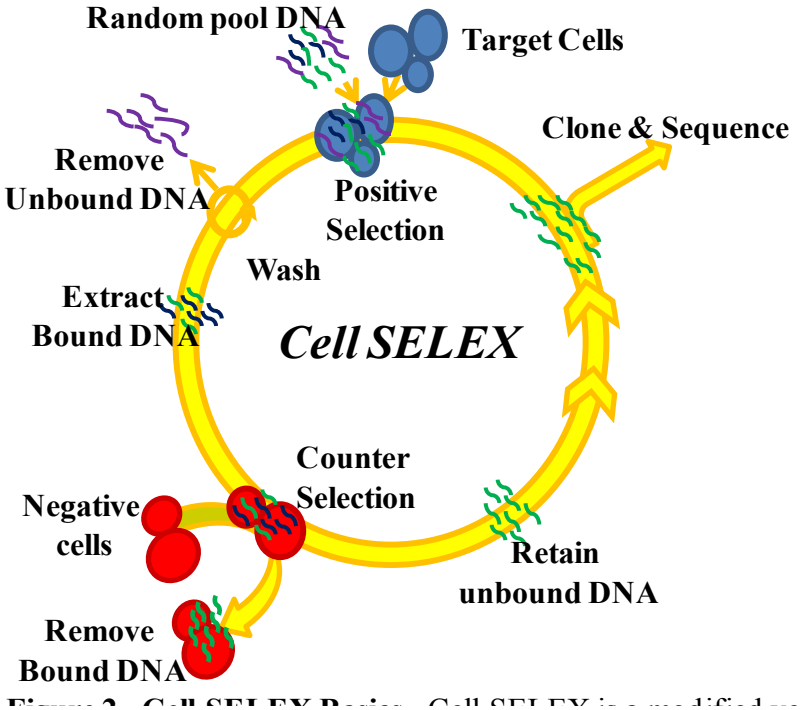

Figure 2. Cell SELEX Basics. Cell SELEX is a modified version of SELEX that is capable of generating apatmers that can differentiate between diseased cells and healthy cells, as well as one type of bacterial cell from another. Cell SELEX consists of several iterative consecutive steps. The live target cells are incubated with the ssDNA library and the cells are washed to remove the unbound DNA. The bound DNA is purified, extracted, and subsequently incubated with negative cells in order to produce counter selection. The bound DNA is removed, whereas the unbound DNA is retained and amplified via PCR. This process is continued for several rounds, until the evolved DNA pool is cloned and sequenced (Kim 2009).

Cell SELEX methodologies combine several different cell types to provide negative, subtractive, and positive selection steps to generate aptamers with the ability to differentiate between the target cell type and other cell types based on the molecular markers found on each cell surface (Kim *et al.* 2009). There are various applications for generating aptamers through this method and include dye profiling, nanoparticle therapy,

drug therapy, target validation, microfluidic device capture, nanoparticle capture, and nanoparticle detection which is illustrated in Figure 3 (Tang *et al*. 2009).

Furthermore, Cell SELEX processes can be used to directly target transmembrane proteins in their natural environment or recombinantly-expressed proteins found on cell surfaces (Hamula *et al.* 2008, Strehlitz *et al.* 2009, Shamah 2009, Chen *et al.* 2007, and Tang *et al.* 2009). For instance, the use of aptamers to stain patient samples and detect cancer using flow cytometry or microscopy can be transitioned into clinical trials. Aptamers can be functionalized in current biotechnologies and analytical methods by integrating the aptamers into microfluidic devices and nanobiosensors to create inexpensive detection devices. One of the most interesting is for molecular profiling of blood or other easily obtainable bodily fluids, because it can give a virtual picture of a patient's current state of health, which could possibly provide clues to a prognosis (Tang *et al.* 2009).

Advantages of Aptamers

Since their inception, aptamers have been found to have an assortment of advantages in comparison to antibodies (Stoltenburg *et al.* 2007, Chu *et al*. 2006, Hamula *et al*. 2008, Kim *et al*. 2009, and Stehlitz *et al.* 2009). This observation maybe attributed to the vast significance of this particular methodology in several research fields; such as, pharmaceuticals, medicine, and environmental analytics (Kim *et al.* 2009 and Strehlitz *et al*.2009). Aptamers tend to bind to targets with affinities and specificities comparable to antibodies. At first is may seem counterintuitive that olgionucleotides can bind to protein targets with high affinity and specificity, but interactions between olgionucleotides and

Table 4. Aptamers generated via Cell SELEX. This table illustrates some of the current research projects that have used Cell SELEX to generate aptamers. The binding affinities of aptamers generative via Cell SELEX are reported in the nM/L range. Four of the experiments illustrated used live bacteria cells to generate aptamers via Cell SELEX.

Cell-SELEX Target	Type of Aptamer	K_D	Reference
U251 gliobalstoma cells	ssDNA	5nM	Daniels *et al.* 2003
Transformed YPEN-1 endothelial cells	ssDNA	Not Specified	Blank *et al.* 2001
Leukemia cells CCRF-CEM	ssDNA	0.8-22nM	Shangguan *et al.* 2006
Differentiated PC12 cells	ssDNA	Not Specified	Wang *et al.* 2003
***Mycobacerium tuberculosis* cells**	ssDNA	Not Specified	Chen *et al.* 2003
***Lactobacillus acidophilus* cells**	ssDNA	13$\pm$3nM	Hamula *et al.* 2008
***Campylabacter jejuni* cells**	ssDNA	292.8$\pm$53.1 nM	Dwivide *et al.* 2010
***Staphylococccus aureus* cells**	ssDNA	Not Specified	Cao *et al.* 2009

proteins arise naturally in numerous biological processes including transcription, translation, and RNA interference (Bouchard *et al.* 2010).

As mentioned earlier aptamers and antibodies have similar binding affinities and specificities for target molecules, although aptamers have several advantages (Bouchard *et al* 2010). For instance, aptamers demonstrate high affinity and exemplary specificity, with K_D values ranging in the nanometer to picometer range, for a wide variety of targets proteins arise naturally in numerous biological processes including transcription, translation, and RNA interference (Bouchard *et al* 2010).

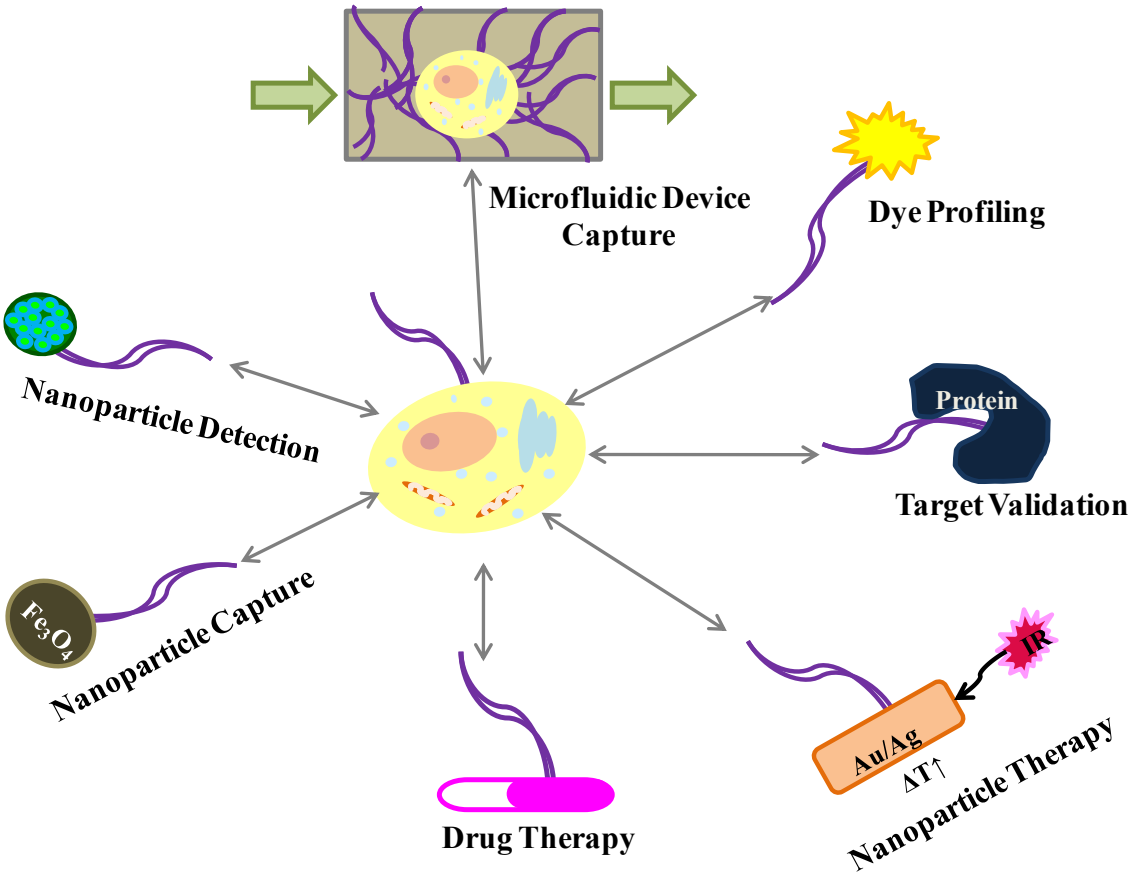

Figure 3. Uses for aptamers generated via Cell SELEX. Aptamers generated via Cell SELEX can and have been used in various fields, such as clinical, diagonisis via dye profiling, nanparticle therapy, drug therapy, and target validation, investigation of protein function via dye profiling, and target validation, bioanalytical recognition via target validation nanoparticle detection, nanoparticle capture, microfluidic device capture, dye profiling, and target validation, and separation systems via target validation. This figure is based on a figure from Tang and Fan (2009).

As mentioned earlier aptamers and antibodies have similar binding affinities and specificities for target molecules, although aptamers have several advantages (Bouchard *et al*. 2010). For instance aptamers demonstrate high affinity and exemplary specificity, with K_D values ranging in the nanometer to picometer range, for a wide variety of targets, some targets that antibodies cannot recognize (Mayor 2009, Zhou *et al.* 2010, Stoltenburg *et al.* 2007, Chu *et al*. 2006, Hamula *et al.* 2008, Kim *et al.* 2009, and Strehiltz 2009).

Unlike antibodies, aptamers are generated via *in vivo* process apart from animals or cell lines. This process can be used to infer effects under both physiological and non-physiological conditions. Therefore, aptamers can be used to identify or inhibit toxic target molecules or molecules that have illustrated no or low immunogenicity (Stoltenburg *et al.* 2007, Chu *et al.* 2006, Hamula *et al.* 2008, Kim *et al.* 2009, and Strehlitz *et al*. 2009).

Another major advantage of aptamers is their small size in comparison to antibodies. Aptamers can access protein epitopes that could otherwise be blocked. As a result, aptamers can be used on intracellular and extracellular targets. Consequently, aptamers can be used in various innovative experiments; such as, investigation of function and interplay of proteins in the environment of living cells or organisms, intracellular detection of target molecules via cytometry or imaging analysis, or exploration of intracellular inhibitors (Stoltenburg *et al.* 2007 and Strehlitz *et al.* 2009).

In contrast to antibodies, SELEX methodologies can be used to guide the generation of an aptamer pool in the direction of a specific target molecule; for instance, counter SELEX. Additionally, adaptations of the selection technique; buffer composition, temperature, binding time, and length of the random region in the template strand, are used to when generate a specific aptamer pool (Stoltenburg *et al*. 2007 and *et al*. 2009).

Post-SELEX modifications are the most important aspect in the aptamer versus antibody debate, because post-SELEX modifications can improve affinity, potency, and metabolic stability of aptamers (Ulrich *et al*. 2006). Modifications can be performed to increase the aptamers specificity and affinity for the target molecules, which increases the

stability in the physiological conditions and bioavailability (Bouchard *et al.* 2010). Some of the most common SELEX modifications include; substitutions of 2'OH group of the ribose backbone of pyrimadines via amino, fluoride. or *O*-methylene groups, and Speigelmer technologies in which RNA generated via SELEX is performed against the unnatural mirror image configuration (D-enamtiomers) of a target peptide. At this point the identified RNA sequence is synthesized in the opposite chirality using mirror image L-nucleotides to produce an aptamer that binds to the natural form of the target. Post-SELEX modifications increase the half life on RNA aptamers in serum or urine from approximately eight seconds to approximately eighty-six hours for flouro-or amino-modified RNA (Ulrich *et al.* 2005 & Bouchard *et al.* 2010). Aptamers resulting from post-SELEX modifications are produced in sizable amounts and are highly accurate and reproducible (Strehiltz 2009, Ulrich *et al.* 2002, and Bouchard *et al.* 2010,). Table 4 highlights the advantages of aptamers in comparison to antibodies.

Current Applications of Aptamers

Since the creation of the SELEX methodology and the isolation of the first protein-specific aptamers, many potential uses have been found in medical fields for therapy and diagnostics and analytical fields for quantification and separation sciences. The two fields tend to merge in diagnostics and quantification areas (Strehlitz *et al.* 2009 and Klussman 2006).

In various aspects, the applications for aptamers as therapeutics seem to parallel those for antibodies even though the technical difficulties have been somewhat different. One of the first aptamers approved for therapeutic purpose was published by Ruckman et

al. in 1998 is the anti-human VEGF (vascular endothelial growth factor) which has an affinity of 130pmol/L. In 2004, the US Food and Drug Administration (FDA) approved pegaptanib (Macugen^{TM}), which an aptamer generated for therapeutic treatment of diabetic macular-edema by Pfizer Inc./OSI Pharmaceuticals (Ruckman *et al.* 1998).

Table 5. Properties of aptamers versus antibodies. The utilization of aptamers in medical and analytical arenas has been exceptionally promising due to their innate ability to bind with high affinity and specificity to target molecules. These factors have lead to a rivalry between antibodies and aptamers, which are described in this table (Klussman 2006).

Aptamers	Antibodies
K_D in low nanomolar to picomolar range.	K_D in low nanomolar to picomolar range.
Selection methodology is completed *in vitro* and can target any protein.	Selection methodology requires a biological system, which generates antibodies that are used against toxins or non-immunogenic targets.
Aptamers can be selected under a wide variety of condition for *in vitro* diagnostics.	Selection is restricted to physiologic conditions for optimizing antibodies for diagnostics.
Specificity of selection methodology is created by iterative rounds against know targets.	Selection methodology for antibodies is time consuming and expensive.
Each batch of a specific aptamer pool is consistent in specificity and affinity.	Each batch of a specific antibody pool varys in consistency in specificity and affinity.
Pharmacokinetic parameters can be altered.	Pharmacokinetic parameters are difficult to modify.
Aptamer binding site can be determined by the investigator.	Antibody binding site is determined by the immune system.
A wide variety of post-SELEX modifications are possible.	Modifications are limited.
Shelf-life is unlimited.	Shelf-life is limited.
No verification of immunogenicity.	Considerable immunogenicity.
Aptamer-specific antidote can be generated to reverse the inhibitory activity of the drug.	Cannot be reversed.

Numerous corporations, such as Archemix Inc., SomaLogic Inc., Noxxon Pharma AG., and Isis Innovation Ltd., are active in research and development field of therapeutic aptamers. All of these companies have aptamer-based therapeutics in product

development or clinical studies. The amassed experience of both academia and industry implies that aptamers are quite capable of binding to targets form virtually any protein class (Sullenger *et al.* 2002). Most of the earliest aptamer applications concentrated mainly on nucleic acid-binding targets (Tuerk *et al*. 1990; Tuerk *et al.* 1992; Tuerk *et al.* 1993), later applications have been used to generate aptamers against a wide variety of proteins; such as antibodies (Kim *et al.* 2003; Lee *et al.* 1997; Wiegand *et al.* 1996), peptide hormones (Lin *et al*. 1996), cell surface receptors (Ulrich *et al.* 2002; Chen *et al.* 2003), tumor markers (Hicke *et al.* 2001; Daniels *et al*. 2003), and viral coat proteins (Huizenga *et al.* 2003). The therapeutic aptamers in clinical use or clinical development as of June 2009 are shown in Table 5 (Ferreira *et al.* 2006, Herr *et al*. 2006, Hicke *et al.* 2001, Jeon *et al.* 2004 and Klussman 2006).

The medical and analytical fields overlap in diagnostics and detection. Early applications utilizing aptamers in diagnostics and detection are in constant demand for effective and sensitive procedures which can provide accurate detection of diseases or infection in the early stages. Clinical diagnostics and detection via aptamers provide an innate ability to identify specific targets in heterogeneous target mixtures via fluorescence, flow cytometry, or biosensor assay (Phillips *et al*. 2006).

Fluorescence and nanotechnology has been paired with aptamer pools to create targeting elements for diagnosis and detection. Pairing fluorescence with nanoparticles provides a fluorescent intensity several hundred times higher in comparison to single organic dyes. These bioanalytical tools are produced using several different types of fluorescence, such as fluorescent nanoparticles, quantum dots, dye doped silica particles, or radioactive decay. In most cases, the fluorophores are covalently linked to either end

or middle of the aptamer. Several techniques can be used as signal mechanisms in bioanalytical tools including, fluorescence anisotropy, fluorescence lifetime, fluorescence quenching, and fluorescence resonance energy transfer (FRET) (Phillips *et al*. 2006).

Flow cytometry is one of the most utilized aptamer-based diagnostic and detection methods produced via Cell SELEX because it recognizes cell surfaces epitopes that are either specifically or differentially expressed in the target cells (Tang *et al.* 2009). Aptamers were used in previous studies to evaluate target recognition as well as for isolation of cell populations that have tumor related biomarkers. Their study analyzed the binding of FITC-labeled DNA aptamer and a complex of mouse antihuman nuetrophil elastase (HNE) antibody and FITC labeled rat antimouse antibody to HNE-labeled beads. The result derived from the flow cytometry analysis demonstrated that aptamers are just as effective as antibodies in HNE detection. Table 6 illustrates other applications of flow cytometry paired with aptamer-based bioassays used as diagnosis and detection methods (Li *et al.* 2008, Guo *et al.* 2006, Li *et al.* 2009, and Chen *et al.* 2009).

Biosensors are another aptamer based methodology used for diagnosis and detection arena. Conventionally, biosensors depend on the encapsulation of an analyte by either an antibody or an aptamer receptor. The biosensor apparatus contains two or more of the following parts: recognition component, linker, localization component, signal receptor, signal amplification component, or signal transducer. Although only two of these components are necessary, the recognition apparatus, which identifies the target and signal transducer, which transforms signal produced by the analyte into a detectable signal. Generally, the recognition components are enzymes, antibodies, or aptamers.

Table 6. Therapeutic aptamers in clinical use or clinical development. In June 2009 there were seven therapeutic apatmers that were approved for use as a marketed product or in clinical development. The table is based one shown in Klussman (2006).

Compound ID	Therapeutic Agent	Disease Indication	Method of Administration	Clinical Phase	Reference
Macugen™	VEGF	Macular degeneration	IV	Market	Ng *et al.* 2006
ARC1779	Von Willebrand Factor	Thrombic microangiopathy adjunct to carotoid endarterectomy	IV infusion	Phase 2	Hamidi *et al.* 2009
REG1	Factor IXa	Coronary artery bypass Percutaneeous coronary intervention	IV bolus	Phase 2	Dkye *et al.* 2006
AS1411	Nucleolin	Acute myeologenous leukemia Renal cell carcinoma	IV infusion	Phase 2	Bates *et al.* 2009
E10030	PDGF-b	Macular degeneration	IV	Phase 1	Jo *et al.* 2006
ARC1905	Complement Factor 5	Macular degeneration	IV	Phase 1	Kurz *et al.* 2005
NU172	Thrombin	Coronary artery bypass	IV infusion	Phase 1	Wagner-Whyte *et al.* 2007

These components directly determine the selectivity, sensitivity, stability, and application of biosensors (Klussman 2006, Mayor 2009, Zhou *et al.* 2010).

All biosensors fall into four basic categories: target induced structure switching, sandwich/sandwich like, target induced dissociation displacement, and competitive replacement. All four categories target specific binding processes between ligands and aptamers into different signal variations in order for the ligands to be effectively detected. Current biosensors have exceptional performance on standard samples, but to date few biosensors have been applied to serum or urine samples (Mayor 2009, Zhou *et al.* 2010).

One example of an aptamer-based biosensor is the immunoglobine E (IgE) disposable electrochemical strip sensors in allergy detection devices. Other electrochemical sensor platforms utilize methylene blue attached to one end of the aptamer that is fixed to an electrode surface via a redox marker, which is used to detect the aptamer-binding reaction. One example of an aptamer-based biosensor is the immunoglobine E (IgE) disposable electrochemical strip sensors in allergy detection devices. Other electrochemical sensor platforms utilize methylene blue attached to one end of the aptamer that is fixed to an electrode surface via a redox marker, which is used to detect the aptamer-binding reaction. This is a target induced dissociation displacement biosensor, which produces a methylene blue signal only when the target is present. Similar technologies are used to detect cocaine, thrombin, and platelet-derived growth factor (PDGF) (Strehlitz *et al.* 2009, Wiengand *et al.* 2006).

Aptamers can also be used for purely analytical purposes, like separation and purification of analytes by chromatography, electrochromatography, or capillary electrophoresis techniques. RNA and DNA aptamers have been used to separate and purify proteins, small molecules, and enamtiomers. Aptamers have also been successfully used to separate species and characterize the affinity of binding interactions

Some scientists believe the possibilities of aptamers in the separation science field are vast, although massive amounts of aptamer must be used in order to compete with current separation methods. Consequently, aptamers can only be used in separation science on micro scale (Ravelet *et al*. 2005, Klussman 2006). The current innovations in these fields can be seen in Table 8.

Table 7. Aptamers and cytometry applications. This table summarized recent developments and applications of RNA and DNA aptamers in cytometry research described by Klussoman (2006). FITC-fluorescein isothiocyannate, PE- phycoerythrin, CM-confocal microscopy, FM- fluorescence microscopy, FCS- fluorescences correlation spectroscopy, and FACS- fluorescence activated cell sorting.

Target	Fluorescence Reporter	Application	Instrument	Reference
Angiogenin	Cy5	Target protein internalization into intracellular organelles of HUVE and human breast cancer cells	CM	Li *et al.* 2008
Biomarker of mesmchymal stem cells	FITC	Sorting mysenchymal stem cells	FACS	Guo *et al.* 2006
Various Tumor cell lines	FITC, PE	Analysis of signal-strength of target recognition and unspecific binding of aptamers to their targets on cells	FM, CM	Li *et al.* 2008

Table 8. Examples of aptamers in affinity chromatography for analyte capture and separation. This table illustrates how aptamers can be used in separation science to separate or capture analytes via liquid chromatography (LC) and capillary electrochromatography (CEC). The table was based on a table in Klussman (2006).

Target	Aptamer Type	Separation Systems	Application	Reference
Proteins				
L-selection	DNA	LC	Capture	Romig *et al.* 1999
HCV RNA polymerase & replicase	RNA	LC/chip	Capture	Cho *et al.* 2004
Non-target proteins	G-quartet DNA	CEC	Separation	Rehder & McGowen 2001
Small Molecules				
Non-target analytes	G-quartet DNA	CEC	Separation	Kotia *et al.* 2000
Adenosine and analogs	DNA	Nano-LC	Separation	Deng *et al.* 2001
Flavin mononucleotide and other molecules	RNA	CEC	Separation/Capture	Clark & Remcho 2003
Enantiomers				
Vasopressin	DNA	LC narrowbone column	Separation	Michaud *et al.* 2003
Adenosine	DNA	Micro-LC	Separation	Micahaud *et al.* 2004
Amino Acid Derviatives	DNA	Micro-LC	Separation	Michaud *et al.* 2004

CHAPTER TWO

Background on Cell Lines Used For Cell SELEX

Chapter Overview

Throughout this chapter the current literature-based knowledge relating to the outer membranes and outbreak details of the three cell lines, *Escherichia coli* DH5α*, Bacillus subtilis,* and *Enterobacter aerogenes,* will be reviewed.

Escherichia coli, is used as the primary target in Cell SELEX for this particular research project, whereas, *B. subtilis* and *E. aerogenes* are utilized as counter Cell-SELEX targets. This process generates an aptamer pool that binds with high specificity and affinity to *E. coli* DH5α.

Escherichia coli

Background and History

Escherichia coli is an uncomplicated in comparison to other bacterial lines, Gram-negative, rod-shaped bacteria, single-celled organism that is approximately one to two microns in length and one half to one micron in diameter. Each *E. coli* cell is surrounded by the inner and outer membrane. The periplasm is located between the outer and inner membrane; the cytoplasm is the space surrounded by the inner membrane. Each of these cellular components has unique contents and properties that provide specific cellular functions (Trun *et al.* 2004, Inouye *et al*. 1986, Lederburg *et al.* 2006).

E. coli can be found in the intestinal tract of mammals or in areas tainted by feces. *E .coli* is able to grow in both in the presence or absence of oxygen, which makes it quite easy to grow in the laboratory using chemically modified media. In this setting each *E. coli* cell divides approximately every twenty to thirty minutes. Numerous *E. coli* genetic variants are non-disease causing; therefore, *E. coli* has been one of the most extensively studied cell lines over the past fifty years (Trun *et al.* 2004, Nedhardt *et al*. 1996, Lederburg *et al*. 2000).

E. coli was first discovered by Theodor Escherich, a German pediatrician in 1885. The first specimen was taken from feces of healthy individuals. From its scientific inception *E. coli* was used as a representative, harmless bacterium that can be safely and easily cultured in synthetic media; however, pathogenic strains have been found (Trun *et al*. 2004, Neidhardt *et al.* 1996, Lederburg *et al.* 2000).

Cell Physiology and Reproduction

The outer membrane is the major cellular barrier that interacts with the extracellular environment. This barrier protects the cell from detergents, dyes, hydrophobic antibiotics, and bile salts from the intestines, using a double layer of lipids with the polar head groups facing outward (Trun *et al*. 2004). The leaflets of the outer membrane are quite different. Therefore, the cell can distinguish inside of the cell from the outside of the cell based solely on the composition of the leaflets (Trun *et al.* 2004, Inoye *et al.* 1986).

The peptidoglycan layer, which is arranged in rings that travel around the short axis of the *E. coli* cell, is attached to the inner face of the outer membrane of the *E. coli* cell. This structure is required for retaining the rod like structure of the cell and protects

the cell from pressure differences between the inside and outside of the cell. When the *E. coli* cell is grown in the presence of penicillin antibiotics, rings that make up the peptidoglycan layer are not connected to each other, which results in cell explosion (Trun *et al.* 2004, Inoye *et al*. 1986).

The periplasm, which is an aqueous partition that is similar to the aqueous environment located outside the *E. coli* cell, comprises proteins that are capable of perceiving the external environment and relaying the information across the inner membrane to the cytoplasm (Trun *et al.* 2004, Neidhardt *et al*. 1996). These particular system permits cellular response to the surrounding environment in either a positive or negative manor. There are several other features of the periplasm; such as, proteases which are used to degrade abnormal proteins and a system that provides the formation of disulfide bonds in proteins that are attempting to enter the cell. One of the most important features of the periplasm is its ability to monitor the outside of the cell and concentrate solutes to the inside of the cell (Trun *et al.* 2004). Figure 4 illustrates some of the basic cellular components of *E. coli*.

Lipopolysaccharides (LPS), which are very specialized carbohydrate side chains, are attached to the outer membrane facing away from the cell, which can be seen in Figure 4. Each LPS molecule as six to seven fatty acid chains per molecule, which are used to produces a very dense barrier. These specialized liquids, composed mainly saturated fatty acids, are vital in preventing toxic compounds from traveling through the outer membrane. LPS generally contains negatively charged side chains which allow the *E. coli* cells growing in intestinal tracts to evade being engulfed by intestinal cells, attacks from the immune system, or degraded by digestive enzymes. This particular

characteristic also allows the bacterial cells to colonize on the surface of human cells in intestinal tracts (Trun *et al.* 2004, Inouye *et al*. 1986).

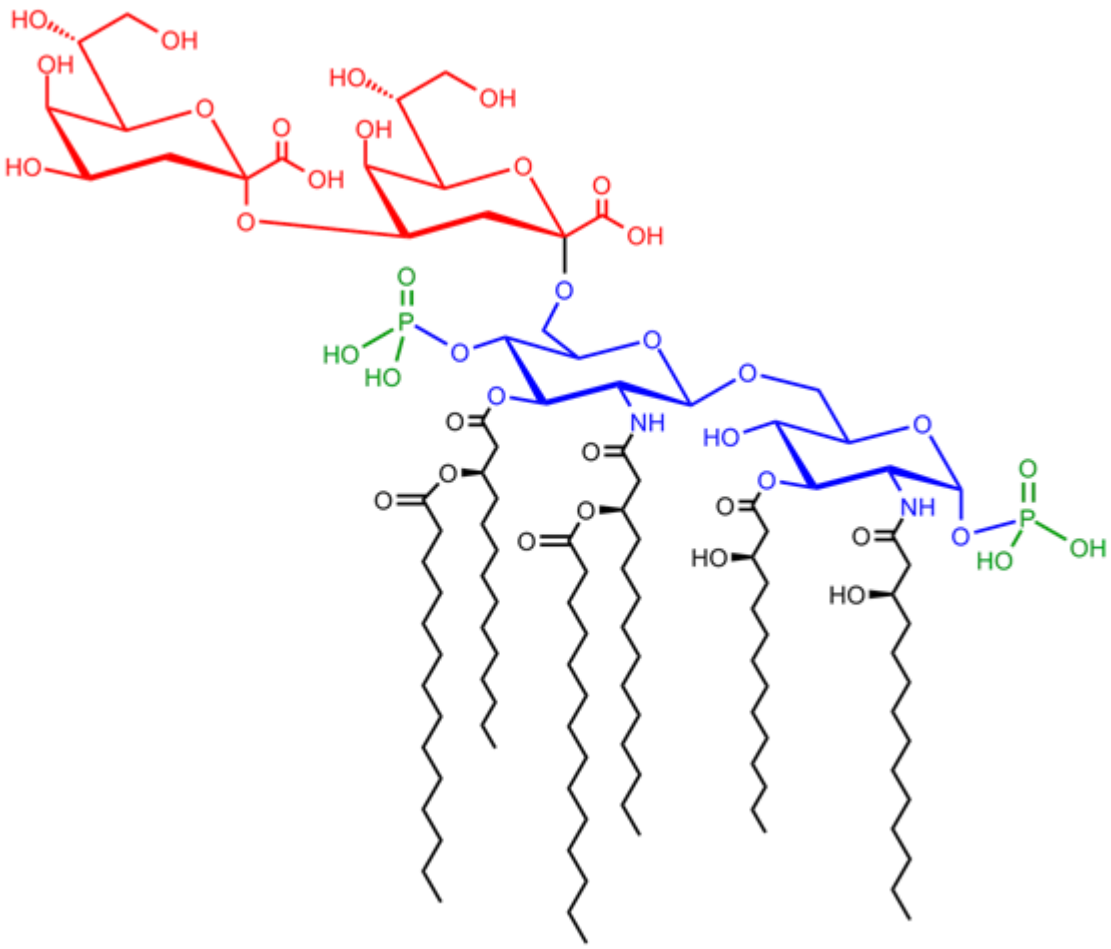

Figure 4. The Lipopolysaccharide of *E. coli*. LPS is one of the chief component of the outer membrane of *E. coli*, which donates to the general structural integrity of the bacterial cell. LPS also protects the membrane form chemical attack and aides increasing the negative charge of the cell membrane. Cell death is imamate if LPS is removed. LPS is also an endotoxin, which induces a strong response from normal mammalian immune systems (Raetz *et al.* 2006).

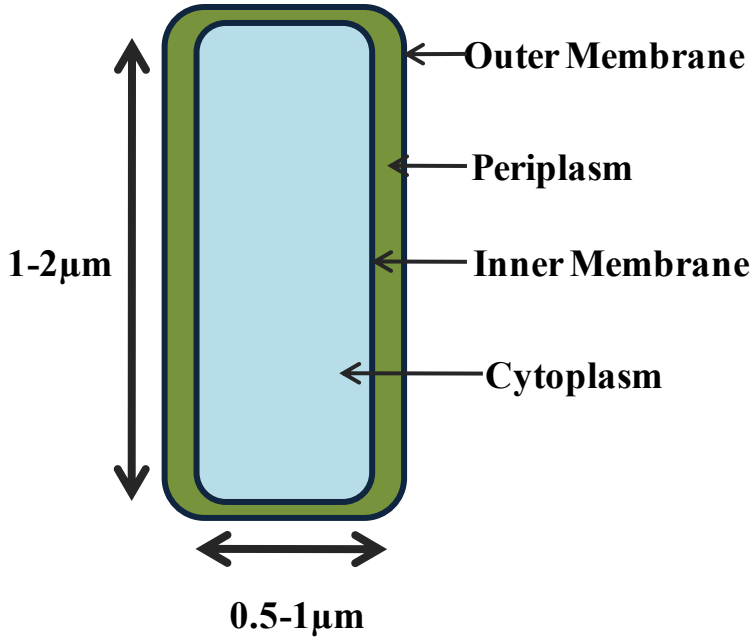

Figure #. Cellular components of *E. coli* cells. *E. coli* is a

Figure 5. Cellular components of *E. coli* cells. *E. coli* is a gram negative bacteria and each cell is approximately 0.5-1 µm wide and 1-2 µm long. Each cell has four cellular components. The outer membrane contains lipids and lipopolysaccharides. The periplasma is an aqueous layer that contains various proteins, such as proteases. The inner membrane acts as a cellular barrier and prevents toxic materials from entering the cell. The cytoplasm is the heart of all the activities of the *E. coli* cell. This illustration is based on a figure in Trun *et al.* (2004).

Research on the *E. coli* cell envelope was originally focused on the function, structure, and biosynthesis of LPS; however, the field evolved into the study of membrane protein function, structure, and secretion, which are still major research topics today (Braun *et al*. 2009). The first protein isolated from the *E. coli* cell envelope was the lipoprotein covalently bound to murein and the amino acid sequence and lipid attachment sites were identified in 1972/1973 (Braun *et al.* 1972, Hantke *et al.* 1973). Soon thereafter TonA was isolated. In 1973 both LamB the receptor of phage λ (Randal-Hazelbauer *et al.* 1973) and BtuB receptor of colicin E3 were isolated (Sabet *et al*. 1973).

Later in the same year, the BtuB protein was found to be engaged in vitamin B_{12} transport and phage BF23, E1, and E3 receptor (Di Masai *et al.* 1973). In 1975, LamB was shown to facilitate the uptake of maltose (Szmelcman *et al.* 1975) and TonA was found to transport ferrichrome, which resulted in a new name FhuA (Hantke *et al.* 1975 and Luckey *et al.* 1975). The characterization of *E. coli* outer membrane protein F (OmpF) paved the way for porin studies in 1976 (Nakae *et al.* 1976). Outer membrane protein G (OmpG) is an example of a porin found in *E. coli,* which forms a 14-stranded β-barrel that seems to function as a monomer. OmpG's central pore is wider that other *E. coli* porins and it thought to be a non-specific channel utilized for the transport of larger oligosaccharides (Subbaro *et al.* 2006).

Specialized hair-like projections anchored in the outer membrane facing the outside of the *E. coli* cell, covering the entire cell surface are known as fimbrae. These specialized proteins allow *E. coli* cells to adhere to eukaryotic cells via blood group antigens, collagens, and cell surface sugars. Each cell can have several types of fimbriae on the cell surface (Trun *et al.* 2004, Inouye *et al*. 1986). Figure 6 depicts these projections.

Flagella, which in its simplest form are corkscrew-like proteins that are five to ten microns in length, have a motor that is imbedded in the inner membrane of the *E. coli* cell. The flagella are capable of rotating in both clockwise and counter-clockwise direction, which results in movement of the cell. This movement is paired with a sensing system located in the cytoplasm which is responsible in the net movement of the cell toward favorable environments. This movement is known as chemotaxis (Trun *et al.* 2004, Inouye *et al.* 1986). These projections can are illustrated in Figure 7.

Male *E. coli* cells contain an extra surface, called the F pilus, structure which has a distinctive function, transferring a single chromosome to the female cell. The transferred DNA is then replicated in two double-stranded DNA molecules and

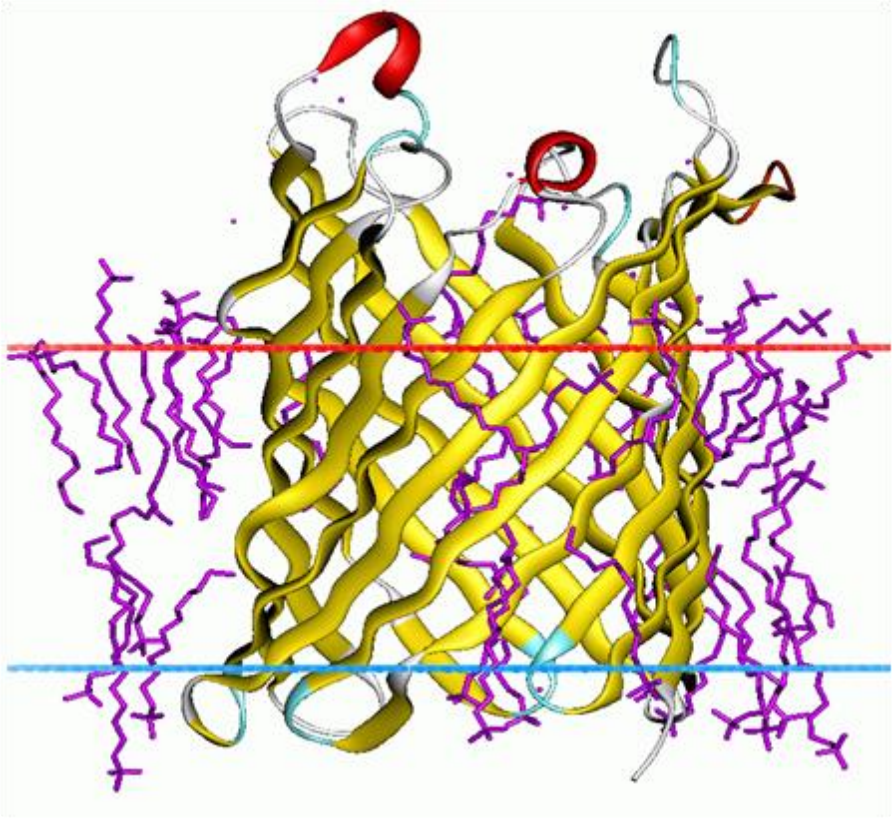

Figure 6. *E. coli* outer membrane protein G (OmpG). OmpG is a porin found in Gram-negative bacteria, which forms a 14-stranded β-barrel that functions as a monomer.

incorporated into the female cell. This whole process is known as conjugation, which has been shown to be utilized to transfer DNA from *E. coli* to other bacterial species and in some cases into eukaryotic cells (Trun *et al.* 2004, Inouye *et al.* 1986). Figure 9 shows the protein composition of the F pilus.

All *E. coli* cells have capsular a polysaccharide lining, known as glycocalyx, which is surface structure composed of polysaccharides and various proteins that are loosely attached to the outer membrane, which can be seen in Figure 10. There are several different distinctions between the various layers of the glycocalyx; for instance,

polysaccharides covalently linked to the cell are known as the capsule and loosely secured polysaccharides discharged from the cell are referred to as the slime layer.

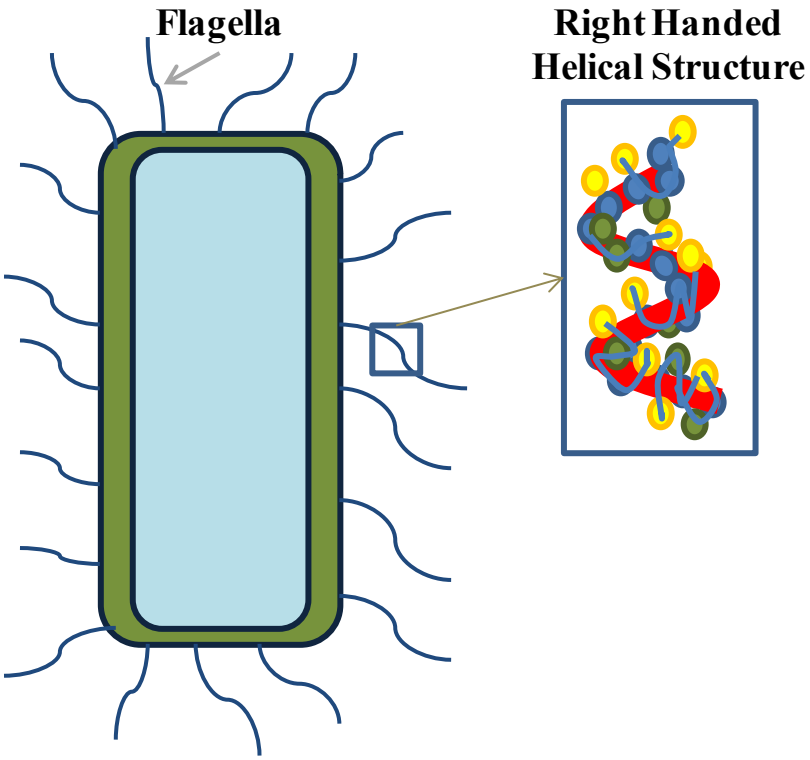

Figure # Structure of fimbriae in *E. coli* cells Fimbriae are hair-like

Figure 7. Structure of fimbriae in *E. coli* cells. Fimbriae are hair-like proteins that project from the outside of the outer membrane, which are used to adhere E. coli cells to eukaryotic cells. These each fimbriae is a single identical protein that exhibits a right-handed helical structure. The figure is based on Trun *et al*. (2004).

The glycocalyx composition can vary from cell to cell, but they all generally contain polysaccharides including polyalcohols, amino sugars, proteoglycans, and glycoprotiens (Lewin *et al.* 2007). Capsular polysaccharides are created by the fusion of monosaccharides into long chain. Interestingly, any monosaccharides can be joined together creating a large diversity in capsular polysaccharides. For instance, more the 80 diverse capsular polysaccharides, known as K antigens have been described for *E. coli*

cells. Particular K antigens are associated with specific infections (Trun *et al.* 2004, Inouye *et al.* 1986).

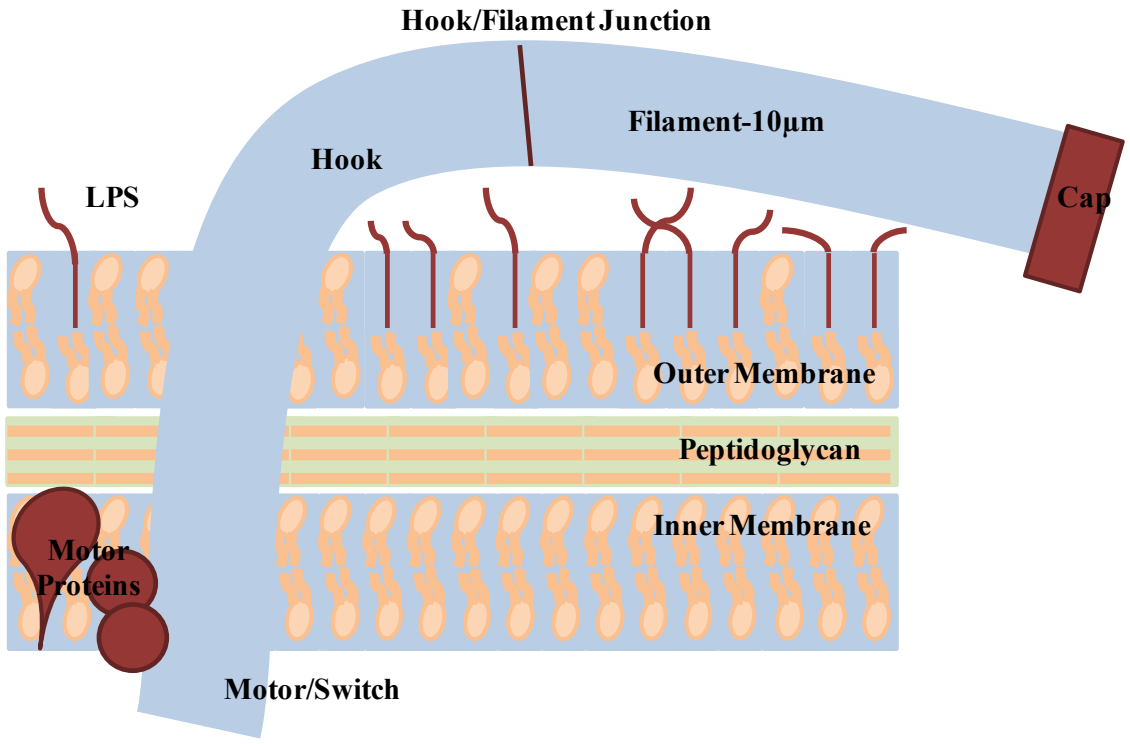

Figure 8. Depiction of the flagella in the E. coli cell's membrane layers. Flagellum are fastened in the inner membrane, travel through the peptidoglycan layer, and break through the outer member of *E. coli* cells. The motor proteins and motor switch are located in the inner membrane and create the clockwise and counter-clockwise movement of the flagellum; which ultimately controls the cell's movement. LPS-lipopolysaccharides. The figure is based on an illustration in Trun *et al.* (2004).

The glycocalyx has several different proposed functions; such as, protection from desiccation, phagocytosis, detergents, bacteriophages, and to help the bacteria evade the host immune system. Capsular proteins can also aide in adherence to other prokaryotes, which can lead to bioflim formation. This thick alginate coat helps protect different species of cells form antibiotics and host defenses (Lewin *et al.* 2007, Trun *et al.* 2004).

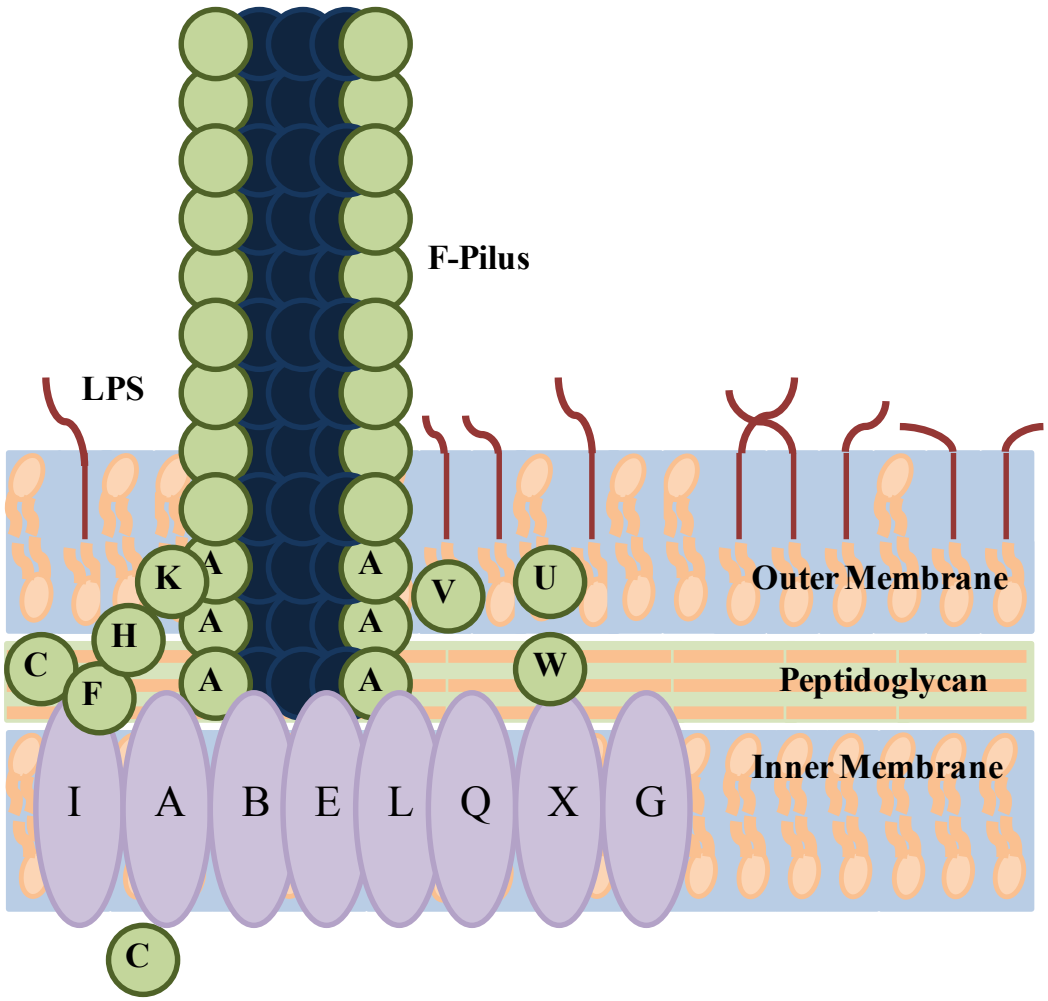

Figure #. Assembly of F-pilus. Thirteen different proteins are required for F-pilus

Figure 9. Assembly of F-pilus. Thirteen different proteins are required for F-plus biogenesis. These proteins are responsible for surface exclusion of the F-plus, mating pair stabilization, and DNA synthesis. The exact function of the individual function for each of these proteins is unknown. LPS- lipopolysaccharides. Based on a figure in Trun *et al*. (2004).

Many distinctive varieties of proteins are imbedded in the outer membrane; some of which act as receptors and are used as transport through the outer membrane via gated holes or pores. The pores provide a gated pathway through the outer membrane for chemicals to passively diffuse. Other proteins located in the outer membrane provide structural support for the *E. coli* cell, which is depicted in Figure 8. (Trun *et al.* 2004, Inouye *et al.* 1986).

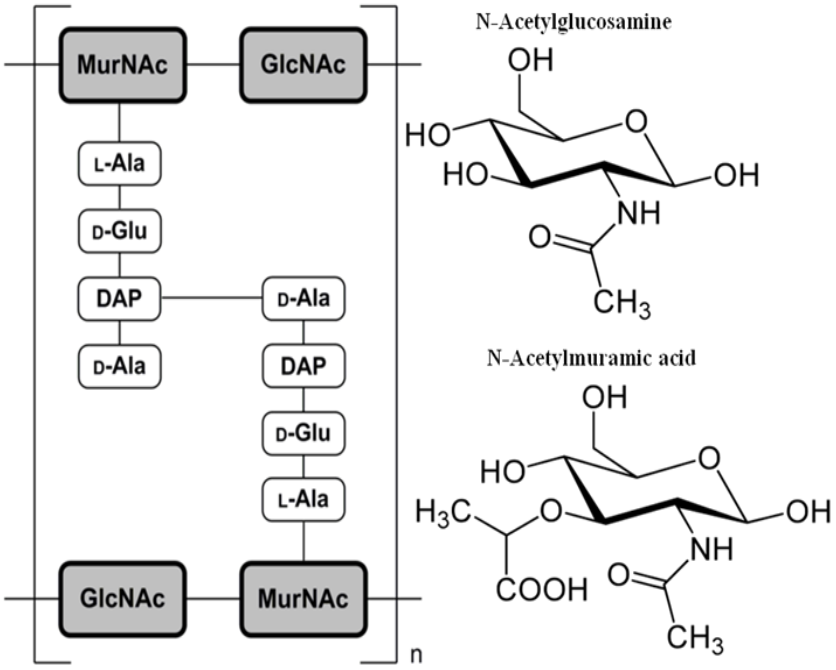

Figure 10. The chemical structure of *E. coli* peptidoglycan. Peptidoglycan, which is also known as a murein, is a polymer containing alternating redisues of β (1,4) linked N-acetylglucosamine and N-acetylmuramic acid. A peptide chain of three to five amino acids are attached to the N-acetylmuramic acid. The peptide layer can often be cross-linked to the peptide of another strand to form a three dimensional structure. The peptidoglycan provides structure to the bacterial cell, counteracts the osmotic pressure of the cytoplasm, and participates in binary fission during bacterial cell reproduction (Salton *et al.* 1996).

Strains

E. coli K-12 is a non-pathogenic strain that contains lysogenic bacteriophage lambda, and is the seat of many plasmids. DH5α is a special strain of *E. coli* that widely used in DNA manipulation, because IPTG is not required to induce expression from the *lac* promoter even though the strain does contain the Lac repressor (Trun *et al.* 2004).. Throughout several generations of cultivation in a laboratory environment the K-12 strains have lost their "O" surface antigens, which imparts further assurance if its harmlessness to people; however, this same characteristic leaves K-12 out of the study of

pathogens, unlike *E. coli* O157, which is closely associated with the *Shigella* toxin (Lederberg *et al*. 2000 and Quinones *et al*. 2009).

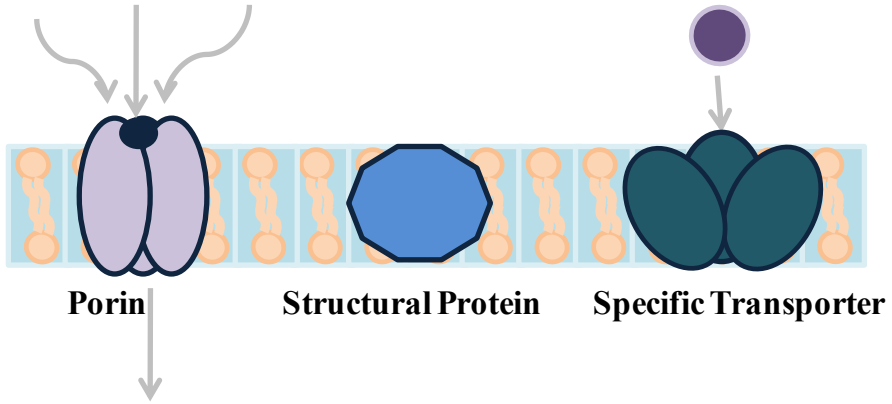

Figure 11. A schematic that illustrates structures located in *E. coli*'s outer membrane as described by Trun *et al.*(2004). Porins, structural proteins, and specific transport receptors that span *E. coli'*s outer membrane. Porins, which are responsible for passive diffusion of small molecules, are compiled of three identical subunits with three channels, one through each subunit. Structural proteins are needed to provide architectural support, when these compounds are absent the outher membrane is destabilized. Specific transporters only allow certain compounds through the outer membrane, such as *Btub*, which transports vitamin B.

Several different subspecies of *E .coli* have been isolated; Kauffman and coworkers described a system, around fifty years ago, utilized to classify *E. coli* based on their outer membrane surface structures that are capable of eliciting an immune response in mammals: the lipopolysaccharide or O-antigen, the flagella or H-antigen, and the capsule or K antigen. At this point, 173 various O antigens, 56 H antigens, and 80 K antigens have been found (Trun *et al.* 2004, Quinoes *et al.* 2009, and Kauffman 2006).

The most commonly used laboratory strains is *E. coli* K12; conversely, outbreaks are of *E. coli* O127:H7 strain, which contains very distinct O and H antigens that continually arise in the United States each year, usually found in contaminated food. An

infection generated from *E. coli* O157:H7 can be lethal in young, elderly, or immunocompromised individuals (Trun *et al.* 2004, Neidhardt *et al*. 1996).

Shiga toxin-producing *E. coli* O157:H7 (STEC) has been known to cause serious food borne disease in humans. These pathogens at low doses may cause diseases ranging from mild diarrhea to haemorrhagic colitis, and the life-threatening haemolytic-uremic syndrome (HUS). STEC virulence is largely accredited to the production of Shiga toxins (Stx), and their innate ability to colonize the human intestine by the attaching and effacing mechanism encoded by the locus of enterocyte effacement (LEE). In every instance Shiga toxins are encoded on bacteriophages, and several toxin variants have been depicted and divided into two main groups, Stx1 and Stx2, and further into subtypes. The STEC variants that are most often associated with serious disease in humans often contain genes for the Stx1 subtype Stx2, and the locus of enterocyte effacement (LEE) (Sekse *et al.* 2008).

E. coli O157:H7 is a quickly evolving pathogen which is highly adaptive to different ecological niches and environmental stresses. The differences among most *E. coli* strains occur in their ability to survive stressful conditions. These diverse phenotypes direct result of the organism's highly plastic and adaptive genome. The *E. coli* O157:H7 strains, most commonly associated with human disease outbreaks, have been grouped into nine distinct phylogenetic clans that display variable resistance to acid exposure in a simulated GI system (Sekse *et al.* 2008).

Outbreaks and Disease

The chief source of Shiga toxin *E. coli* is the intestines of domestic ruminants, like sheep and cattle. Human transmission is normally food borne, although fecal-oral

transmission from person to person and animals to humans may also occur. In the spring of 2006, a national disease outbreak was caused by Stx2-producing *E. coli* was investigated in Norway. Seventeen patients were recorded in the outbreak; ten with diarrhea-associated HUS, including one fatal case, six with diarrhea, and one person with an symptomatic infection. Patient stool samples retrieved isolates at the Norwegian Institute of Public Health (NIPH) revealed *E. coli* belonging the serogroup O103:H25 from 11 patients. All of the patient isolates contain the gene encoding intimin (*eae)*, located in LEE. However, only two isolates obtained from two different patients contained the gene encoding Stx (stx_2), the remaining isolated were *stx*-negative. (Sekse *et al.* 2008).

Interestingly, Shiga toxin-producing *E. coli* O157:H7, the leading cause of bloody diarrhea and HUS is responsible for the highest proportion (21%) of all produce-linked *E. coli* outbreaks in the United States. Recent *E. coli* outbreaks connected to fresh produce demonstrated an increased severity of infections amid susceptible populations over a widespread geographic location. In the 2006, contaminated baby spinach lead to approximately 200 cases over 26 states, and more than 30 cases of hemolytic-uremic syndrome and three deaths were reported (Sekse *et al*. 2008).

Enterobacter aerogenes

Background and History

Enterobacter aerogenes is Gram negative pathogen generating bacteria responsible for many hospital-acquired respiratory tract infections. The presence of a prevalent resistance clone of *E. aerogenes* has been found in France, Belgium, and

Spain. Antibiotic resistance of *E. aerogenes* was linked with a high crude fatality rate in infected patients in a Belgian hospital. This tragic response is the result of the pathogens ability to harbor an assortment of antibiotic resistance mechanisms; such as, the modification of outer membrane permeability and a porin deficiency associated with the expression of cephalosporinase activity (Chen *et al*. 2008, Bornet *et al*. 2004).

Cell Physiology and Reproduction

The outer membrane of Gram negative bacterial cells, such as *E. coli* and *E. aerogenes,* provide an obstruction that is utilized to defend against cellular components exterior forces like, heavy metals, detergents, or other damaging substances. Essential nutrients are allowed passage into the cell via porins, which are proteins that form hydrophilic channels. The production of porins is upregulated and downregulated based on osmolarity, temperature, the concentration of pressure reducers, or the presence or absence of various molecules within the media. Other forms of regulation may occur through the response to antibacterial or aromatic compounds, which have been shown to decrease the number of porins in the outer membrane through various regulatory cascades utilizing *mar* or *sox* operons (Thiolas *et al.* 2004). Antibiotic interaction with Gram negative bacteria begins when the antibiotic tries to cross the outer membrane. At this moment science has a difficult time explaining the exact method of uptake across this lipid bilayer by hydrophobic compounds. It is known that outer membrane is interspersed by porins, which are known as outer membrane proteins (Omp) that form water-filled canals permitting diffusion through the membrane. Several clinical studies illustrate that the general diffusion Omps of various enterbacteriaccal species provide a key entrance for the passage of β-lactams and fluoroquinolones (James *et al.* 2009).

The most significant porin in *E. aerogenes* is Omp36 is a major outer membrane protein which aides in the stabilization of the bacterial envelope. This protein consists a β-barrel with three loop domains in which there is an elevation of preserved charged residues when compared to outer membrane proteins from *E. coli*, which may correspond to an rising bacterial drug resistance strategy used to hamper antibiotic influx (James *et al.* 2009 & Thiolas *et al*. 2004).

Strains

The Enterobacteriaceae family contains *Escherichia, Shilgella, Salmonella, Enterobacter, Klebsiella, Serratia, Proteus*, and others. These bacteria can be found in soil, water, dairy products, and in the gastrointestinal tract of humans and animals. The rod shaped forms of Enterobacteriaceae come in various sizes, are not spore forming, exist in motile and non-motile forms, and can grow aerobically and anaerobically (Brooks *et al.* 2007 and Lederberg *et al*. 2000).

Outbreaks and Disease

The *Enterobacter* genus is a nosocomical opportunistic pathogen which seems to be one the chief causes for infections second only to *E. coli*. Infections generally caused by *E. aerogenes* are respiratory, gastrointestinal, urinary tract infections, wound, bloodstream, and central nervous system infections. Additionally, *E. cloacaea* and *E. aerogenes* are generally associated with adult cases of meningitis (Sankaran *et al.* 2000).

E. aerogenes is known to have drug-resistant characteristics. Recently antibiotics have provided significant success dealing with infections. However, the rapid insurgence of multidrug resistance has become an escalating dilemma (Sankaran et.al 2000). These

multiresistant strains have been the source of outbreaks in Belgium, France, Austria, and the United States. Current studies have demonstrated that *E. aerogenes* is resistant to ampicillin and imipenem (Baily *et al*. 1974, Brooks *et al.* 2007).

Bacillus subtilis

Background and History

The Gram-positive, spore forming bacterium, *Bacillus subtilis*, is one of the cell lines used in the Cell SELEX process as a counter SELEX target. *B. subtilis* is a naturally transformable soil bacterium. Competency is established in this particular cell line when the cells reach a high density towards the end of exponential growth, at which point vital nutrients such as carbon, nitrogen, or phosphorous has been depleted. During this period two separate pathways can activate the expression of a specific set of genes, which is known as *com* genes, whose products are needed for the establishment of competency. Both of the genes involved in establishing competency are capable of establishing cell density; however, only one of these pathways is responsible for inducing sporulation. The elegant process *B. subtilis* utilizes to induce competency will be elaborated on throughout this thesis chapter because of the importance surface proteins play in this process (Trun *et al.* 2004, Dubnau *et al.* 1993).

Cell Physiology and Reproduction

Most Gram-positive bacteria utilizes a mechanism for DNA binding and transport that is quite similar to the mechanism that *E. coli* employs. DNA binding receptors, which are assembled and placed on the cell surface, are encoded by the *com* genes.

These receptors remove the dsDNA from the pilus; at this point, the dsDNA undergoes a conversion form loosely bound DNA, which can be removed via a phenol-detergent treatment, to irreversibly bound DNA. The DNA is impervious to a phenol-detergent treatment while it is bound to the receptor. It has been projected that approximately 50 DNA binding receptors on the surface of every *B. subtilis* cell, and all of the receptors can be saturated with bound DNA simultaneously (Trun *et al.* 2004, Dubnau *et al*. 1993).

When exposed to nutrient limitation, *B. subtilis* starts an extraordinary cell division process that produces two unequal-sized compartments, the mother cell and the forespore, from one cell, which can be seen in Figure 12. These compartments lie adjacent to one another. The division septum, known as the polar septum, curves as the mother cell surrounds the forespore in a phagocytic-like process (Trun *et al*. 2004). Eventually, the edges of the surrounding membrane fuse, producing a double membrane bound forespore. A thick layer of peptidoglycan, known as the cortex, is produced between the two membranes encompassing the forespore, and the mother cell deposits a proteinaceous shell on the surface of the outer membrane of the forespore. This mechanism is largely unknown (Trun *et al*. 2004). Ultimately, the ripened spore is set free into the surrounding environment via a lysis of the mother cell (Ramamurthi *et al.* 2006). A general depiction of the two common pathways *B. subtilis* use to achieve competence can be seen in Figure 13.

The first pathway used in *B. subtilis* in sensing the density of cells is a small peptide or competence factor called ComX which is excreted by actively growing cells. ComX is manufactured in the cytoplasm as a bulky precursor molecule, which is cleaved and modified by the ComQ protein, after which the mature ComX is transported out of

the cell. The concentration of ComX in the environment reaches high levels when cells are at a high density (Trun *et al*. 2004, Dubnau *et al.* 1993).

High levels of ComX in the environment, which is depicted in Figure 14, are sensed via a two-component signal transduction system which consists of two proteins, the sensor-kinase protein and the response-regulator protein. The sensor-kinase protein is a surface protein that spans the membrane, with a portion of the protein exposed in the other environment and the other portion is exposed in the cytoplasm. The external portion of the sensor-kinase protein senses the signal produced within the environment, then relays them to the response regulator; whereas, the kinase portion of the protein adds phosphoryl groups to a specific place on a molecule or substrate. This important process proceeds via phosphorylation and dephosphorylation. Initially, the response to the environmental signal that the sensor-kinase phosphorylates is to the specific histadine that is conserved among all sensor-kinases. This phosphate is then conveyed by the sensor-kinase to an aspartate, which is conserved in the response-regulator protein. At this point, the phosphorylated response-regulator is able to turn on or turn off the genes, which allows the cells to respond to the specific change in the environment (Trun *et al.* 2004, Provvedi *et al.* 1999).

This very elegant *B. subtilis* two-component signal transduction system that senses the levels of ComX which is complied of ComP, the sensor-kinase, and ComA, the response-regulator. At high levels ComX binds to ComP which causes ComP to autophosohorylate. This causes the phosphate to be transferred from ComP to ComA. When ComA is phosphorylated it functions as a regulatory protein used to activate the

transcription of *comS*, which leads to the expression of the competence genes. This complex system is known as a quorum sensing system due to ComP and ComA's

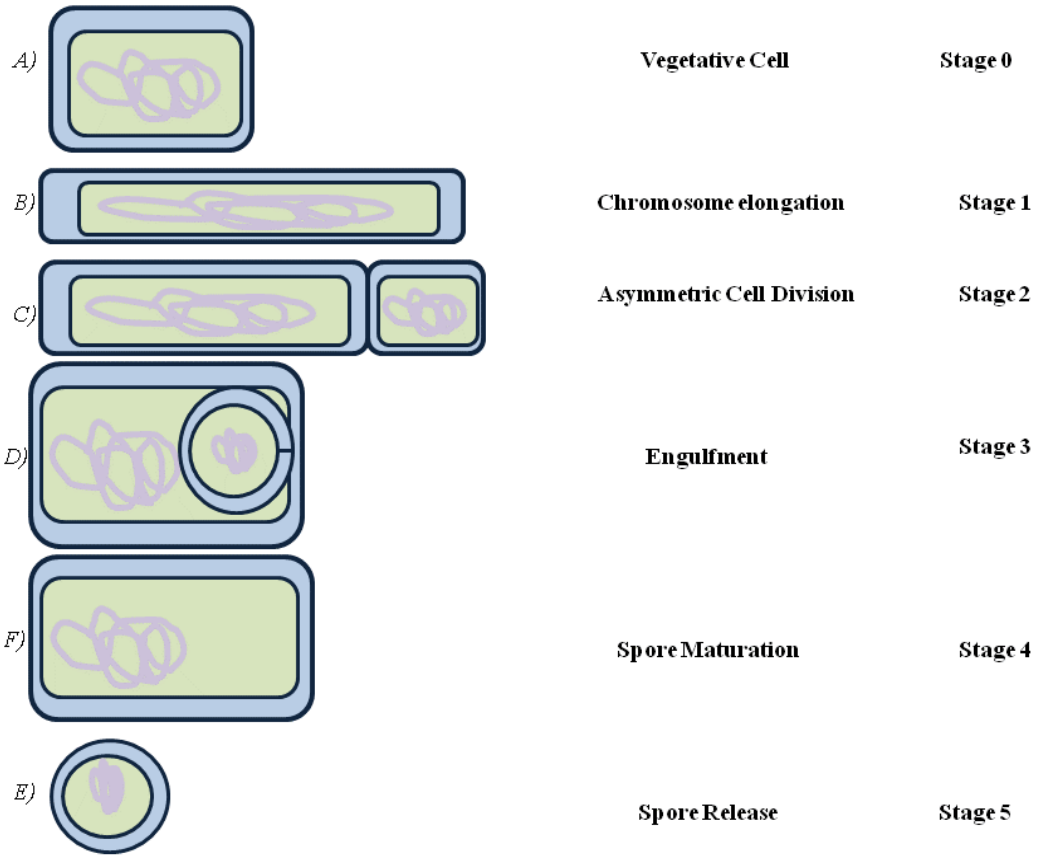

Figure 12. Sporulation basics as described by Trun *et al*. (2004). A) Vegetative cells are actively growing and dividing cells. The conversion from vegetative growth to definitive morphological changes found in the sporulation process is known as Stage 0. B) During Stage 1, the cellular chromosomes are elongated. C) Cells divide asymmetrically to produce polar septum. In this process, known as Stage 2, one chromosome is left in the mother cell and the other is relocated into the spore. D) The polar septum bulges out to include the spore in engulfment, which is known as stage 3. E) In Stage 4, the spore is matured by addition of a protective layer used to surround the dormant chromosome. F) Stage 5, the mother cell lyses the spore into the environment.

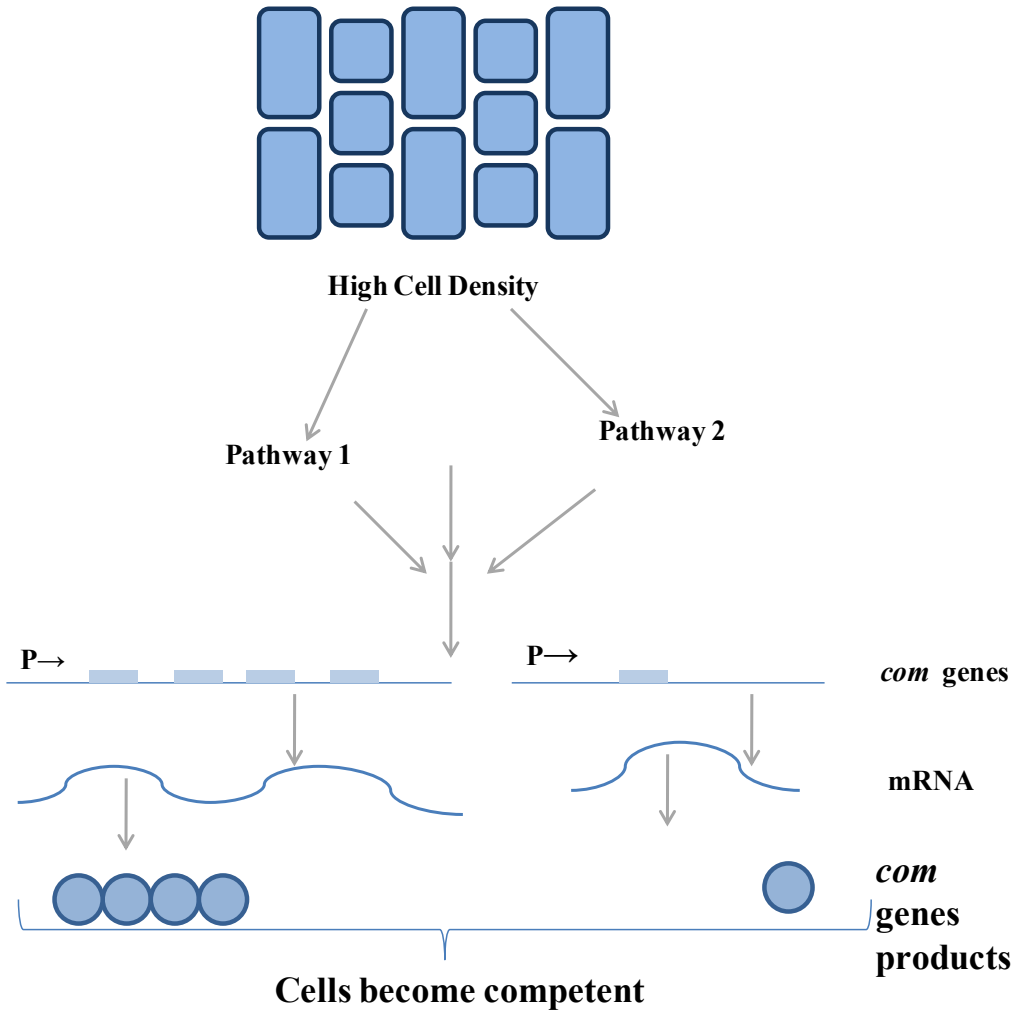

Figure #. Two activation pathways that are responsible for the activation of competence in *B. subtillis* cells.

Figure 13. Two activation pathways that are responsible for the activation of competence in *B. subtilis* cells. Pathway 1 detects an increased cell density and signals the induction of com genes; whereas, Pathway 2 monitors nutrient availability. When limited nutrients are detected, Pathway 2 signals the induction of *com* genes. Based on a figure from Trun *et al*. (2004).

response to the levels to ComX, and ComX's dependence on the number of cells. Quorum sensing systems detect a change in cell density in order to influence the expression of genes and cellular behavior; therefore, this system is used by many different bacteria to control assorted processes; such as, virulence, conjugation, antibiotic

production, sporulation, and motility (Trun *et al.* 2004, Provvedi *et al*. 1999). This pathway is shown in Figures 15, 16, and 17.

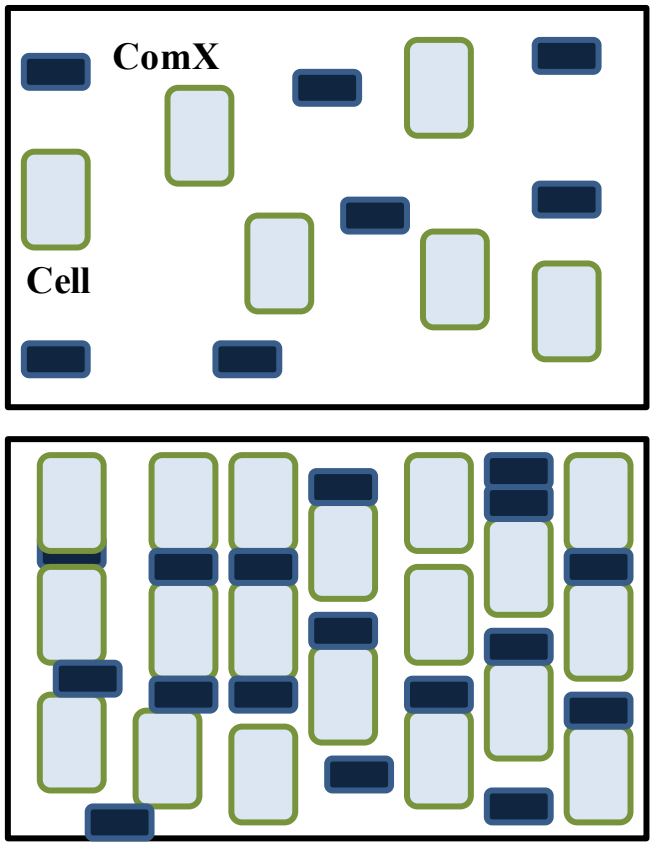

Figure #. ComX concentration. All of the cells in the

Figure 14. An illustrations based on Trun (2004) that emphases the importance of ComX concentrations. All of the cells in the population produce ComX. ComX density is directly proportional to cell density. Cells repond to high ComX density. Based on a figure from Trun *et al.* (2004).

The second pathway for induction of the *com* genes in *B. subtilis* is affected by a second developmental process, the formation of spores. Sporulation is regulated by high cell density and depletion of nutrients. This process starts when normal, vegetative bacterial cell growth is stopped and the cells undergo progressive changes that result in the

production of a spore, which contains a chromosome completely surrounded by protective layers.

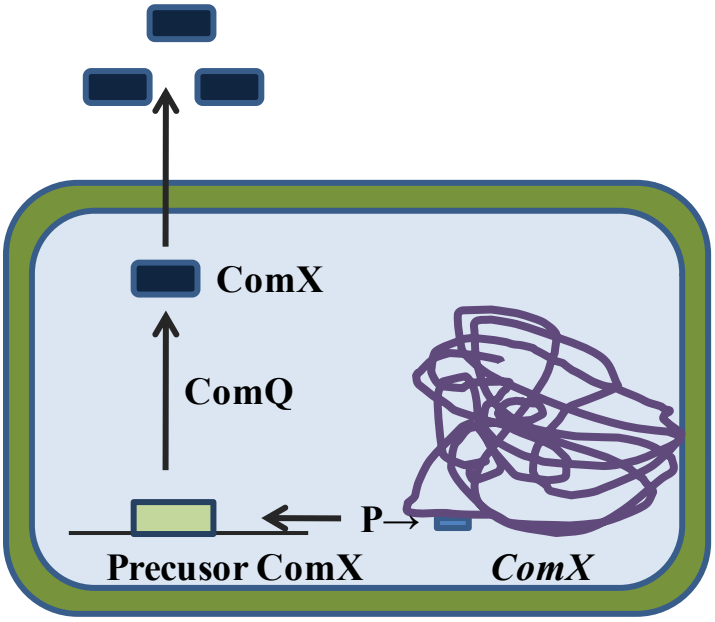

Figure #. In depth review of Pathway 1 in *B.*

Figure 15. An detailed review of Pathway 1 in *B. subtilis*. Pathway 1 detects increased cell density to induce com genes. In this process ComX, which is produced in the cytoplasm, induces the com genes via the ComQ protein process, which modifies ComX to make a smaller active peptide. At this point the active ComX is excreted from the cell. Based on a figure in Trun *et al.* (2004).

The spore, which is the dominate form of *B. subtlis* cells, does not actively grow or metabolize nutrients and can survive many harsh conditions that a *B. subtillis* cell cannot, including high levels of radiation, lack of food and water, high temperatures, and increased levels of toxic chemicals. Spores have been known to survive for decades in some instances; but, whenever the spore comes in contact with a nutrient-rich environment, it germinates into an actively growing bacterium (Trun *et al.* 2004, Dubnau *et al.* 1993).

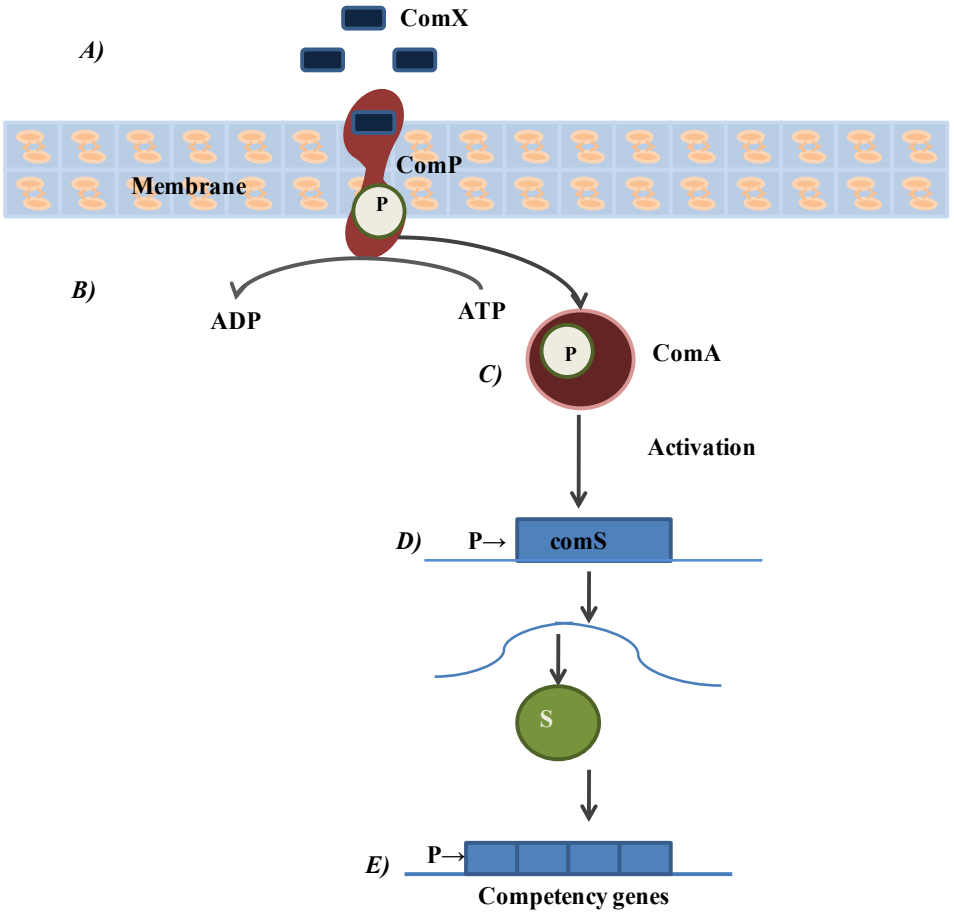

Figure 16. Two-component signal transduction system in *B. subtilis* for the induction of the competency genes. A) ComX binds to ComP, B) which induces autophosphoryaltion of ComP. C) Then the phosphoryl group is relayed to ComA, D) this leads to the phosphorylation of ComA, which activates the comS gene. E) Finally, ComS leads to the induction of the competency genes. Based on a figure from Trun *et al*. (2004).

A second pathway in *B. subtilis* bypasses ComX and ComP, but feeds on ComA to activate competency and sporulation. A second competence peptide, competence and sporulation factor (CSF), cooperates with a second membrane protein called Spo0K, which is a permase or hole through the outer membrane. Spo0K is used to transport small peptides, like CSF, inside the cytoplasm. Cytoplasmic levels of CSF determine the activation of competency and sporulation; however, these acts are not simultaneous. At low cytoplasmic levels, CSF inhibits RapC, which removes the phosphoryl group from

ComA and prevents it from activating *comS*; therefore, activating competency. At high cytoplasmic levels ComS is inhibited; therefore, inhibiting competency and inhibition of RapB, which leads to the production of the genes needed for sporulation.

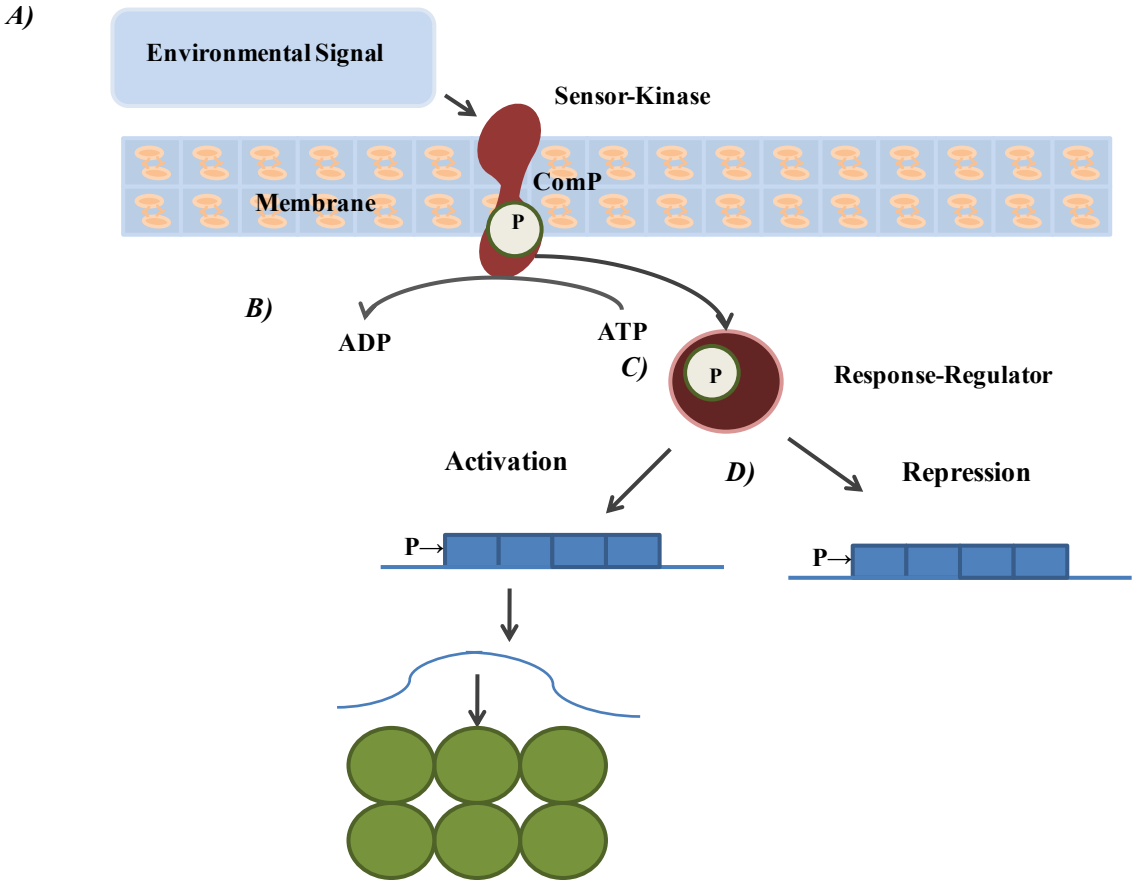

Figure 17. Basic two-component signal transduction system. Two-component signal transduction systems utilize a sensor-kinase to detect environmental signals. A) Sensor-kinases autophosphorlate on a certain histidine residue in the protein. B) Then the sensor-kinase transfers the phosphate to the response-regulator. C) The response-regulator is activated, which means it can induce or repress genes at this point. D) These genes permit cellular response to the original environmental signal in the appropriate way. Based on a figure in Trun *et al.* (2004).

There are different pathways in which competency is activated in *B. subtillis*, however only 20% of the cells are capable of competency, but these cells remain competent for several hours (Trun *et al.* 2004, Provvedi *et al*. 1999). An in depth depiction of the second pathway *B. subtilis* utilizes for competence can be shown in Figures 18 and 19.

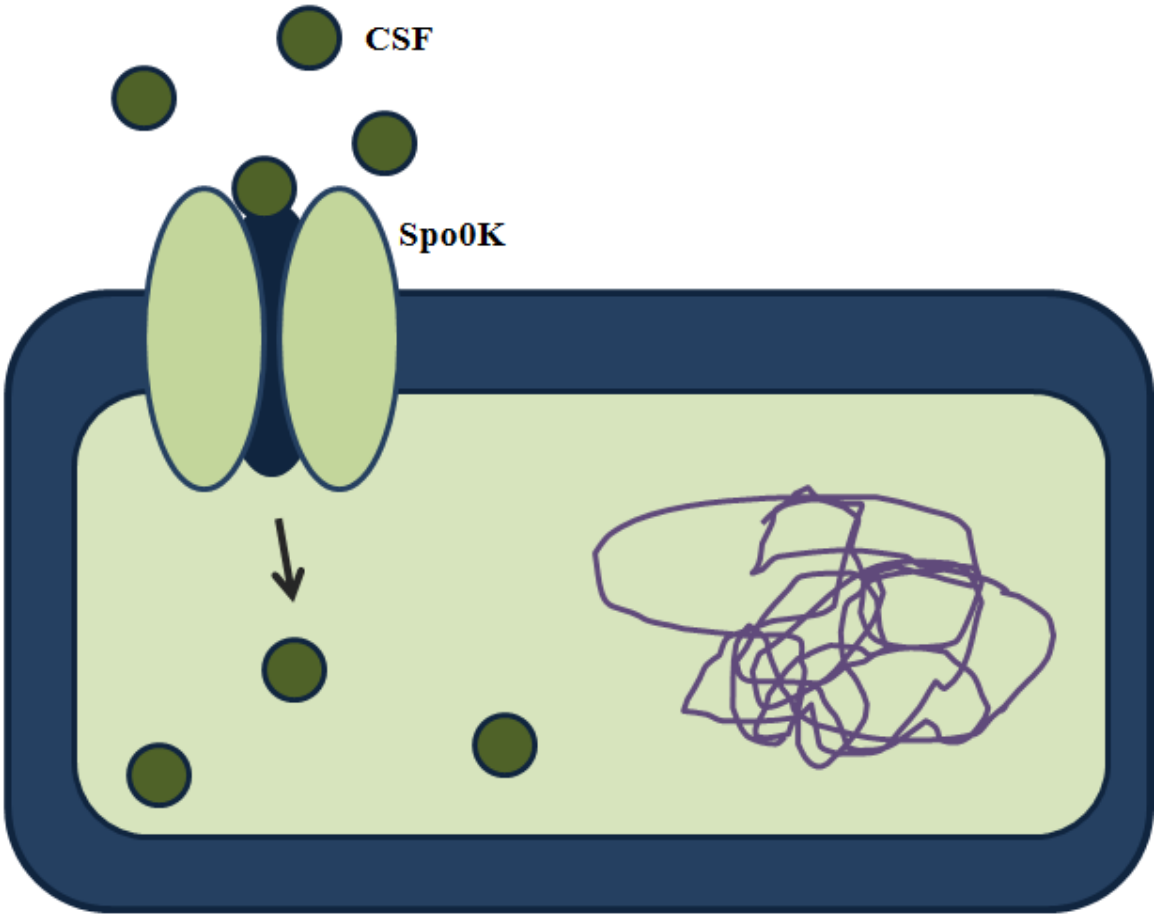

Figure 18. *B. subtilis* secondary pathway utilized to induce competency. The second pathway to induce competency genes in B. subtillis employs a small peptide known as competence and sporulation factor (CSF). CSF is transported through the cell via Spo0K, a membrane protein. Based on a figure in Trun *et al.* (2004).

B. subtilis contains the germination-related lipase LipC within the spore coat. This particular enzyme hydrolyses *p*-nitrophenyl ester substrates in to various acyl-chain lengths. Studies have shown that LipC cleaves the fatty acids at the *sn*-1 and *sn*-2 positions of phospholipids. LipC shows no selectivity for the polar head groups of the lipid molecules. This information suggests that *B. subtilis* LipC plays a significant part in the degradation of the outer spore membrane during sporulation (Masayama *et al.* 2010). In *B. subtilis*, many different types of pilli, known as type IV pilli, bind to the DNA first. The DNA binding receptor in *B. subtilis* is known as ComEA. ComEA utilizes ComG proteins in order to access dsDNA. After the DNA is irreversibly binds to ComEA, it is susceptible to degradation via endonucleases. ComI is a membrane bound protein that is responsible for degrading one strand of the dsDNA that is irreversibly bound to ComEA. At this point, the remaining DNA strand is transported into the cell, via ComEA and ComEC. Once the DNA is inside the cell, the ssDNA is protected via a *com* encoded DNA binding protein (Trun *et al*. 2004, Provvedi *et al.* 1999).

Strains

There are many strains of *B. sublis*, but the most common *B. subtillis* strains are QST 713, which is used as a natural fungicide and biological control agent, and pBE2C1 and pBE2C1AB which are utilized in the production of polyhydroxyalkanoates (Ryan *et al.* 2004).

Outbreaks and Diseases

B. subtillis is not generally found to be a human pathogen; however, it can contaminate food sources. In most cases this bacterium is seldom responsible cases of

food poisoning. In most cases, *B. subtilis* is responsible for the sticky consistency in spoiled bread which is the product of bacterial production of long chain polysaccharides (Ryan *et al.* 2004).

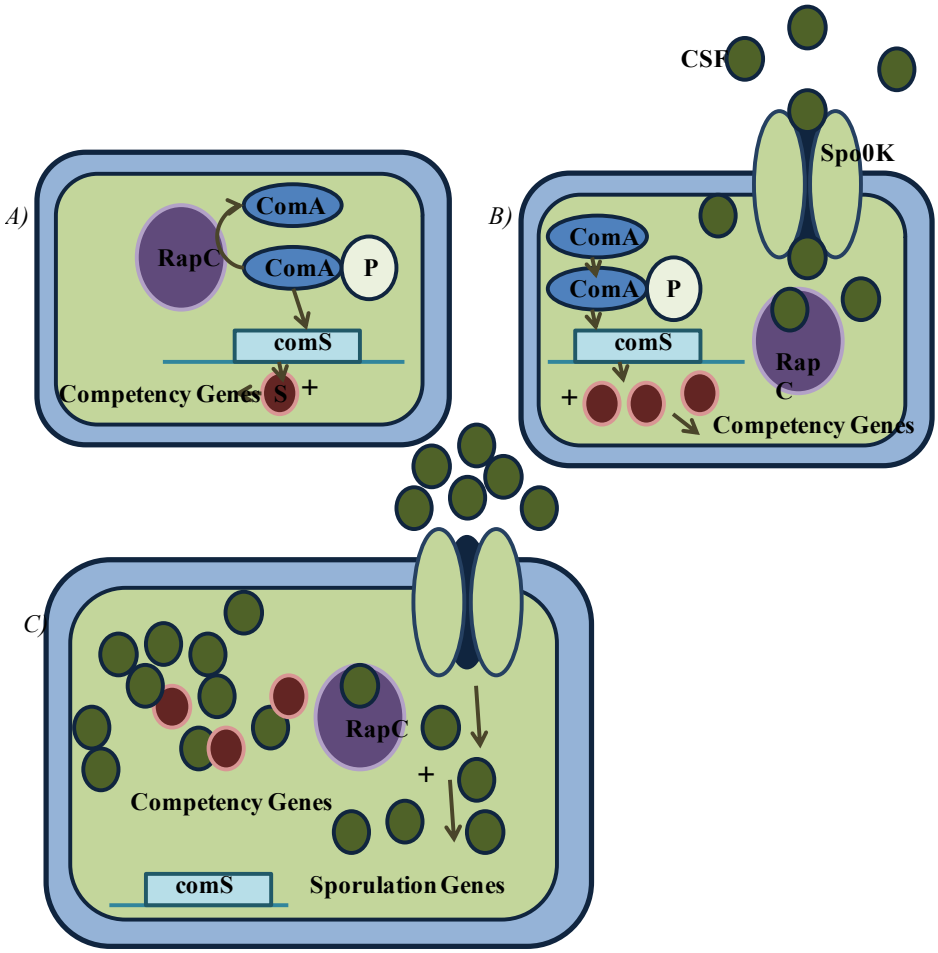

Figure # Regulation of the competency genes and the sporulation genes of *B*

Figure 19. Regulation of the competency genes and the sporulation genes in *B. subtillis* by CSF. A) Synthesis of the competency genes is induced by phosphorylatioin of ComA. The phosphoryl group (RapC) removes the phosphoryl group from ComA and inhibits the activations of comS, therefore, preventing the activation of the competency genes. B) RapC is inhibited at low intracellular levels of CSF, because CSF binds to RapC. This reaction allows the ComA to remain phosphorylated and activates comS and the competency genes. C) CSF binds directly to ComS, which prevents the expression of the competency genes. This reaction also activates sporulation genes. The figure is based on an illustration in Trun *et al.* (2004).

CHAPTER THREE

Cell SELEX on Living Bacteria Cells

Study Objectives

This study will focus on dsDNA olgionucleotides to develop a selection technique to produce aptamers which bind to the molecules on the outside of *E.coli* DH5α cells as targets. Two aptamers were created using eight rounds of Cell SELEX and two rounds of counter Cell SELEX. These aptamers will be tested via flow cytometry in order to determine their binding affinity. (Hamula *et al*. 2008, Chen *et al.* 2007, Strehiltz 2007, Qwivedi 2010, and Huizenga *et al.* 1995).

Although Cell-SELEX has many applications which were mentioned before, it has not been used to detect live *E. coli* cells in non potable water, which is the purpose of this study. *B. subtilis* and *E. areogens* were introduced through counter cell SELEX rounds to introduce specificity of the aptamer for *E. coli* DH5α cells.

As mentioned before, *E. coli* is a nonpathogenic Gram negative bacteria that possess a wide variety of potential targets on the outer membrane. These targets include lipopolysaccharides, fimbriae, F pilus, capsular polysaccharides, glycoprotein's, lipoproteins, among other proteins (Olsen *et al*. 1998, Bayer *et al.* 1965, Trun *et al.* 2004, Inouye *et al.* 1986, and Neidhardt *et al.* 1996). Whole live *E. coli* cells were incubated in suspension with a dsDNA library, the partition of tightly bound aptamers from loosely bound aptamers as well as non-bound aptamers was accomplished by multiple

centrifugation and washing steps. The aptamers that survive this process were cloned, sequenced, and were tested for specific binding to the target cells.

Materials and Methods

Bacterial Strains and Culture Media

In this experiment, *Escherica coli* DH5α*, Bacillus subtilis* and *Enterobacter aerogenes,* from Carolina Biological, were used as SELEX targets to produce aptamers that should bind specifically to molecules residing on the outside of *E. coli* DH5α cells based on methods used by Hamula *et al*. 2008, Chen *et al*. 2007, and Kim *et al.* 2009. Cultures of *E. coli, B. subtillis,* and *E. aerogenes* were grown under aerobic conditions at 37°C in Luria-Bertani (LB) media in a New Brunswick Scientific Excella E24 Incubator Shaker Series and a 50 ml Falcon tube to a density of approximately 0.6 at $OD_{600,}$ which was taken on an Eppendorf BioPhotometer Plus. All bacteria were harvested during the logarithmic phase of growth via centrifugation in a Beckman Coulter Allegra X-115R centrifuge. *E. coli* JM109 high efficiency competent cells were used for all transformations.

DNA Library

A 60-bp oligonucleotide double-stranded DNA library consisting of a 30-bp randomized region bordered on either side by 15-bp primer hybridization sites was used to generate aptamers against *E. coli* DH5α cells.

PCR Amplification and Gel Electrophoresis. The primers and template (From IDT), which contained approximately 4^{30} sequences, used to create the dsDNA library and successive aptamer pools contained the following sequences:

Forward: 5'-3'GCGCGGGATCCCGCGC

Reverse: 5'-3'GCGCAAGCTTCGCGC

Template: 5'-3'GCGCGGATCCCGCGC-N_{30}-GCGCGAAGCTTGCGC

The PCR conditions, which are based on Hamula *et al.,* for each round are as follows: *Taq* DNA polymerase, dNTPs, $MgCl_2$ (*GoTaq* from Promega), 10 µM concentration of each primer, and approximately 2000 ng of DNA library or approximately 2000 ng concentration of fraction supernatant. Thermocycling parameters were 94ºC for 4 minutes denaturation, followed by 20 cycles of denaturation at 94ºC for 40 seconds, annealing at 55ºC for 55 seconds, and extension at 72ºC for 20 seconds. A final extension step for 5 minutes at 72°C is followed by a final cycle where the temperature is dropped to 4°C (Hamula *et al*. 2008). All PCR reactions where done in an Eppendorf Mastercycler.

Following PCR, the reaction products were partitioned on a 1.0 mm 12.0% native polyacrylamide gel electrophoresis (PAGE) in 1x TA buffer at 100 V. The gels were stained with ethidium bromide and photographed under UV light. All products were purified using the crush and soak method.

Viable Cell Count

Cultures of *E. coli* DH5α, *B. subtillis, E. aerogenes* and cells were grown in a 50ml Falcon Tube in LB media overnight in a New Brunswick Scientific Excella E24 Incubator Shaker Series until an OD_{600} of approximately 0.5 was obtained with an Eppendorf BioPhotometer Plus. Serial dilutions from 1-1000 pM of cells were plated on agar plates made with agar and LB media from VWR in order to determine what concentration of cells would provide 10^5 -10^8 cells. Viable cell counts were preformed on

each set of cells in order to approximate how many cells were needed in the incubation mixture.

Gel Preparation

All gels were made using a 10 mL mixture of 3.31 mL autoclaved deionized water, 4.0 mL of 30% acrylamide mix made from a 40% acrylamide mix, 2.5 mL 1.5M Tris (pH 8.8), 0.1 mL 10% Ammonium persulfate, and 0.004 mL of TEMED. All materials were obtained from VWR. All gel solution was pipette into Bio-Rad glass plates. These gels were ran on a Bio-Rad Power Pac 3000 at 100 V in TA buffer. All gels were stained using 1 μL of ethidium bromide in 10 mL of deionzed water for 10 minutes. The gels were destained in 10mL of deionzed water for 10 minutes. Pictures were taken using Sony digital camera with a UV filter and a FirstLight UV Illuminator.

Crush and Soak and Ethanol Precipitation

The DNA sample and a 20 base pair marker from VWR were 12% native polyacrylamide gels were run in a Bio-Rad Power Pac 3000 in TA buffer. The DNA was located by examination of ethidium-bromide stained gel in long-wavelength (302 nm) UV light and a picture was taken. A clean sharp scalpel was used to extract the segment that contains the 60 bp band. Place the gel slice into a PCR tube with a hole in the bottom. This tube should be place in 1.5 mL microfuge tube and was centrifuged at 5000*g* for 5 minutes in a Beckman Coulter microfuge 16-centrifuge. At this point all of the gel should be in the 1.5 mL microfuge tube. Approximately 10-50 μL of a mixture of 0.5 mL ammonium acetate, 1 mM EDTA (pH 8.0), and low salt TE (pH 8.0) was added to the extracted gel. This mixture was incubated at 45°C for 3hours in a Isotope 202.

The sample was centrifuged at 5000*g* for 1 minute at 4°C in a Beckman Coulter microfuge 16-centrifuge. The supernatant was transferred to a fresh 1.5 mL microfuge tube which 100 µL of 100% ethanol along with 5 µL of 0.5m ammonium acetate was added. This mixture was refrigerated overnight at 4°C. The sample was then centrifuged at 5000*g* for 5 minutes at 4°C in a Beckman Coulter microfuge 16-centrifuge. Approximately 95% of the supernatant was removed via pipette. The rest was removed an Eppendorf vacufuge at 45° for 5-10 minutes. The resulting DNA was resuspended in 10 µL of autoclaved deionzed water, and 1µL was added to 99 µL of deionzed water and the concentration was taken in the Eppendorf BioPhotometer Plus at 260 nm. The resulting concentration was then converted from ng to pM.

Aptamer Selection

The method used for aptamer selection was based on the procedure describe by Hamula *et al.* Cells were pelleted at 4750*g* in a Beckman Coulter Allefra X-115R centrifuge at 4°C then washed twice in 1x binding buffer (1xBB) (50 mM Tris-HCl (pH 7.4), 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$) at room temperature. 200-500 pM DNA pool and approximately 10^5-10^8 cells were used in these experiments. A mixture of 2 µL of 200-500 pM of random pool dsDNA or aptamer pool, 10 µL of *E. coli* DH5α, *B. subtilis,* or *E. aerogenes* cells, 8 µL of 10mg/mL BSA, and 480 µL 1xBB were incubated for 45 minutes at room temperature. After incubation, the cells placed in 4°C for 10 minutes and then were centrifuged at 5000*g* and 4°C for 5 minutes, the supernatants were removed, and the cells were washed twice in 250 µL of 1xBB using resuspension and centrifugation. Finally, the cells were resuspended in 100 µL of 1xBB and heated at 94°C for 10 minutes and placed on ice for 10 minutes. This step denatures and elutes

cell-bound aptamers, and kills the cells. The mixture was centrifuged at 5000g and 4°C for 5 minutes and the supernatant was isolated. The resuspended cell solution was transferred to a new microcentrifuge tube to eliminate aptamers bound to the tube wall, after each step.

Ethanol precipitation was then used on all fractions collected to concentrate and desalt the aptamer pool. The fractions were amplified through PCR after every round. The crush and soak product from the elution step of Cell SELEX using *E. coli* DH5α was then used for the next round of selection. In counter Cell SELEX rounds the crush and soak product form the incubation and both washing steps are used for subsequent rounds. Each round of selection was performed using fresh aliquots of cells.

Eight rounds of Cell SELEX using *E. coli* DH5α as a target were performed along with one counter Cell SELEX round using *B. subtilis* as a target, and a counter cell SELEX round using *E. aerogenes* as a target. These two rounds were used to increase specificity of the aptamer pool towards molecules that reside on the surface of *E. coli* DH5α. The elution product from the fifth round was divided into two aliquots, one aliquot was used to continue through Cell SELEX using *E. coli* DH5α as a target for eight rounds; whereas, the other aliquot was exposed to *B. subtilis* and *E. aerogenes* in counter Cell SELEX rounds and subsequently went through a total of eight cell SELEX rounds with *E. coli* DH5α as a target. In rounds in which *E. coli* DH5α was used as a target the aptamers from the elution step were taken to the next round; whereas, in the rounds in which other bacteria was used as a target the incubation step and/or the washing steps were selected to participate in subsequent rounds.

Cloning, Sequencing, and Structured Analysis of Aptamers

Sequencing analysis was done on the aptamers that survived eight rounds of Cell SELEX with *E.coli* DH5α as a target , because they should demonstrate higher affinity and specificity for the target cells in question than the aptamers resulting from previous rounds. Aptamer pools were cloned using a pGem-TEasy vector which was used because the purified aptamer had 3'A and the vector contained 5'-T, and transformed using *E. coli* JM109 high efficiency competent cells. The colonies containing the vector were selected by overnight incubation at 37°C on LB plates containing 50 μg/mL ampicillin. Ten colonies from each aptamer pool were chosen for screening. The plasmid DNA was purified using mini prep using the Wizard Plus-SV kit from Promega and analyzed for the presence of a 60-bp insert via digestion with 1 U of *Eco*RI at 37°C overnight, followed by 1.0% agarose gel.

K_D value Measurement via Microplate Reader

After the aptamers were sequenced PCR was done with the mini prep product as a template and the following primers (FAM is two fluorescent dyes with an excitatioan and emission of 492 nm and 517 nm, which can be seen in Figure 20):

Forward: 5'FAM-GCGCGGATCCCGCGC3'

Reverse: 5'-GCGCAAGCTTCGCGC3

The PCR products were then purified via crush and soak. The concentration of the crush and soak product was $1.7x10^{-8}$ pM. Fourteen samples ranging from 1-1365 pM were incubated with 10 μL of *E. coli* DH5α cells at room temperature for 45 minutes, placed in -20°C for 5 minutes, and centrifuged at 5000g for 10 minutes. The supernatant was then removed and the cells were then washed with 100 μL 1xBB, the sample was

then heated at 94°C for 10 minutes to separate the aptamers and cells, and then was placed in -20°C for 10 minutes to restore the aptamers into the α-helix form. The samples were then centrifuged at 5000*g* for 10 minutes, the supernatant was removed and the cell solution was resuspended in 400 µL of 1xBB. Subsequently, the sample was subjected to nitrocellulose filtration. The last two steps were used in order to remove cellular debris from the sample. The samples absorption was then measured at 485nm and emission was measured at 535 nm on Thermo Flouroskan Ascent FL . The K_D values for this experiment is approximately 96 pM.

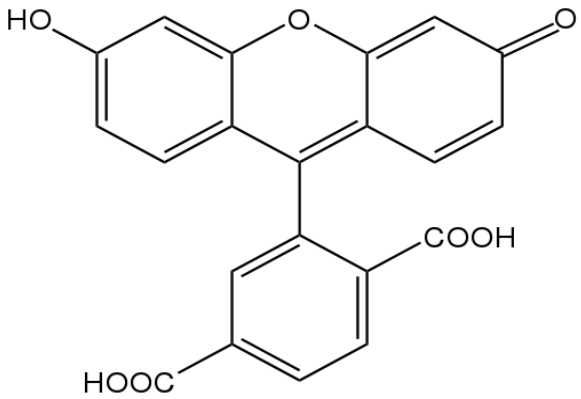

Figure 20. The chemical structure of FAM. FAM is known as carboxyfluorescein, which was placed at the 5' end of the forward primer (Parish 1996).

Results and Discussion

Selection of DNA Aptamers against Live E. coli cells

The technique for the selection of aptamers against live bacterial cells consists of five main steps; incubation of the DNA library with live bacterial cells, portioning of the bound aptamers from the unbound random DNA sequences via centrifugation, liberation of the bound DNA from the cell surface, amplification of the previously bound DNA sequences, and characterization of the bound DNA for binding affinity as a new library

for the following round of selection, as well as for sequencing. *E.coli* DH5α was selected as a model for *E.coli* O157:H7, which continues to occur in the United States each year in contaminated food or water sources (Trun *et al.* 2004). *E. coli* DH5α was chosen because it is simple to maintain and robust to handle. Eight rounds of cell SELEX and two rounds of counter Cell SELEX were performed. During the incubation process using *E. coli* DH5α as a target, most of the aptamers remained bound until the elution step; however, when *B. subtilis* and *E.aerogenes* were used as a target the aptamers were found in higher abundance in the elution and washing steps. The observation of a 60-bp band on the gel after each round of selection and PCR amplification of the eluted fraction suggests that the cells were able to bind to a pool of aptamer sequences. The PCR products of the elution fraction were subjected to desalting and elution, and afterward were used in subsequent rounds of selection. Figures 21-35 illustrate this process.

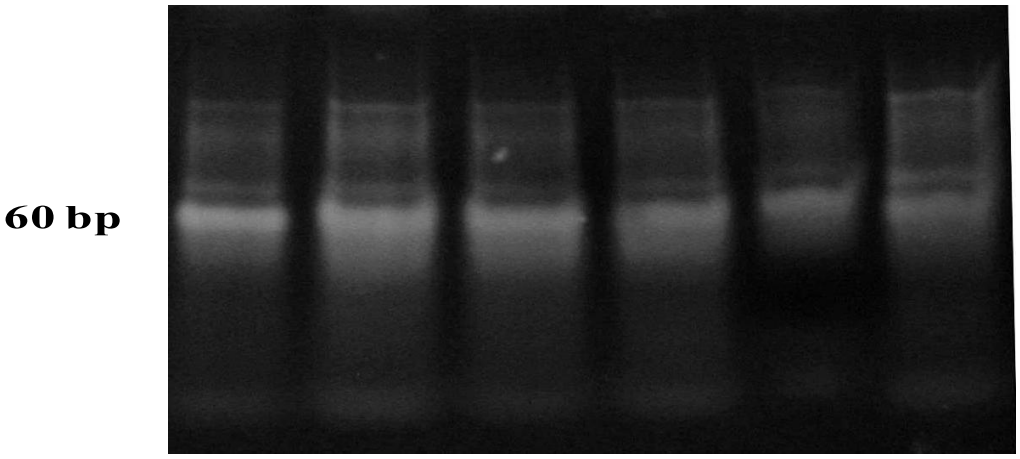

Figure 21. Template DNA. Nine 20 µL samples of template DNA, forward and reverse primers, and *GoTaq* after twenty cycles of PCR. These samples were ran in 12% native gel, then purified via crush and soak.

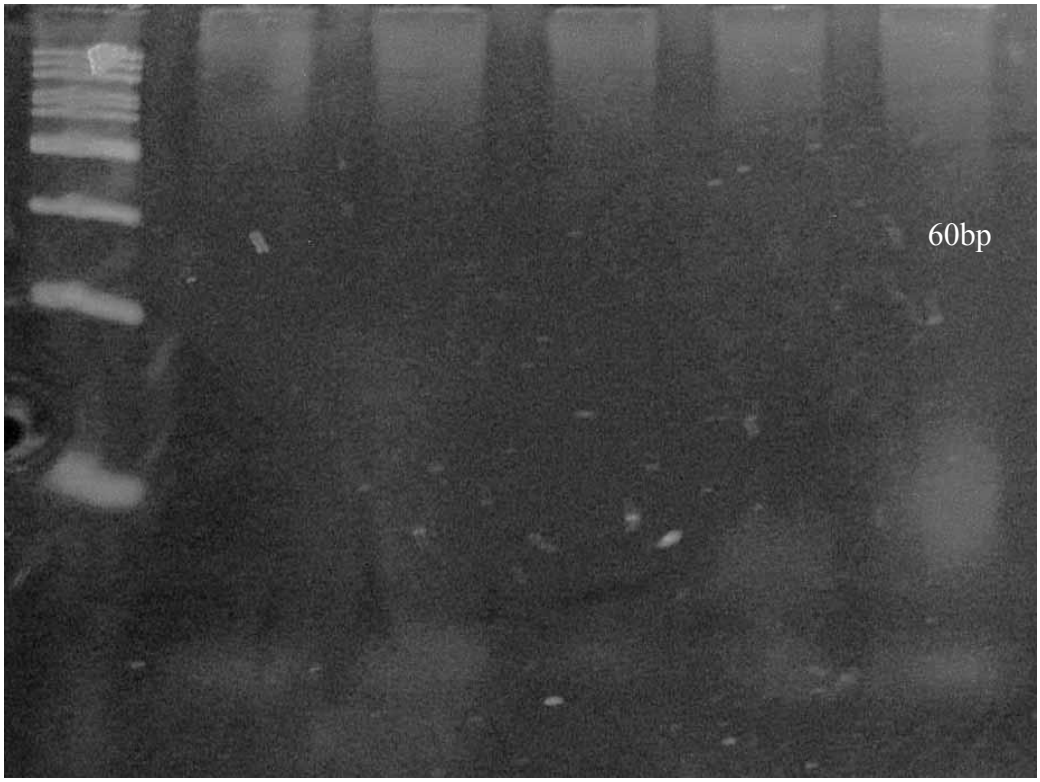

Figure 22a. Round 1 of Cell-SELEX. A) The 2 µL of ~500 pM purified DNA template was incubated with 10 µL of ~10^8 *E. coli* cells at room temperature for 45 minutes in the presence of 480 µL of 1x BB and 8 µL 10 mg/mL BSA. Subsequently, this reaction was refrigerated at 4°C for 10 minutes then centrifuged at 5000*g* for 10 minutes. The supernatant went through ethanol precipitation to produce I (Incubation). The precipitate produced from centrifugation before ethanol precipitation of I was resuspended in 250 µL 1xBB and refrigerated at 4°C for 10 minutes then centrifuged at 5000*g* for 10 minutes. The supernatant went through ethanol precipitation to produce 1 (Wash 1). The precipitate from centrifugation before ethanol precipitation of 1 was resuspended in 250 µL 1xBB and refrigerated at 4°C for 10 minutes then centrifuged at 5000*g* for 10 minutes. The supernatant went through ethanol precipitation to produce 2 (Wash 2). The precipitate from centrifugation prior to ethanol precipitation of 2 was resuspended in 100 µL of 1xBB and heated for 10 minutes at 94°C then in 4°C for 10 minutes. At this point, the sample was centrifuged at 5000*g* for 10 minutes. The supernatant was subjected to ethanol precipitation to produce E (Elution). The precipitate from centrifugation of E prior to ethanol precipitation was resuspended in 100 µL of 1xBB and subjected to ethanol precipitation to produce C (Cells). All of the product of ethanol precipitation steps I, 1, 2, E, and C were subjected to PCR where ran through a 12% native gel along with a 20bp marker (M) to produce figure A. This figure illustrates that at this point in Cell SELEX most of the random-pool DNA remains attached to *E. coli* cells at the elution step.

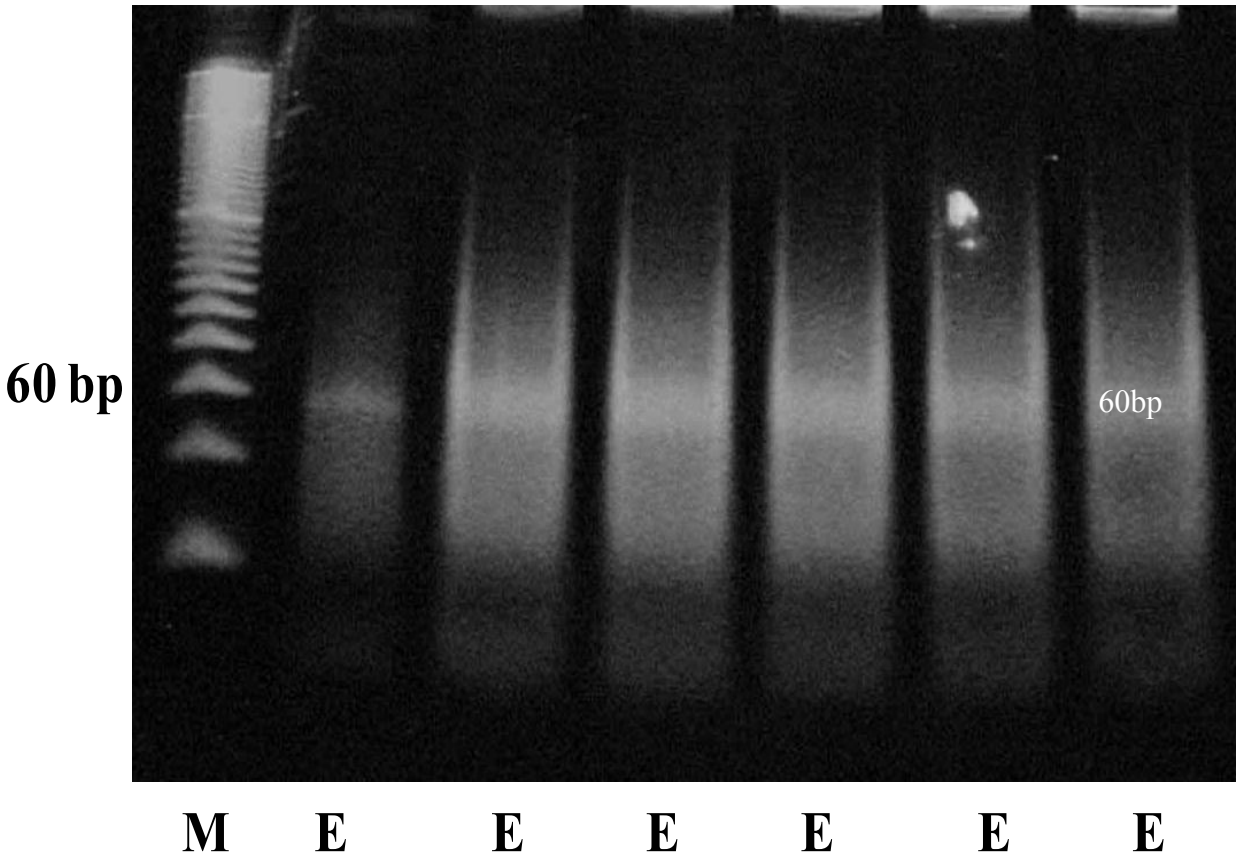

Figure 22b. Nine PCR reaction of the elution step were ran through a 6% native gel and then subjected to crush and soak in order to produce the random pool DNA used for Round 2 of Cell SELEX.

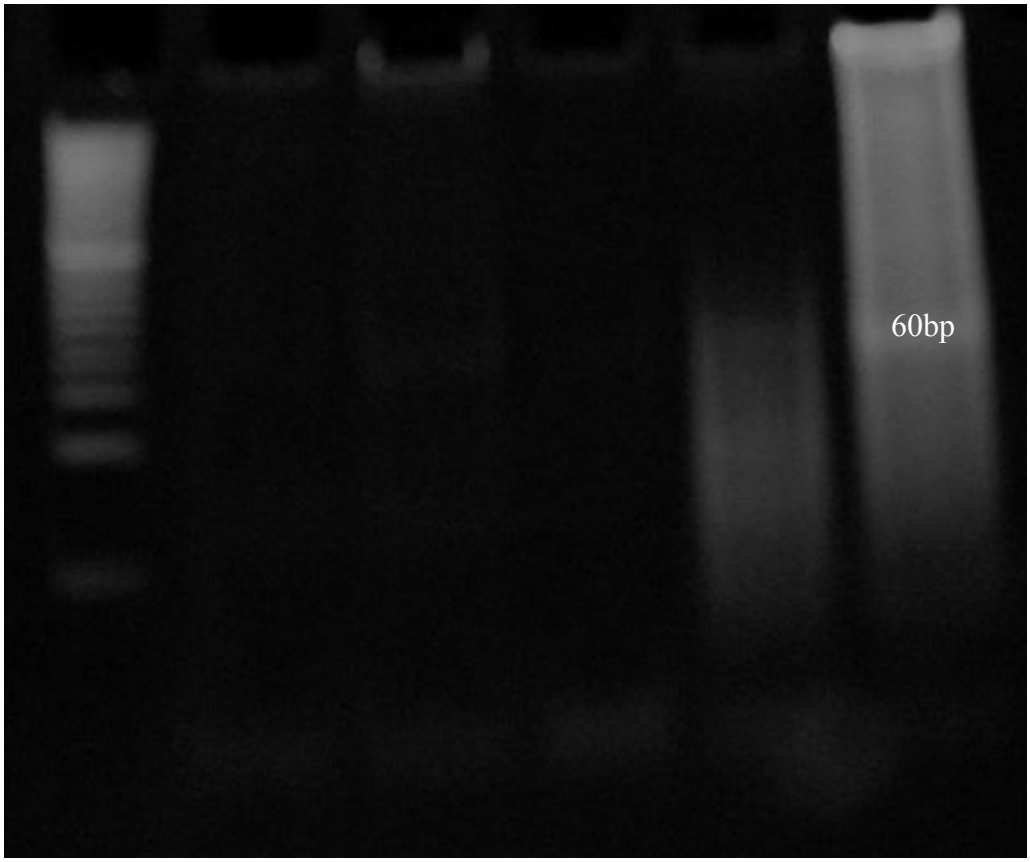

Figure 23. Round 2 of Cell SELEX. A) 2 µL of ~500 µL of crush and soak product from Round 1 of Cell-SELEX was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation, and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in Round 3 Cell-SELEX. B) A clear picture of a 12% native gel produced with a 20 bp marker (M) in lane one, incubation (I) DNA in lane two, wash 1 (1) DNA in lane three, wash 2 (2) DNA in lane four, cell (C) debris in lane five, and elution (E) DNA in lane six.

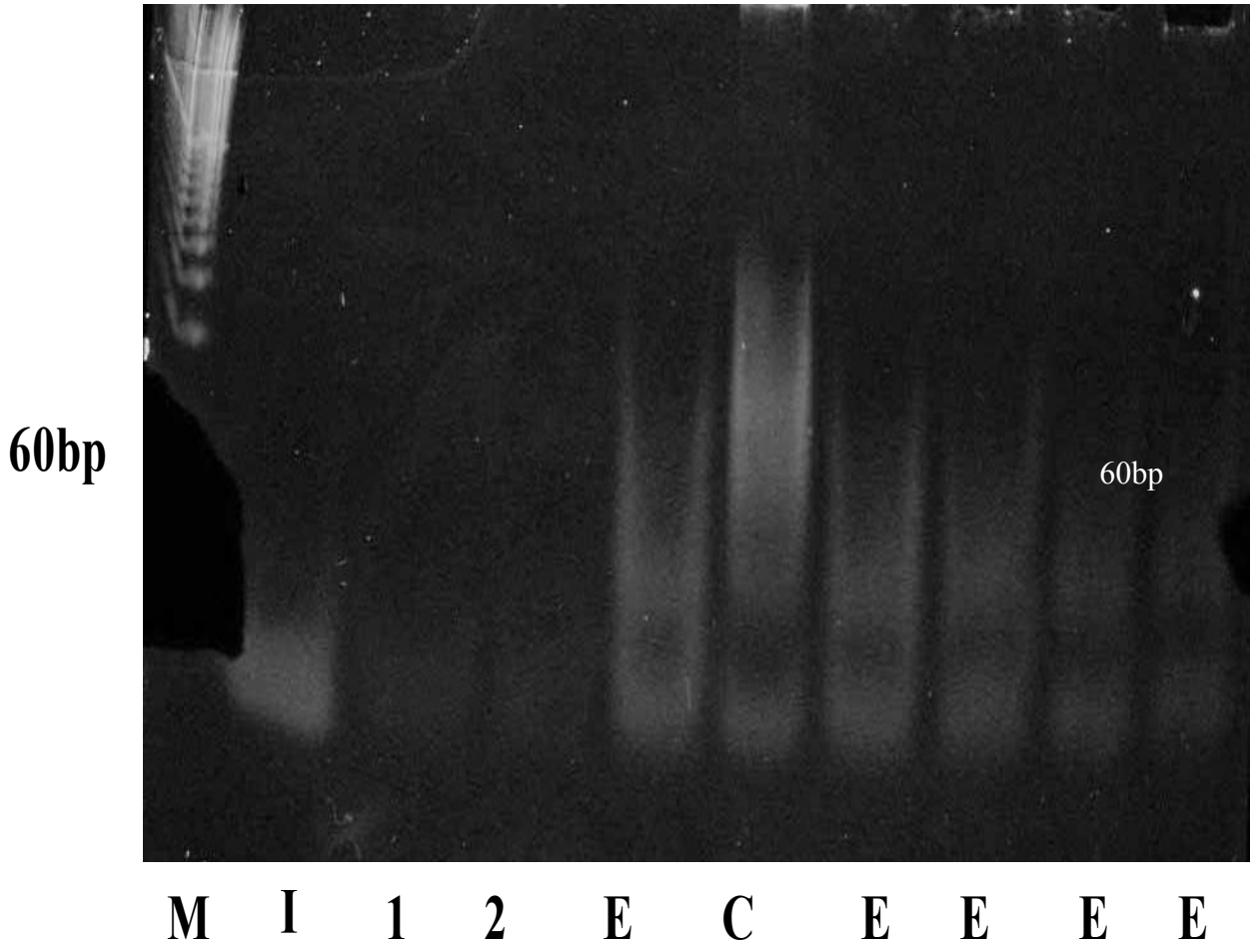

Figure 24. Round 3 Cell SELEX. 2 µL of ~500 µL of crush and soak product from Round 2 of Cell-SELEX was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation ,and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in Round 4 of Cell-SELEX.

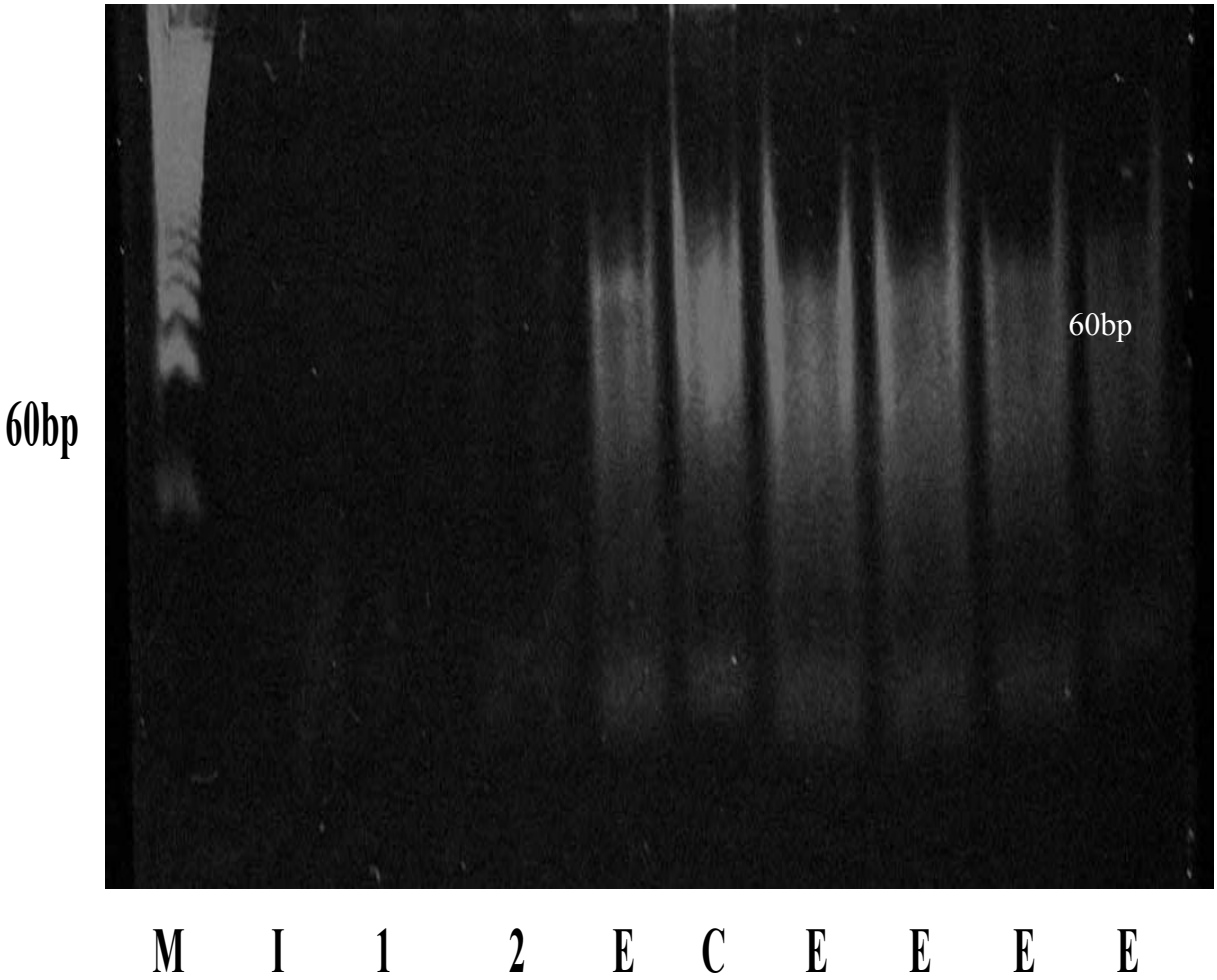

Figure 25. Round 4 Cell SELEX. 2 µL of ~500 µL of crush and soak product from Round 3 of Cell-SELEX was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation, and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in Round 5 of Cell-SELEX.

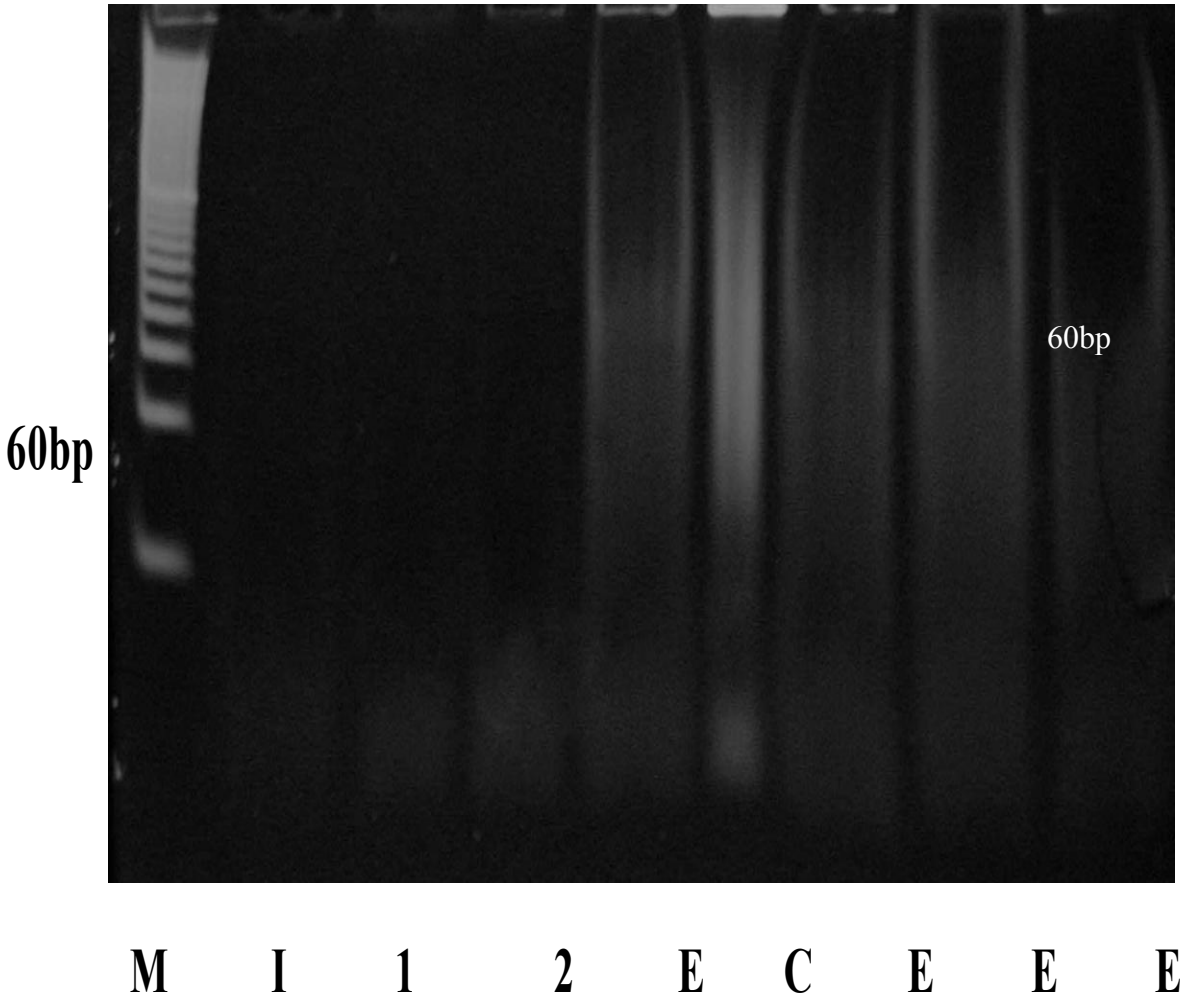

Figure 26. Round 5 Cell SELEX. 2 µL of ~500µL of crush and soak product from Round 4 of Cell-SELEX was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation, and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in Round 6 of Cell-SELEX and counter Cell-SELEX against *B. subtilis*.

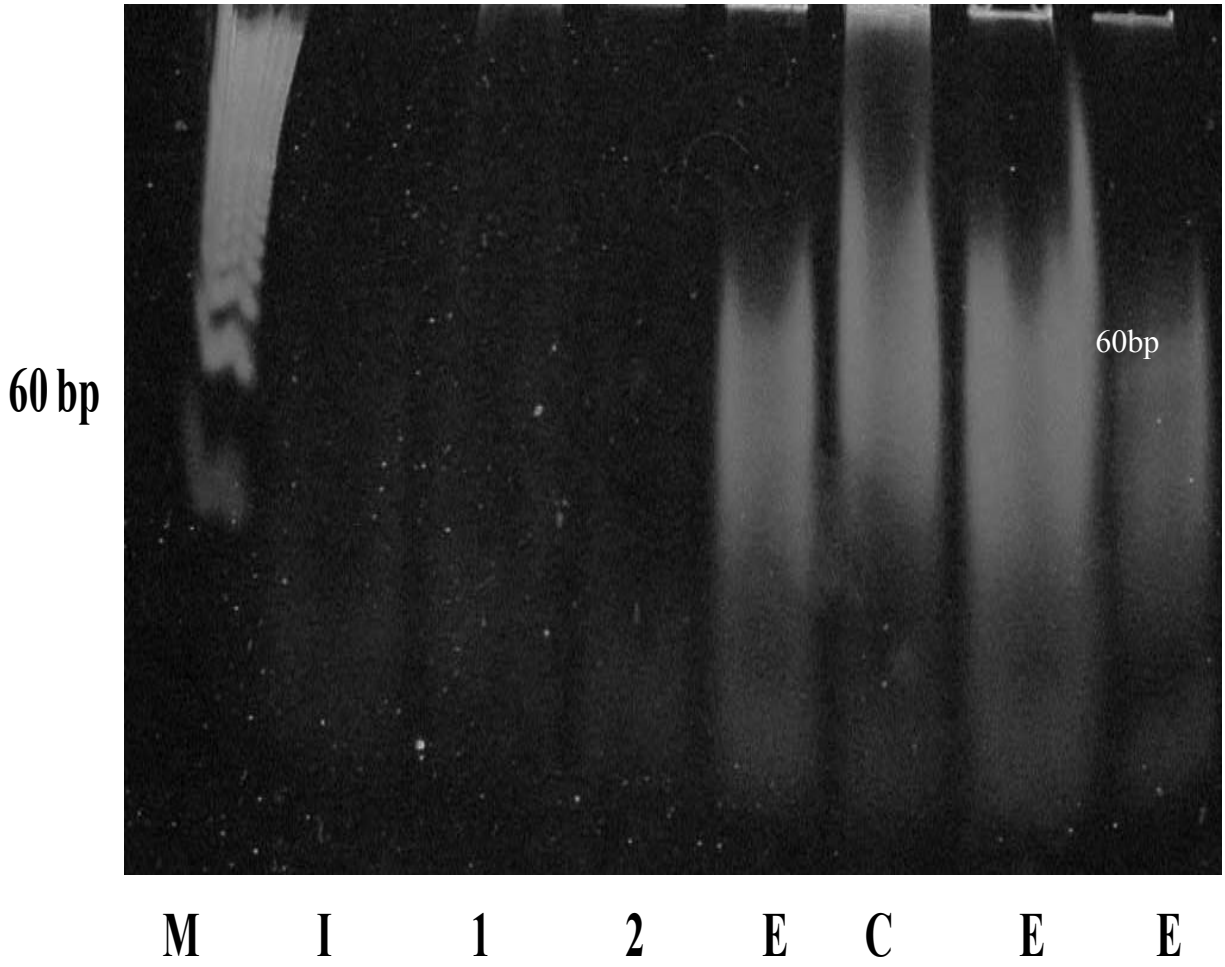

Figure 27. Round 6 Cell SELEX. 2 µL of ~500 µL of crush and soak product from Round 5 of Cell-SELEX was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in Round 7 of Cell-SELEX.

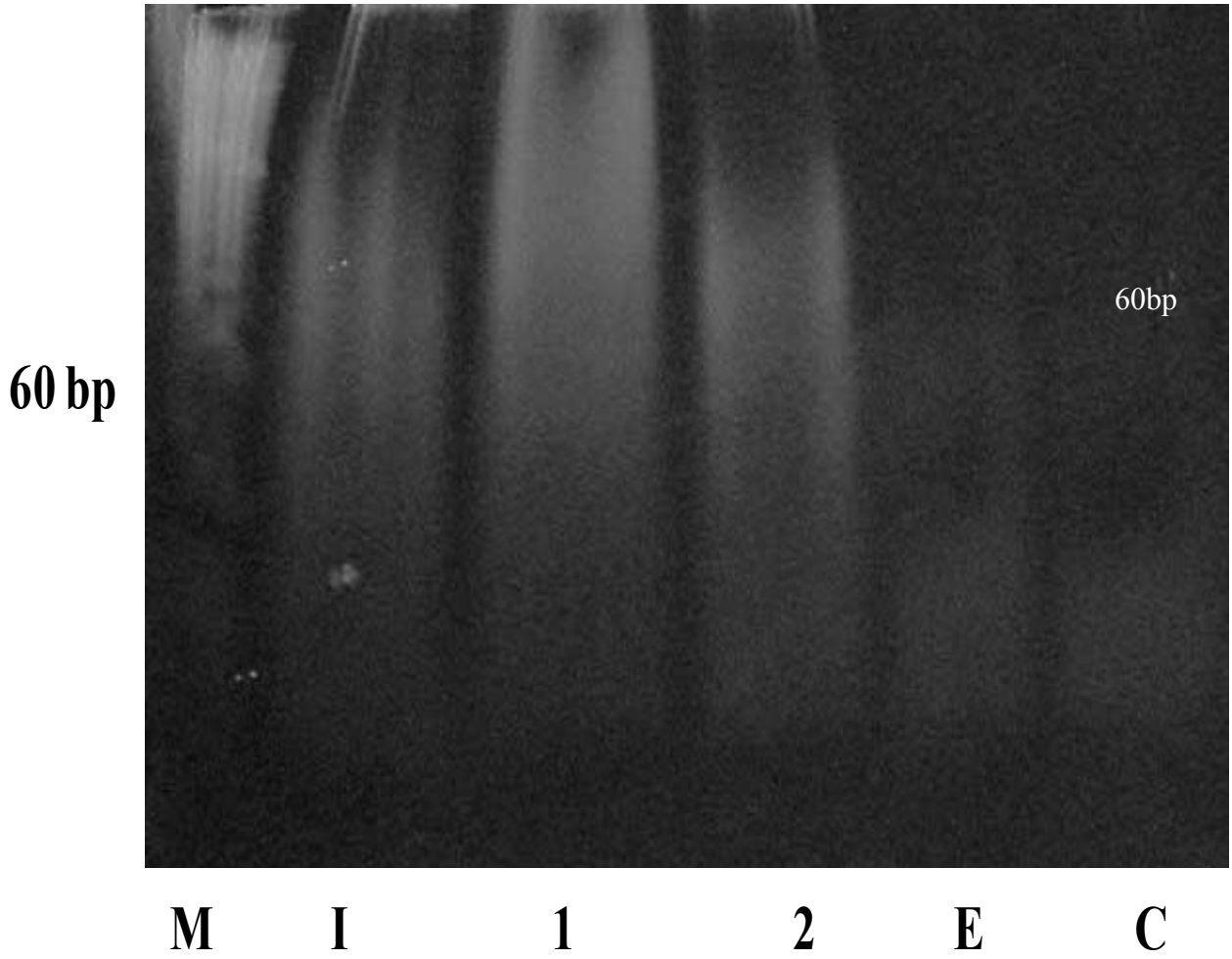

Figure 28. *B. subtilis* counter Cell SELEX round. 2 µL of ~500 µL of crush and soak product from Round 5 of Cell-SELEX was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation, and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the gel tore in the picture taking process, the DNA from the incubation, wash 1, and wash 2 steps were extracted via crush and soak and used in Recovery Round 6 of Cell SELEX.

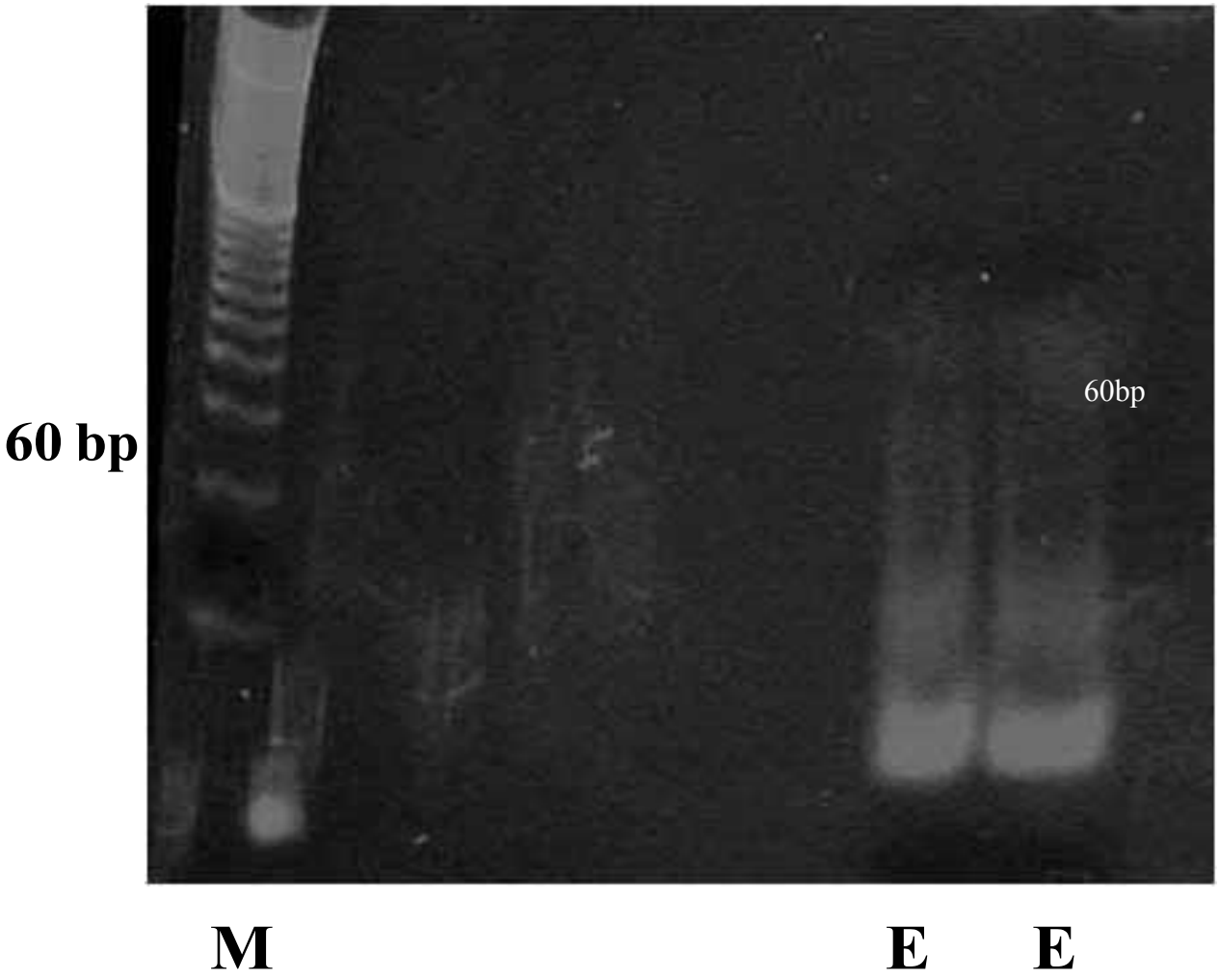

Figure 29 The PCR products from the elution steps were re-ran on a 1.5 mm 12% native gel due to the unclear nature of Figure 28.

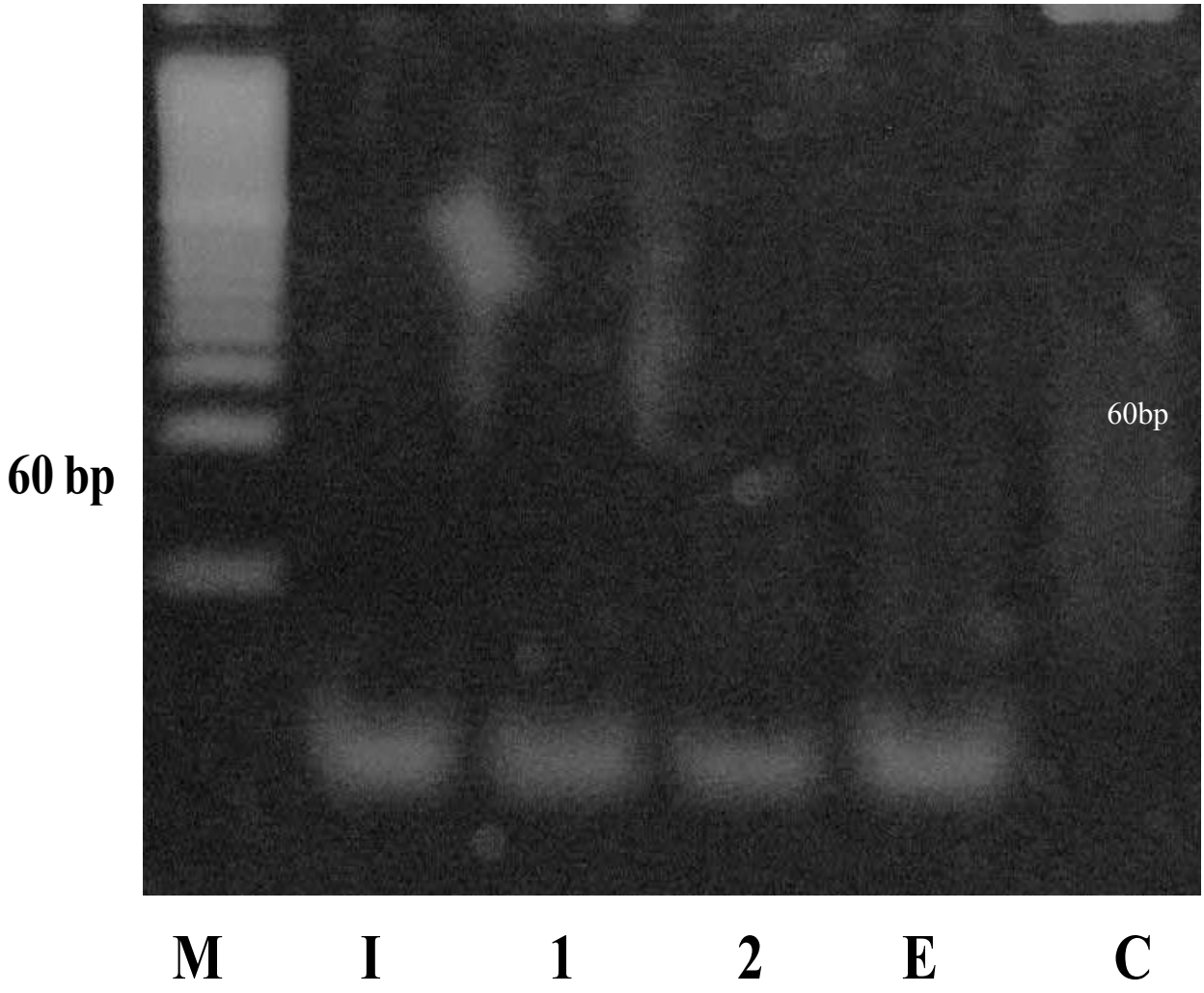

Figure 30. Recovery Round 6 Cell SELEX. A) 2 µL of ~500 µL of crush and soak product from *B. subtillis* counter Cell-SELEX round was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation, and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in *E. aerogenes* counter Cell-SELEX round.

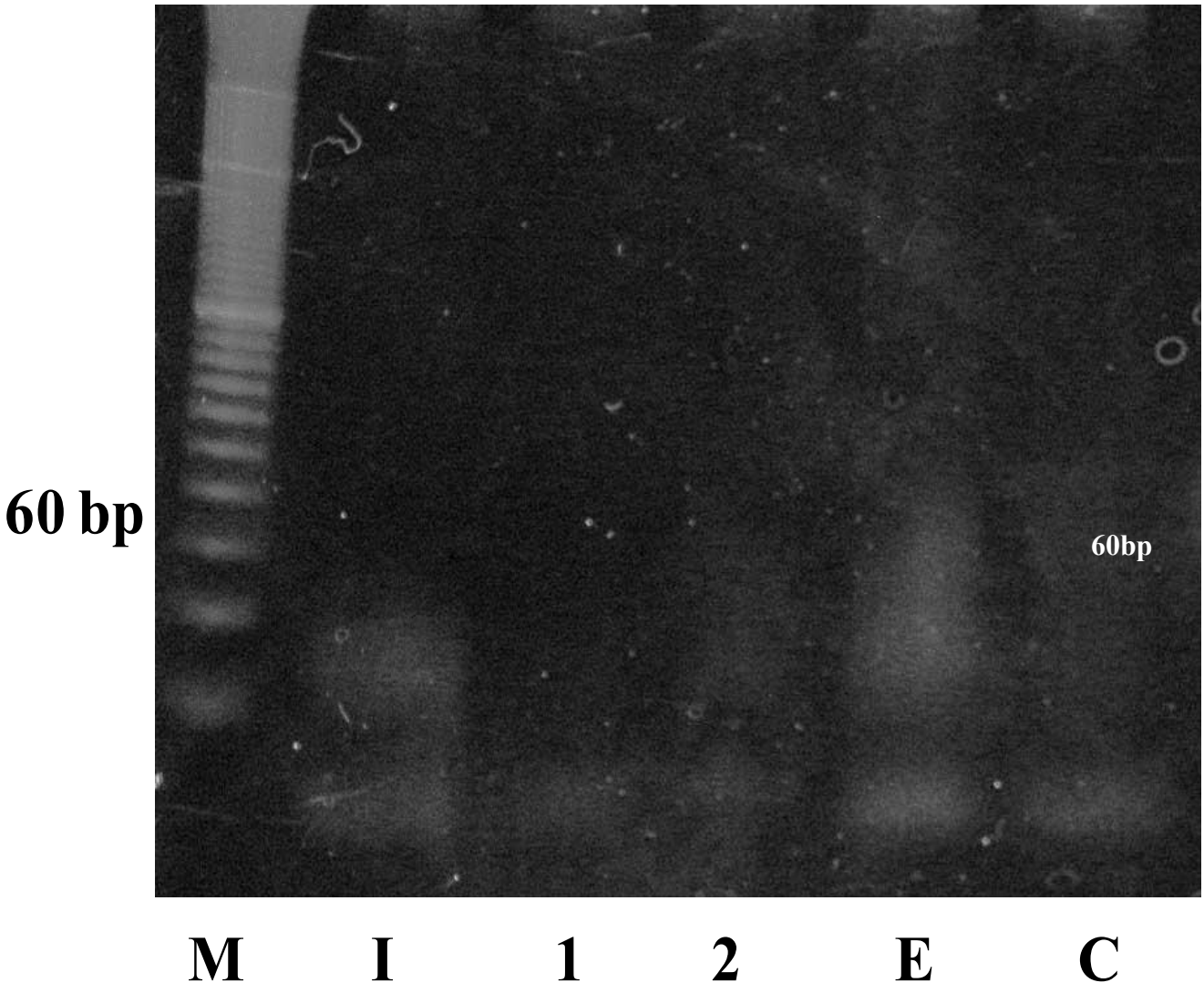

Figure 31. *E. aereogenes* counter Cell SELEX Round**.** 2 µL of ~500 µL of crush and soak product from Recovery Round 6 was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the incubation step was extracted via crush and soak and used in Recovery Round 7.

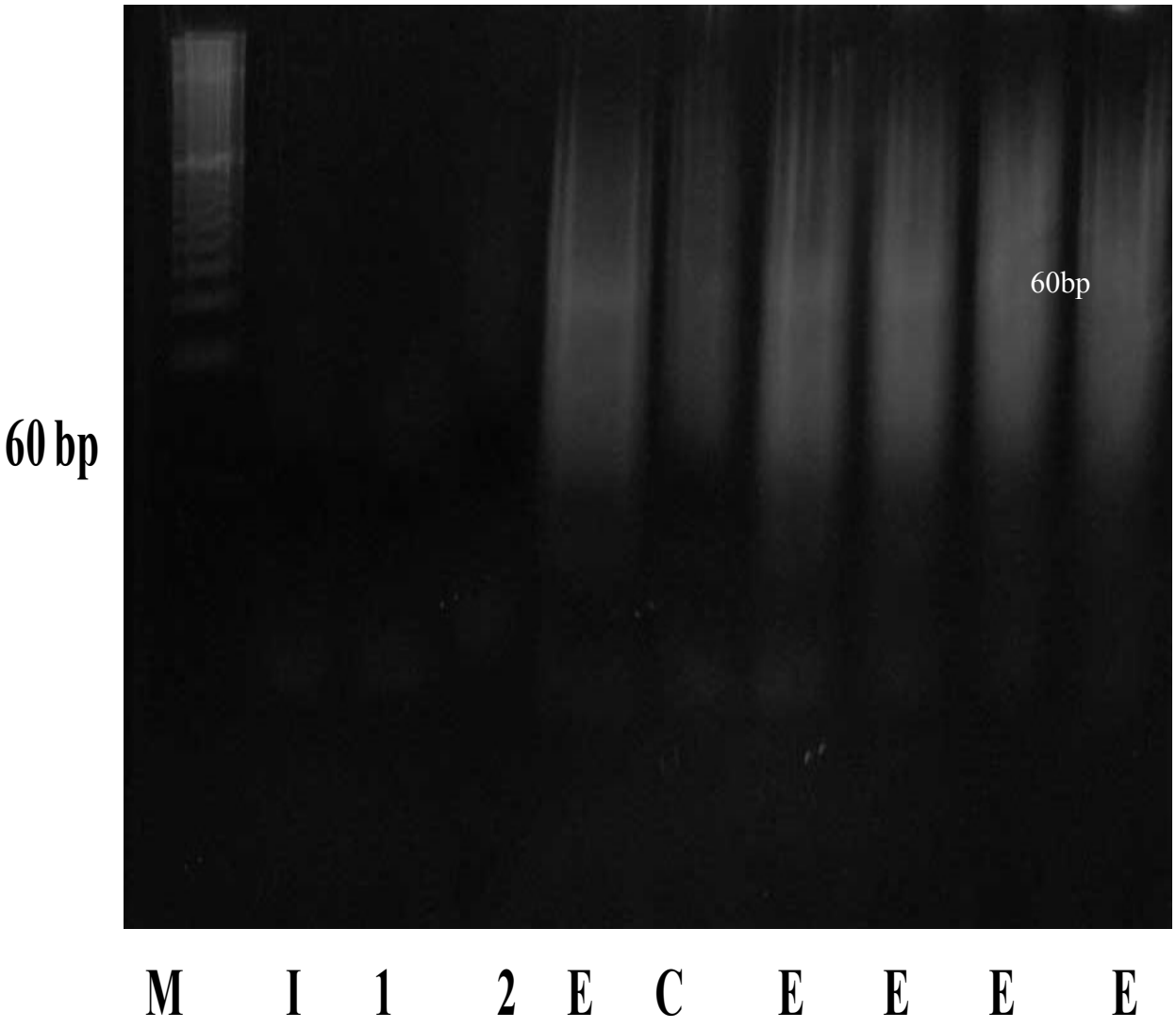

Figure 32a. Recovery Round 7 of Cell SELEX. A) 2 µL of ~500 µL of crush and soak product from *E. aerogenes* counter Cell-SELEX round was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation, and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in *E. aerogenes* counter Cell-SELEX round.

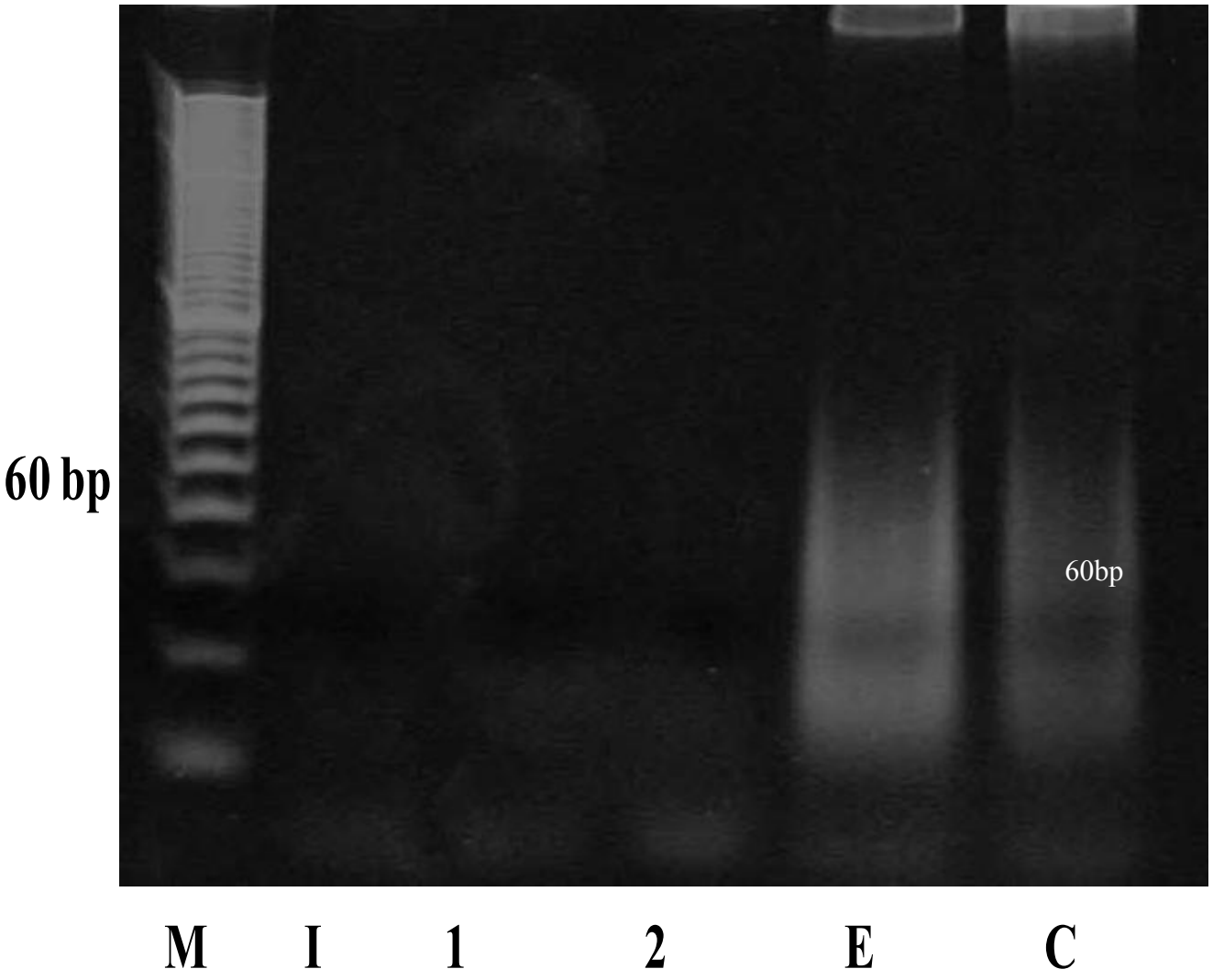

FIGURE 32b. The PCR products from the elution steps were re-ran on a 6% native gel due to the unclear nature of Figure 32a.

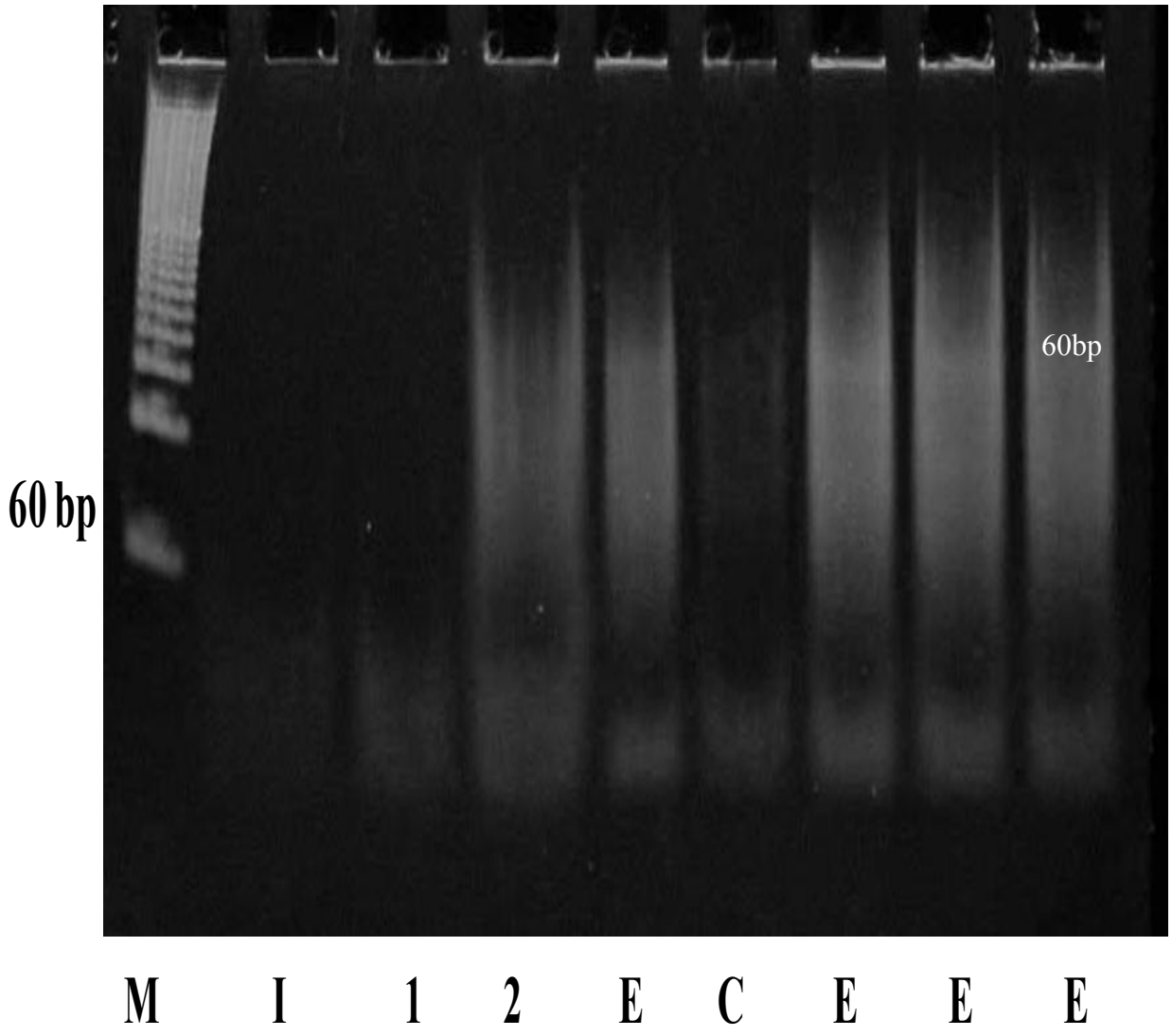

Figure 33. Round 7 of Cell SELEX. 2 µL of ~500 µL of crush and soak product from Round 6 of Cell-SELEX was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation ,and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in Round 7 of Cell-SELEX.

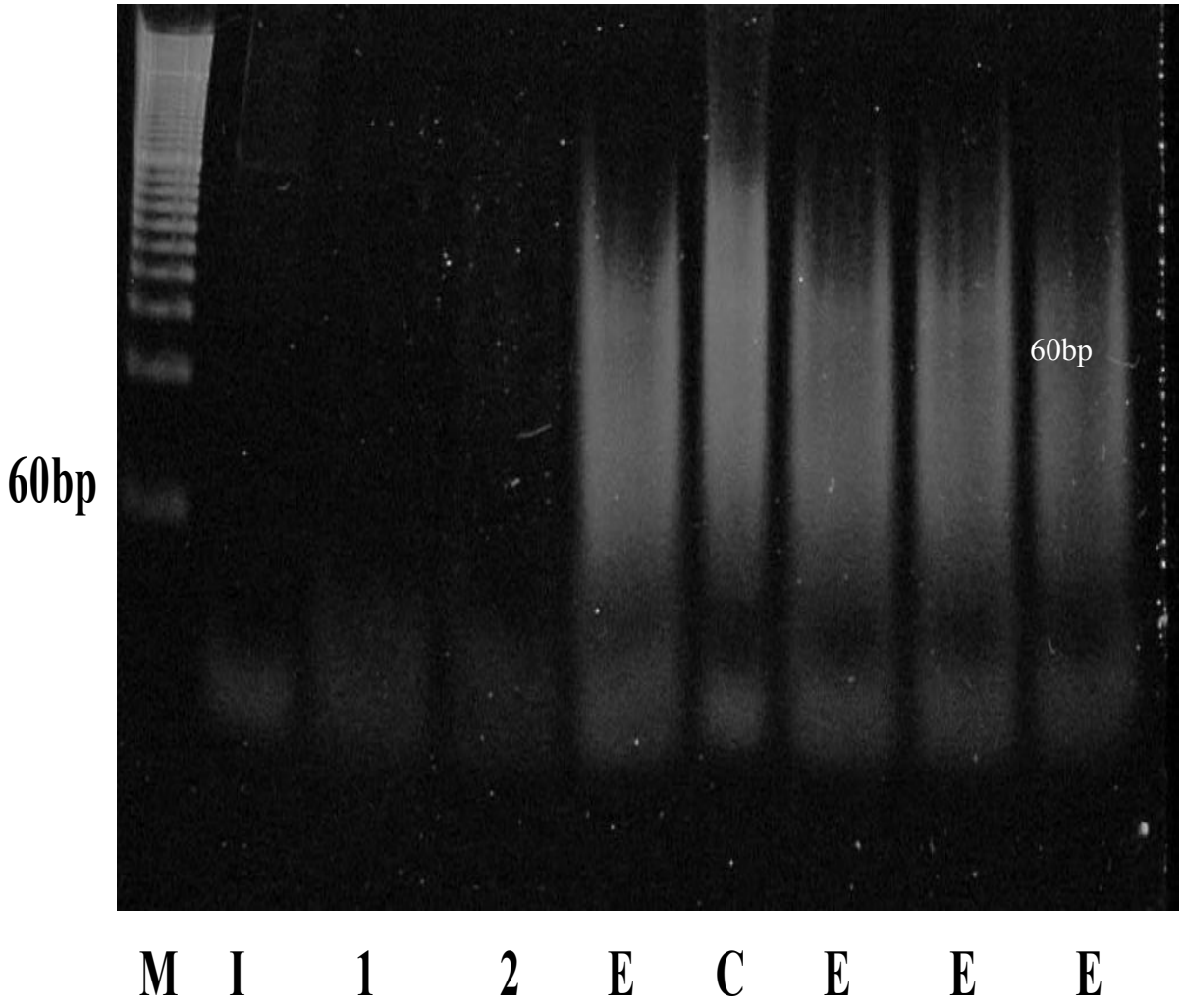

Figure 34. Round 8 of Cell SELEX. 2 µL of ~500 µL of crush and soak product from Round 7 of Cell-SELEX was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation, and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step. Although the cell tore in the picture taking process, the DNA from the elution step was extracted via crush and soak and used in ligation.

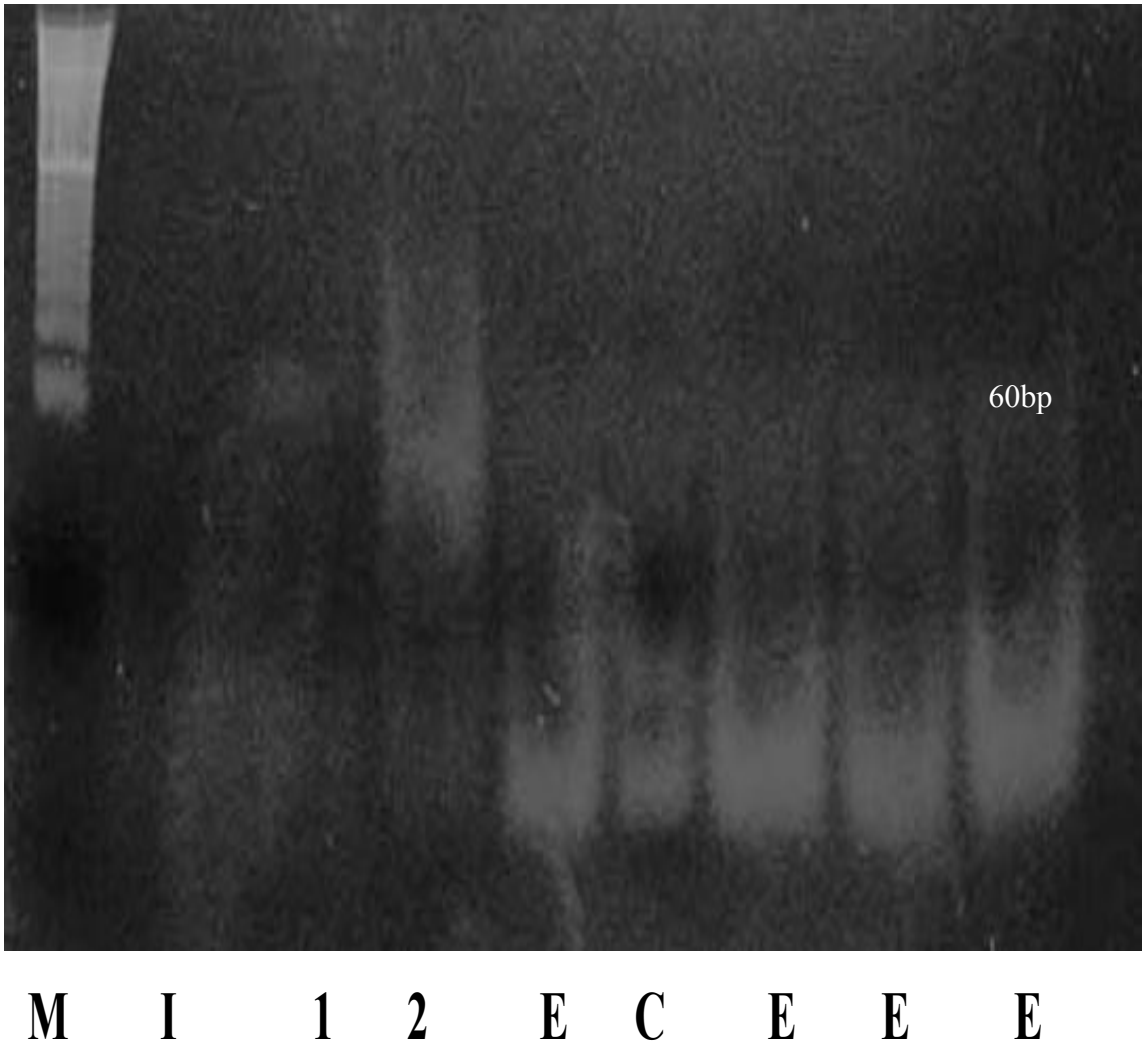

Figure 35. Recovery Round 8 of Cell SELEX. 2 µL of ~500 µL of crush and soak product from Recovery Round 7 was incubated (I) with 10 µL of ~10^8 *E. coli* cells, 480 µL of 1xBB, and 8 µL of 10 mg/mL BSA for 45 minutes at room temperature, chilled at 4°C for 10 minutes, then centrifuged at 5000*g* for 10 minutes, and the DNA is extracted from the supernatant via ethanol precipitation. The sample is put through two washing steps (1, 2), an elution step (E), which consists of heating, cooling, and centrifugation ,and a final wash that consists of all cells (C). All of the supernatants taken from each step were subjected to ethanol precipitation, PCR, and ran through 12% native gel. This gel contains a 20 bp marker (M) in lane one, incubation (I) in lane two, wash 1 (1) in lane three, wash 2 (2) in lane four, elution (E) in lane five, seven, eight, and nine, and the cell (C) supernatant in lane six. This gel illustrate that most of the random pool DNA remained attached to the *E. coli* cells until the elution step.

Cloning and Sequence Analysis of Aptamer Pools and Binding of Individual Sequences to Target Cells.

Aptamer pools were cloned and sequenced after eight rounds of cell SELEX using *E. coli* DH5α as a target, which can be seen in Figure 32. Two aptamers were found. Each aptamer sequence was fluorescently labeled with 5'FAM via PCR with 5'FAM synthesized on the forward primer, a K_D value of approximately 96.98 pM was found, which is depicted in Figure 36.

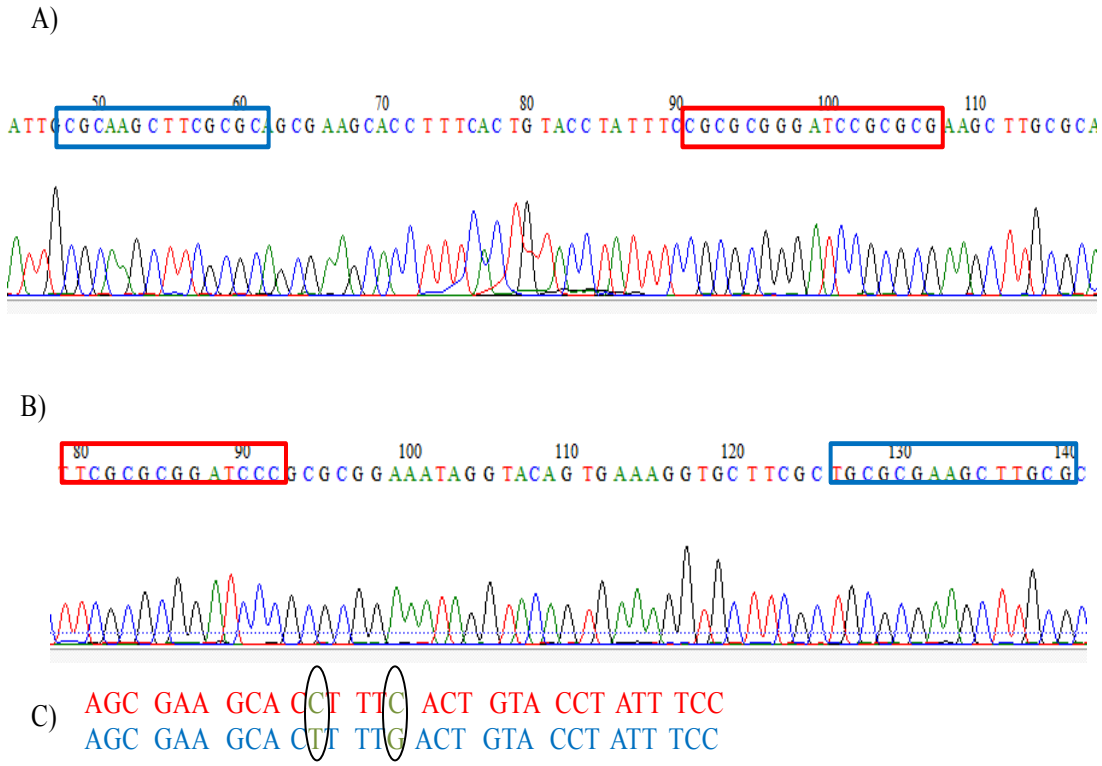

Figure 36. Aptamer sequences. Two aptamers were generated against live E. coli DH5α cells via Cell SELEX. The restriction enzyme sites are shown *Bam*HI (Red) and *Hind*III (Blue). A) This particular aptamer was more abundant and was used in all of the KD studies. B) This aptamer was not used in any K_D studies and was less abundant. This aptamer was sequenced using SP6 Promoter. C) Illustration in differences of sequences: Aptamer A (Red), Aptamer B (Blue) changed sequences (Green).

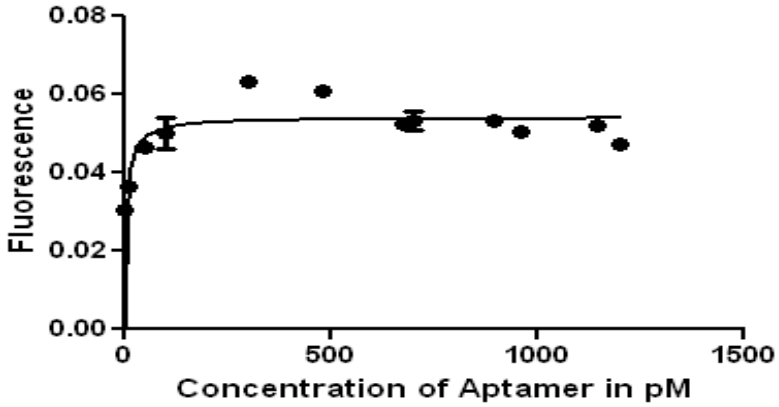

Figure 31. K_D Plot of 5'-FAM labeled aptamer. This plot depicts aptamers at

Figure 37. K_D plot of 5'- FAM labeled aptamer. This plot depicts aptamers at various concentrations incubated with 107 E. coli cells versus the fluorescence of the 5'-FAM at 485 nm taken by a Thermo Flouroskan Ascent FL 2.6. The K_D vales for this experiment was 96.98 pM.

Conclusions

Through this experiment two aptamers were generated and were found to have a K_D value of 96.98 pM.

CHAPTER FOUR

Conclusions

Aptamers contain inherent characteristics which make them an appealing class of olgionucleotides that are quite capable to meeting and exceeding the properties of antibodies as therapeutic compounds (Doudna *et al.* 1995). Aptamers are also able to act as biosensors and separation devices. All of these applications are based on the aptamers ability to fold into a three-dimensional structure based on its nucleic acid sequence that binds to target molecules (Sullenger *et al.* 2002). Aptamers have been found to bind to their targets with dissociation constants (K_D values) in the low picomolar ($1x10^{-12}$ mol/L) to low nanomolar ($1x10^{-9}$ mol/L) range. The interaction between the aptamer and its target is a very specific reaction that distinguishes between related targets that share characteristics (Rusconi *et al*. 2002).

In this chapter research strategies utilized to create aptamers against live bacterial cells will be reviewed in order to determine future studies for the aptamers created against live *E. coli* cells. In a similar study to the research project at hand, Hamula *et al.* utilized ssDNA aptamers generated against *Lactobacillus acidophilus* cells, in which 27 aptamers were created. After each round of selection aptamer pools were evaluated for binding affinity and specificity toward the target cell line via flow cytometry and microscopy. A K_D value of 13 ± 3 nM was determined via flow injection coupled to fluorescence detection and flow cytometry (Humula *et al.* 2008).

Chen *et al.* employed whole Cell SELEX to create a therapeutic agent against virulent *Mycobacterium tuberculosis*. After 10 rounds of selection against *M. tuberculosis* H37Rv 20 potential aptamers were sequenced, resulting in two aptamers. Isothermal titration calorimetry and flow cytometry was utilized to determine the affinity and specificity towards the target cell line; however a specific K_D value was not reported (Chen *et al*. 2007).

In a recent study by Dwivedi *et al.* used Cell-SELEX to produce an aptamer based biosensor with binding selectivity for *Camplobacter jejuni*. Following the seventh and tenth rounds of SELEX flow cytometry was used to determine which aptamer pools would be candidates for subsequent rounds of counter Cell SELEX. After ten rounds of SELEX and two rounds of counter Cell SELEX the aptamer pool was cloned and sequenced, which resulted in a single aptamer. The K_D value of 292.8 ± 53.1 nM was determined via flow cytometry (Dwivedi *et al.* 2010).

Cao *et al.* utilized a panel of ssDNA aptamers to detect *Staphylococcus aureus*. Aptamers were generated via a modified Cell-SELEX strategy, counter Cell-SELEX was initiated from the third round of selection. The ssDNA pool was incubated with approximately 10^7 cells of *Streptocococcus* A5005 for the third, fourth, and seventh rounds and *S. epidermidis* 26069 for the fifth and sixth rounds. The enrichment of aptamer pools was monitored via radiolabeld binding assays which found that the fifth round was more radioactivity than subsequent rounds. This anomaly is due to the loss of specifically structured aptamers through PCR. The aptamer pool from the fifth round was cloned and sequenced and eleven candidate sequences were chosen for further characterization. Binding assays were performed via flow cytometry to screen the

specific aptamers for *S. aureus* 8325.4. These studies determined that the vast majority of the aptamers produced could distinguish between *S. aureus* from *streptococcus*. The aptamers with the highest specificity and sensitivity were used in further investigations. Five aptamers were found to be exceptionally specific for *S. aureus*. These aptamers had similar EC50 values in the low nanomolar range and were not cross reactive to *Streptococcus* A5005, *S. epidermis* 26069, or *E. coli* DH5α, however K_D values were not reported (Cao *et al.* 2009).

The detection of bacteria via Cell-SELEX based on a single aptamer can be produce a false negative result due to space-temporal antigenic expression models and the high antigenic variation of bacteria. Thus theoritically combining several different aptamers each targeting different sites would provide multiple molecular bacterial characteristics and enhance sensitivity. Cao *et al.* utilized five highly specific to *S. aureus* combined and tested them via flow cytometry and confocal microscopy. The results from this study indicated that the aptamer cocktail maintained the specific properties of an individual aptamer, specifically, the ability to determine one strain of *S. aureus* from another, *S. epidermis, Streptococcus*, and *E. coli*. Interestingly, the aptamer cocktail demonstrated enhance fluorescence intensities in comparison to the fluorescence intensitiy of a single aptamer, and therefore provided a higher rate of binding capacity against various strains of *S. aureus* in the early growth state (Cao *et al.* 2009).

The K_D value for the aptamers generated through this project, which should be confirmed by flow cytometry, will provide the exact specificity and affinity of the aptamers generated via Cell-SELEX against live *E. coli* cells. A comparative study of binding affinity values (K_D) using the two found aptamers against *E. coli* DH5α, *B.*

subtilis, E. aerogenes, and *E. coli* K12 UT5600 will demonstrate the specificity and affinity of the aptamer pool created through this selection process. This can be done by incubating ~100 pM 5'FAM modified aptamers with 10^7 of cells for 45 minutes in 1xBB and 0.05% BSA. The cells should then be resuspened in 1xBB for immediate flow cytometric analysis. Forward scatter, side scatter, gated fluorescence intensity, and gated fluorescence above background should be quantified (Hamula *et al.* 2008).

Confocal imaging of *E. coli* DH5α cells bound to the aptamers to provide illustrative images. Approximately 240 nM of the 5'-FAM labeled aptamers should be incubated with roughly 10^7 *E. coli* DH5α cells at 37°C for 45 minutes in 500 μL of 1xBB. This mixture should then be centrifuged to separate the unbound aptamers from the bound aptamers. The bacterium-aptamer complex sediment should them be washed and resuspended in phosphate buffered saline solution (PBS). The sample should be dropped on a glass slide to make a thin smear of the bacteria sample. Pass the slide three or four times through the flame of a Bunsen burner to fix the bacteria to the slide. Imaging of the bacteria should be performed with a confocal microscope under 488nm exciting light. The K_D value could be taken by performing the exact same procedure multiple times with approximately 10^7 cells and various concentrations of aptamer. The concentration of aptamer versus the number of aptamers bound to the cell could be used to extrapolate the K_D value (Cao *et al.* 2009).

At this point the aptamers could be made into a microfluidic device, similar to ELISA plates. These plates could be used in a comparison study with the real-time PCR method developed by Lee *et al*, which is based on three basic steps. First, *E. coli* DH5α cells were captured via antibody coated magnetic beads. Then, the RNA aptamers were

attached to the *E. coli* DH5α cell in a sandwich-like fashion. Finally, the heat-released aptamers were amplified via real-time reverse-transcriptase PCR (Lee *et al.* 2010). Both methods could be simultaneously tested using different strains of *E. coli* in order to determine which method is the most efficient.

A clinical trial could also be performed on an aptamer cocktail, which contains the aptamer generated against live *E. coli* DH5α cells, the aptamers generated against *Staohylococcus aureus* (Cao *et al.* 2009), and the aptamers generated against *Campylobacter jejuni* (Dwivedi *et al.* 2010). This particular aptamer cocktail could be used to detect pathogens in both food and environmental samples.

What has *in vitro* selection of functional oligonucleotides taught us? Synthetic oligonucleotides have been used to cleave and form phosphodiester bonds, carbon-carbon bond formation, alkylation, a Diels-Alder condensation, and various acyl-transfer reactions (Joyce *et al.* 2002). RNA aptamers have been used to bind to numerous small molecules and protein targets including, ATP, GTP, B12, caffeine, and HIV-1 (Wilson *et al.* 1995 and Herman *et al.* 2003). The extensive research accomplishments in the selection of functional RNAs raised the issue of whether related nucleic acids could also produce functional sequences. DNA aptamers have been used for many different targets. With the help of metal cofactors DNA should be able to catalyze many different chemistries, similar to RNA (Silverman *et al.* 2004). DNA is made of the same sequence as a functional RNA motif, with a few exceptions, and the lack of 2'OH does not lessen the capability of DNA to form aptamer (Huizenga *et al.* 1995). Therefore, aptamers can be used for various purposes and industries including, therapuedics, separation sciences, and detection (Stoltenburg *et al.* 2007).

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